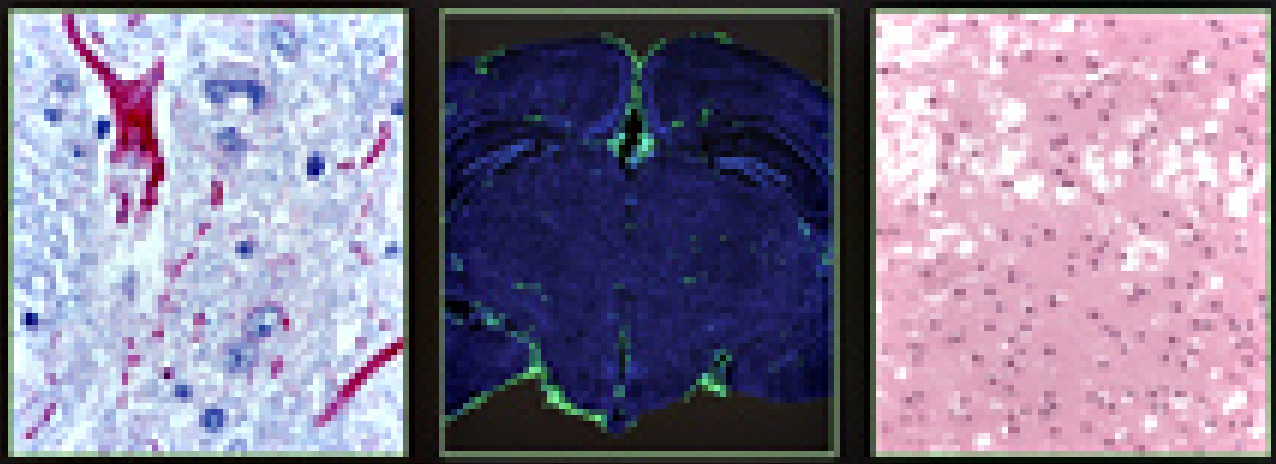
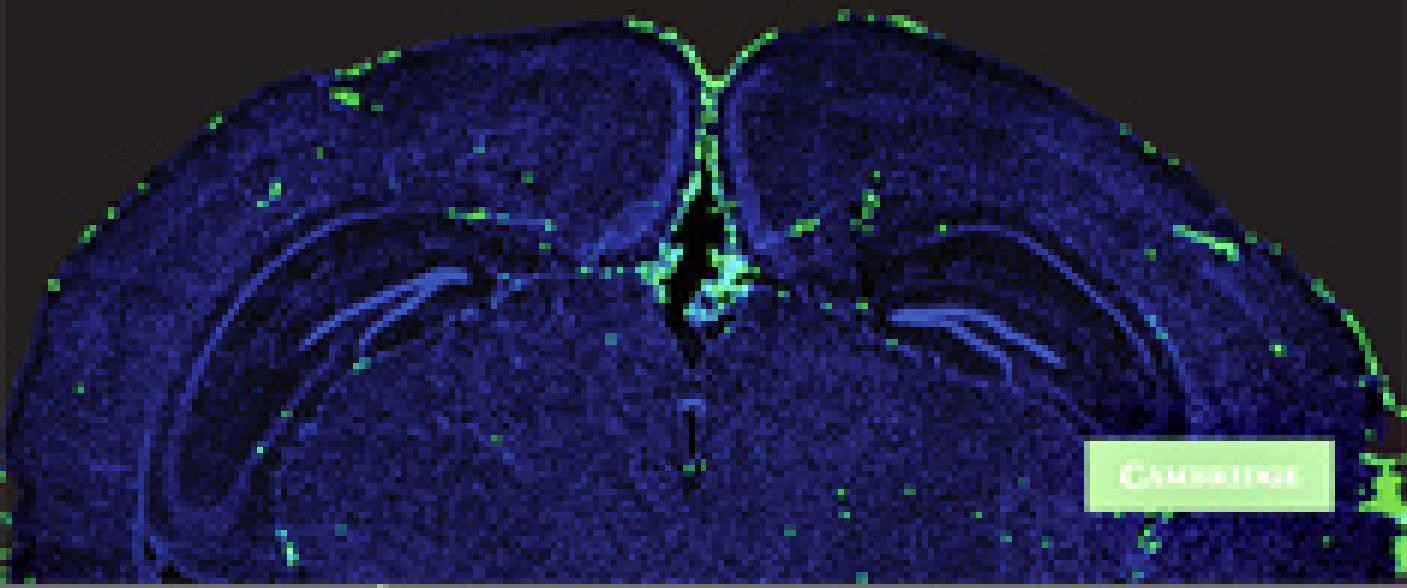


NEUROTROPIC VIRAL INFECTIONS

Edited by

CAROL SHOSHKES REISS



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Neurotropic Viral Infections

Neurotropic viruses infect the brain, often causing lethal disease in people. These diseases range from polio, to rabies and AIDS. This book is a depiction of each of these individual viruses. It discusses the diseases they cause, the mechanisms by which they cause and spread those diseases, the detection and treatment of these illnesses, and their prevention. There is also discussion of novel and beneficial uses of these neurotropic viruses for gene therapy and tumor lysis. It has been written in a style that is appealing for a very wide professional audience, ranging from graduate students, to postdocs, scientists, clinicians, and public health professionals.

Carol Shoshkes Reiss is a Professor in the Department of Biology at New York University. She was Editor-in-Chief of *Viral Immunology* from 1999–2006.

". . . a cohesive and comprehensive overview of viral infection(s) of the nervous system . . . destined to become a great reference and text for some time to come."
Michael J. Goldblatt *President and CEO, Functional Genetics, Inc.*

"The authors did an amazing job of walking that fine line between too much detail for non-specialists and not enough sophistication for the cognoscenti."
Richard M. Ransohoff *Director, Neuroinflammation Research Center*

"I can't imagine a better place to learn than this very well-thought out and comprehensive volume by leading authorities on this important subject."
Stephen S. Morse *Founding Director, Center for Public Health Preparedness*, NCDP

Neurotropic Viral Infections

Edited by

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Foreword

Howard Lipton

Neurotropic Viral Infections is a timely compendium of reviews by noted experts in the field of neurotropic viruses[1], which cause serious neurological disease in humans. It is timely for a number of reasons. First, it is timely because an increasing number of stories of neurotropic virus infections have played out in the world news over the past decade. These include the spread of West Nile virus infection to New York State and across the North American continent; the emergence in Malaysia of two new paramyxoviruses that cause fatal encephalitis; the resurgence of paralytic poliomyelitis in sub-Saharan Africa as the WHO campaign to eradicate poliovirus mopped up the remaining cases; the sudden outbreak of Chikungunya fever, an alphavirus with neurological potential, in the Southwest Indian Ocean and India; the threat of mad cow disease spreading across the border from Canada into the Western United States; the surprising occurrence of progressive multifocal leukoencephalopathy (PML) in several multiple sclerosis patients and a Crohn's disease patient treated with a powerful new immunosuppressive agent, Natalizumab (Tysabri^{TM}), a humanized monoclonal antibody against the integrin $\alpha 4$ on lymphocytes; and, finally, from a therapeutic standpoint, the marketing of a new varicella-zoster virus (VZV) vaccine that promises to substantially reduce the risk of painful shingles in the elderly, as well as the first successful treatment of a young girl with rabies encephalitis in Fond du Lac, Wisconsin. Second, the

[1] Including prion diseases.

continuing HIV-AIDS epidemic and its neurological sequelae and rampant measles virus infections in Africa continue to be of great international concern. Third, the success of genotypic approaches to new virus discovery are being applied to neurological diseases of suspected viral etiology. Finally, the unrelenting (dizzying) progress in the biomedical sciences has had a profound impact on the conduct of research into the pathogenesis of all classes of neurotropic viruses, including the host immune response to these agents.

This book covers the gamut of neurotropic virus infections and is divided into five sections. The first section covers neurotropic RNA viruses causing human disease and includes chapters on such classic infections as poliomyelitis, subacute sclerosing panencephalitis, and rabies; chapters on West Nile, and Japanese B, encephalitides, as well as chapters on other infections.

Retroviral diseases – Human T-lymphotropic virus-1 (HTLV-1) and human immunodeficiency virus encephalitis – and several neurotropic DNA virus infections, such as PML and nervous system involvement by varicella-zoster and herpes simplex viruses and, in addition, transmissible spongiform encephalopathies are the focus of the second section.

Innate immunity in response to neurotropic virus infections is covered in the third section. The section includes chapters on Toll-like receptors and vaccines, as well as how the hypothalamic-pituitary-adrenal axis (i.e., neuro-endocrine networks), influences host immune responses. In addition, the third section of the book covers clinical aspects of neurotropic virus infections and includes chapters on the epidemiology of viral encephalitis, the clinical management of viral encephalitis, infectious disease discovery, and viral oncolysis of glioblastoma multiforme. Because many neurotropic virus infections are spread to humans by arthropod vectors and by bats, chapters on the ecology of vectors, virus-vector life cycles, and interventions are included in this section.

Clearly, viral infections of the brain remain one of the most frightening and mysterious of all human maladies. A better understanding of neurotropic viruses is still needed, and the time is propitious to update our knowledge in this area.

Preface and Acknowledgments

The idea for this book came from Martin Griffiths of Cambridge University Press, who approached me in October 2005. My initial reaction had been that I was too busy to undertake this project. I surprised myself by taking up the challenge. After giving the choice of topics and potential investigators much thought, I contacted the authors whose contributions you see here. Contracts from the Press were circulated in early 2006, and the manuscripts were submitted in the Spring of 2007, except by one very eager team, who completed their paper in October 2006.

We have attempted, in this volume, to put together a comprehensive survey of the principal viruses that cause disease in the central nervous system, the mechanisms by which they cause pathology and evade host responses, the host responses to these infections, and the tools available for diagnosis and treatment of the infections. Vaccine strategies, drug development, and the important questions concerning investigators and clinicians today are described. Given the rapid appearance of new agents and accompanying diseases, and the power of the scientific community to design new tools, we expect this field to continue its rapid changes. The need to provide information in a compact and up-to-date format is essential for students, faculty, clinicians, and public health personnel.

I could not have written my own chapter without the luxury of time from teaching and committee work, made possible by a yearlong sabbatical leave from New York University. For this leave, I am very grateful to Gloria Coruzzi, Chair of Biology,

Dan Stein, Dean for Science; Dick Foley, Dean of the Faculty of Arts and Sciences; and David McLaughlin, Provost. Passing along the responsibility for the Editor-in-Chief position of *Viral Immunology*, after 7 years of devotion, to David Woodland also freed me from other professional obligations during this period.

At Alice Huang's suggestion, I applied to the Rockefeller Foundation for a residency at its Villa Serbelloni in Bellagio on Lake Como in Italy and was privileged to be selected from among hundreds of applicants for an idyllic 4 weeks in May 2007. I am grateful to Tom Braciale, Peter Openshaw, and Peter Doherty, who wrote letters of support for the application. It is there at Bellagio, in the incredible Lombard setting, where the editing of the book was accomplished. I was able to walk the steep hills to think, engage the fantastic minds of other residents and their accompanying people, and focus almost exclusively on this project.

In this book, as in all of my professional work, the contributions of my students and postdocs as well as my collaborators and other professionals who freely exchanged their findings and thoughts, critiques, suggestions, and comments, have been enormously important. I am also grateful for the support and encouragement of my sons, Steven and Joshua Reiss, and for my parents, Lila and Milton Shoshkes.

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SECTION I

Introduction: RNA viruses

Carol Shoshkes Reiss

Viruses that infect the central nervous system may cause acute, chronic, or latent infections. In some cases, the diseases manifested are attributable to viral damage of neurons or supporting parenchymal tissues; in other cases, to immune attack on virally infected cells. They can be spread by excretion, by respiratory droplets or fomites, or, alternatively, by bites of insects or animals. These viruses range from those such as polio (Chapter 1) or rabies (Chapter 3), whose history in man is as old as the earliest records, to those that emerge from animal reservoirs to human hosts for the first time, such as SARS, Hendra, and Nipah viruses (see Chapter 21).

In this section of *Neurotropic virus infections*, viruses with an RNA genome are described, starting with the simplest, picornaviruses (Chapter 1), to the most complex, alphaviruses (Chapter 6) and flaviviruses (Chapter 7). RNA viruses require an enzyme not found in host cells: RNA-dependent RNA polymerases to generate both sense (mRNA) and antisense RNA copies. Because of the lack of the host cell proofreading capacity for genome copying, errors are frequently introduced. Some of these errors are neutral, others may be deleterious (and are selected against) or, alternatively, potentially beneficial in evading host immune responses ranging from innate immune recognition to host adaptive immune recognition by Th1 or CD8 cells of epitopes expressed by host MHC or MHC molecules, respectively, or of antibody recognition of native proteins expressed by virions or infected host cells.

These chapters are designed to describe the viruses, the diseases they cause, the epidemiology and transmission, medical treatment, as well as outline the areas for future advances.

Neurotropic picornaviruses (Chapter 1) are important pathogens causing gastroenteritis and then acute paralytic diseases. Polioviruses were virtually extinguished from human populations (their only hosts) by the efficacy of the Salk and Sabin vaccines. But poliomyelitis is now undergoing a re-emergence due to issues of religion, politics, and third-world sanitary deficiencies.

Measles virus (Chapter 2) normally causes an acute respiratory and then systemic infection accompanied by a characteristic rash; the attenuated live virus vaccine for this disease is highly effective. However, in a few people who develop natural infections, a chronic infection develops in the central nervous system (CNS), which is identified by its accompanying pathology: subacute sclerosing panencephalitis (SSPE).

Unlike the last two infections, which are spread from person to person, rabies is transmitted by the bite of a rabid animal or bat (see Chapter 21) and replicates locally before crossing the neuromuscular junction and entering peripheral nerves (Chapter 3). Retrograde transport brings the virus to the CNS, where the characteristic disease is manifest. It is among a growing group of viruses that have survived due to the development of proteins that block host responses.

Like measles, coronaviruses are spread by the respiratory route (Chapter 4). While most human

coronaviruses cause exclusively respiratory disease, the emergence of severe acute respiratory syndrome (SARS) virus in Asia in the winter of 2002/2003 was associated with gastrointestinal and central nervous system involvement. Much more is known about murine neurotropic coronaviruses, and insights into these are presented here.

Viruses that are endemic in rodent populations can jump the species barrier to man at times, especially when the host is immune compromised. Arenaviruses fall into this category (Chapter 5). Host immune responses and the mechanisms(s) of disease pathogenesis, including disruption of the blood-brain barrier, have been studied extensively in rodents.

Insect transmission of the alphaviruses (Chapter 6) causing eastern and western equine encephalitis to horses and humans by mosquito bites have led to efforts to suppress the arthropod populations (see also Chapter 20). In contrast to infection by picornaviruses, where older hosts are more severely affected, very young hosts are more likely to die of alphavirus disease. These have been extensively studied in mouse models.

West Nile virus is now the most important cause of human viral encephalitis in North America (Chapter 7). Imported by a bird or mosquito larvae in 1999, this virus has been transmitted to avian species, horses, and humans throughout the continental United States, Canada, and the Caribbean. This flavivirus has caused more attention to insect infestations than any other arbovirus in North America, where dengue and malaria are largely absent. In man, this is a severe disease of the elderly.

1

Neurotropic picornaviruses

Vincent Racaniello

Introduction

The family *Picornaviridae* includes many important human and animal pathogens, such as poliovirus, hepatitis A virus, foot-and-mouth disease virus, and rhinovirus. All picornaviruses are small, nonenveloped viruses with a single-stranded RNA genome of positive polarity, properties that are reflected in the name of the virus family: pico, a small unit of measurement [10^{-12}], and the nucleic acid of the viral genome, RNA. Poliovirus is the best-studied picornavirus that causes disease of the nervous system, but the family also contains other neurotropic viruses. This chapter will focus on the biology and pathogenesis of picornaviruses that cause neurological disease.

Members of the family *Picornaviridae* are classified into nine genera, four of which contain neurotropic viruses (Table 1.1). Poliovirus, coxsackievirus, echovirus, and other members of the enterovirus genus replicate in the alimentary tract and are resistant to low pH. The cardiovirus genus includes the encephalomyocarditis-like viruses, which cause flaccid paralysis in mice, and the Theiler's murine encephalomyelitis viruses. Different strains of Theiler's viruses cause either a poliomyelitis-like disease in mice or a chronic demyelinating disease similar to multiple sclerosis. Members of the parechovirus genus, previously classified as echoviruses, have been associated with flaccid paralysis and encephalitis. The teschovirus genus consists of porcine teschovirus, which may cause polioencephalitis in pigs.

Virus structure

Picornavirus virions are 30-nm spherical particles that consist of a protein shell surrounding the naked RNA genome. The virus particles lack a lipid envelope, and consequently their infectivity is insensitive to organic solvents. Cardioviruses, enteroviruses, parechoviruses, and teschoviruses are acid stable and remain infectious at pH values of 3.0 and lower. These viruses pass through the stomach to gain access to the intestine and therefore must be resistant to low pH.

The capsids of picornaviruses are composed of four structural proteins: VP1, VP2, VP3, and VP4. The exception is the parechoviruses, which contain only three capsid polypeptides: VP1, VP2, and VP0, the uncleaved precursor to VP2 + VP4. The picornavirus capsid contains 60 structural proteins arranged into an icosahedral lattice (Figure 1.1) [1]. The basic building block of the picornavirus capsid is the protomer, which contains one copy of each capsid protein. The shell is formed by VP1, VP2, and VP3, while VP4 lies on its inner surface. VP1, VP2, and VP3 have no sequence homology; yet, all three proteins form a wedge-shaped, eight-stranded antiparallel β-barrel. The wedge shape facilitates the packing of structural units to form a dense, rigid protein shell. The main structural differences among VP1, VP2, and VP3 lie in the loops that connect the β-strands and the N- and C-terminal sequences that extend from the β-barrel domain. These amino acid sequences give each picornavirus its distinct morphology and antigenicity.

Table 1.1. Members of *Picornaviridae*

Genus	Type species	Other species	Disease
Enterovirus	Poliovirus	Coxsackievirus, echovirus	Paralysis, aseptic meningitis, meningoencephalitis, encephalitis, hand-foot-and-mouth disease, myocarditis
Rhinovirus	Human rhinovirus		Common cold
Hepatovirus	Hepatitis A virus		Hepatitis
Cardiovirus	Encephalomyocarditis virus (EMCV)	Theiler's murine encephalomyelitis virus (TMEV)	Encephalomyelitis, myocarditis, demyelination
Aphthovirus	Foot-and-mouth disease virus (FMDV)		Foot-and-mouth disease of livestock
Parechovirus	Human parechovirus		Respiratory disease, gastroenteritis, paralysis, encephalitis, myocarditis
Erbovirus	Equine rhinitis B virus		Respiratory disease
Kobuvirus	Aichi virus		Gastroenteritis
Teschovirus	Porcine teschovirus		Encephalitis, paralysis

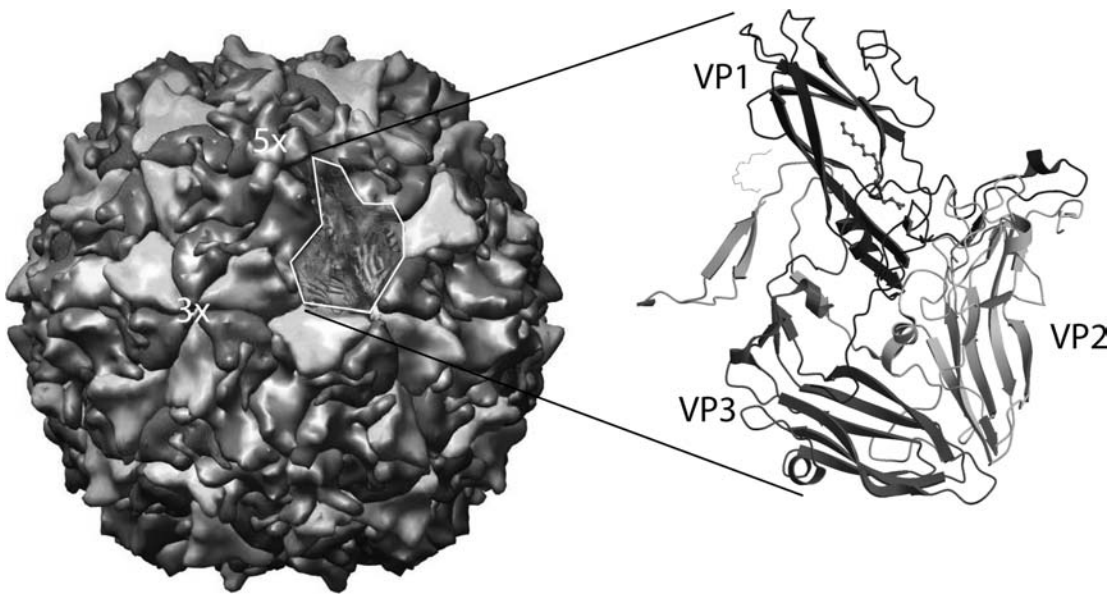

Figure 1.1. Structure of poliovirus. At left is a model of poliovirus type 1, Mahoney strain, based on the X-ray crystallographic structure determined at 2.9 Å [2]. The five-fold and three-fold axes of symmetry are labeled. At the five-fold axis is a star-shaped mesa surrounded by the canyon, which is the receptor-binding site. A propeller-shaped feature surrounds the three-fold axis. A single protomer is shown as a ribbon diagram and expanded to the right, showing the locations of capsid proteins VP1, VP2, and VP3. Image at left courtesy of VIPERdb [207].

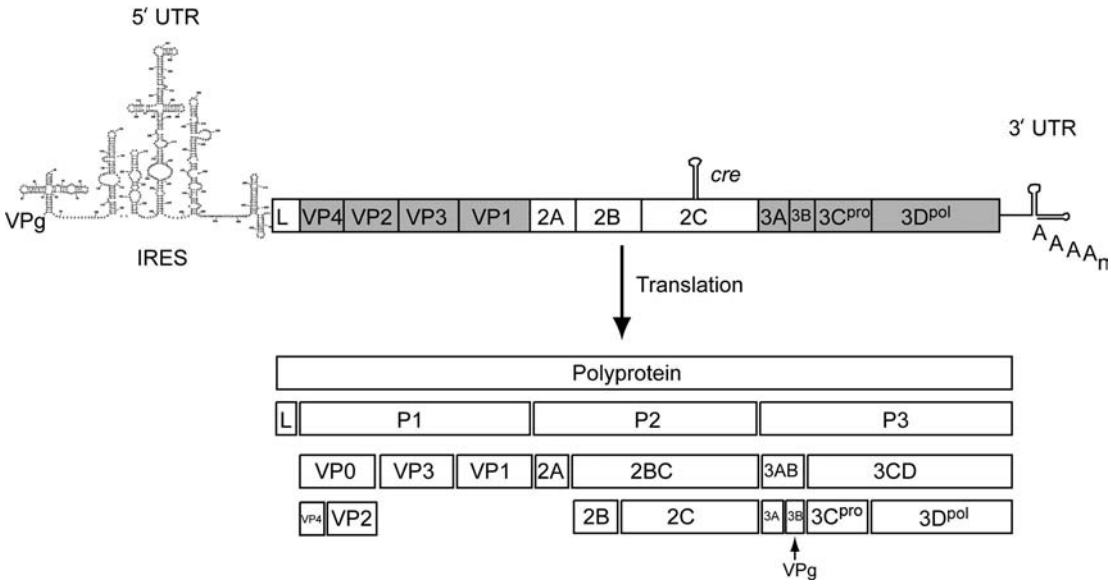

Figure 1.2. Schematic of the picornavirus genome. At top is a diagram of the viral RNA with coding regions labeled. RNA structural elements include an enterovirus IRES within the 5′ untranslated region, the poliovirus *cre*, and the pseudoknot within the 3′ untranslated region. Below is the processing pattern of picornavirus polyprotein. The coding region is divided into P1, P2, and P3, which are separated by nascent cleavage by viral proteinases. Intermediate and final cleavage products are shown. The L protein is encoded by the apthovirus and cardiovirus genomes.

Resolution of the atomic structure of poliovirus revealed that the virus surfaces have a corrugated topography; there is a prominent star-shaped plateau (mesa) at the five-fold axis of symmetry, surrounded by a deep depression (canyon) and another protrusion at the three-fold axis [2] (Figure 1.1). It was originally proposed that the canyon is the receptor-binding site, and this hypothesis has been proved for poliovirus and other picornaviruses [3,4]. The surfaces of cardioviruses lack canyons and have a much smoother appearance [5]. These viruses' other features serve as receptor-binding sites.

The viral genome

The genome of picornaviruses, a single positive-stranded RNA molecule, is infectious because it is translated upon entry into the cell to produce all the viral proteins required for replication. The genomes vary in length from 6500 to 9500 bases and are covalently linked at the 5′ end to a protein called VPg (Virion Protein, genome linked) [6,7], which serves as a primer for viral RNA synthesis [8,9]. Picornavirus genomes have a similar organizational pattern (Figure 1.2). The long (624 to 1,199 nucleotides) and structured 5′-noncoding regions contain sequences that control genome replication and translation. The 5′-noncoding region contains the internal ribosome entry site (IRES) that directs translation of the mRNA by internal ribosome binding. The 5′-untranslated regions of cardioviruses contain a poly(C) tract that varies in length among different virus strains. Following the 5′-noncoding region is a single open reading frame on the viral RNA that is translated into a polyprotein that is processed to form individual viral proteins. The polyprotein is cleaved during translation by virus-encoded proteinases so that the full-length product is not normally observed. At the 3′ end of the picornavirus genome is the 3′-noncoding

Table 1.2. Receptor and co-receptors for neurotropic picornaviruses

Virus	Receptor	Co-receptor	Reference
EMCV	VCAM-1		[195]
Poliovirus	CD155		[15]
Coxsackieviruses A13, A18, A21	ICAM-1		[196]
Coxsackievirus A21	CD55	ICAM-1	[14]
Coxsackievirus A9	$\alpha_v\beta_3$, $\alpha_v\beta_6$ integrin		[197,198]
Coxsackievirus B1-B6	CAR (coxsackievirus-adenovirus receptor)		[32]
Coxsackievirus B1, B3, B5	CD55	$\alpha_v\beta_6$ integrin	[199,200]
Echovirus 1, 8	$\alpha_2\beta_1$ integrin	β_2-microglobulin	[33,201]
Parechovirus 1	$\alpha_2\beta_1$ integrin, $\alpha_v\beta_3$		[202]
Echovirus 3,6,7,11–13,20,21, 24,29,33	CD55	β_2-microglobulin	[33,203,204,205]
Enterovirus 70	CD55		[206]

region (47–125 nucleotides), which has been implicated in controlling viral RNA synthesis [10], and a 3′ stretch of poly(A) [11] that is required for viral infectivity [12].

Viral replication

Virus entry into cells

The first step in picornavirus infection is attachment to a cell receptor. Different types of cell surface molecules serve as cellular receptors for picornaviruses (Table 1.2), and some are shared among picornaviruses and members of other virus families. For example, the cell surface protein CD55 is a receptor for certain coxsackieviruses A and B, echoviruses, and enterovirus 70. The poliovirus receptor, CD155, is also a receptor for alphaherpesviruses [13]. For some picornaviruses, such as poliovirus, a single type of receptor is sufficient for virus entry into cells. A second molecule, or coreceptor, is needed for entry of other viruses. For example, coxsackievirus A21, which attaches to CD55, requires intercellular adhesion molecule 1 (ICAM-1) for entry into cells [14].

The cell receptor for all three poliovirus serotypes is CD155, a glycoprotein that is a member of the immunoglobulin superfamily of proteins [15]. CD155 is composed of three extracellular immunoglobulin-like domains: a membrane-distal V-type domain that binds poliovirus, followed by two C2-type domains. The first Ig-like domain contains the site that binds poliovirus [3,4,16 to 23]. Alternative splicing of mRNA leads to the synthesis of two membrane-bound isoforms, CD155α and CD155δ, and two isoforms that lack transmembrane domains and are secreted from the cell [15,24]. The secreted isoforms' function is unknown. The membrane-bound isoforms are adhesion molecules, participating in formation of adherens junctions by interacting with nectin-3, an immunoglobulin-like protein related to CD155 [25]. CD155 is also a recognition molecule for natural killer (NK) cells and interacts with CD226 and CD96 on NK cells to stimulate cytotoxic activity [26,27]. Cytomegalovirus evades NK cell-mediated killing because the viral UL141 protein blocks surface expression of CD155 [28].

After attachment to a cellular receptor, the picornaviral capsid dissociates, releasing the RNA genome, which then enters the cytoplasm, the site of replication. For some picornaviruses, interaction with a cell receptor tethers virions on the cell surface; release of the genome is achieved by other means, such as low pH or coreceptor activity. For other picornaviruses, the cell receptor initiates conformational changes in the virus that lead to release of the genome.

Interaction of poliovirus with domain 1 of CD155 causes a conformational change in the capsid; these particles, called altered (A) particles, contain the viral RNA but lack the internal capsid protein VP4. The N-terminus of VP1, which is normally on the interior of the capsid, is on the surface of the A particle [29]. In one hypothesis for poliovirus entry, the exposed lipophilic N-terminus of VP1 inserts into the cell membrane, forming a pore through which the viral RNA can travel to the cytoplasm [30,31].

Many enteroviruses, including echoviruses, coxsackieviruses, and enterovirus 70, bind to decay-accelerating factor (DAF, or CD55), a member of the complement cascade (Table 1.2). Interaction with CD55 is usually not sufficient for infection because it does not lead to conformational changes in the virus as observed after poliovirus binding to CD155. Similarly, echovirus type 1 binds to the integrin $\alpha 2\beta 1$, but this interaction does not lead to conformational changes in the particle. Some coxsackieviruses bind to CD55, but conformational changes occur upon subsequent binding to Ig superfamily molecules such as ICAM-1 [14] or the coxsackievirus and adenovirus receptor (CAR) [32]. Additional coreceptors are believed to be required for conformational alteration of echoviruses. Candidate molecules include β2-microglobulin [33], complement control protein CD59, and heparan sulfate [34,35].

A specific role for coreceptors in virus entry is illustrated by coxsackievirus B3 infection of polarized epithelial cells [36]. CAR, which mediates cell entry of all coxsackie B viruses [37], is not present on the apical surface of epithelial cells that line the intestinal and respiratory tracts, but it is a component of the tight junction and is inaccessible to virus entry. Coxsackie B viruses also bind to a second receptor, CD55, which is present on the apical surface of epithelial cells. Coxsackievirus B3 binding to CD55 activates Abl kinase, which in turn triggers Rac-dependent actin rearrangements leading to virus movement to the tight junction where it can bind CAR and enter cells [36]. Virus binding to a coreceptor therefore allows access to the receptor required for cell entry.

Uncoating of the poliovirus genome probably occurs either at the plasma membrane or from within endosomes. Drugs that block acidification of endosomes do not inhibit poliovirus infection [38], and arrest of the clathrin-dependent endocytic pathway using dynamin mutants that prevent clathrin-coated pit budding have no effect on poliovirus replication [39]. Endocytosis alone is not sufficient to trigger poliovirus uncoating, because antibody-coated poliovirus particles cannot effectively infect cells expressing Fc receptors, which are efficiently endocytosed [40,41]. CD155-mediated conformational changes in poliovirus are clearly important for the uncoating process.

Internalization of echoviruses that bind $\alpha 2\beta 1$ integrin or CD55 initiates in lipid rafts followed by endocytosis into caveolae or caveosomes [42]. It is not known how the EV1 genome reaches the cytoplasm from these membranous vesicles.

Translation and proteolytic processing

After the positive-stranded viral RNA enters the cytoplasm, it is translated to provide viral proteins essential for genome replication and the production of new virus particles. The positive-stranded picornavirus RNA genomes lack 5′-terminal cap structures and cannot be translated by 5′-end dependent mechanisms. The 5′-untranslated region of the positive-strand RNAs harbors an internal ribosome entry site (IRES) that promotes internal binding of the 40S ribosomal subunit and allows 5′-end independent translation (Figure 1.2). Picornavirus IRESs have little nucleotide sequence identity, but they contain extensive regions of RNA secondary structure that are crucial for ribosome binding. Translation initiation mediated by the IRES of enteroviruses involves binding of the 40S ribosomal subunit to the IRES and scanning of the subunit to the AUG initiation codon. The 40S subunit probably binds at the AUG initiation codon of the encephalomyocarditis virus (EMCV) IRES. It is believed that the 40S ribosomal subunit is recruited to the IRES through interaction with eIF3 bound to the C-terminal domain of the translation initiation protein eIF4G, which binds directly to the IRES.

Ribosome binding to the picornavirus IRES requires cell proteins other than the canonical translation proteins. Such proteins are identified by their ability to bind the IRES and restore internal initiation in reticulocyte lysates, in which IRES-mediated translation is inefficient. Cell proteins required for IRES-mediated initiation include the La protein, which binds to the 3′ end of the poliovirus IRES [43] and stimulates the activity of the poliovirus and EMCV IRES [43,44]. Other proteins include polypyrimidine tract-binding protein, a regulator of pre-mRNA splicing [45,46]; unr, an RNA-binding protein with five cold-shock domains [47]; and ribosome-associated poly r(C)-binding proteins [48,49,50]. A common property of cellular proteins needed for IRES activity is that they are RNA-binding proteins that can form multimers with the potential to contact the IRES at multiple points. This observation has led to the hypothesis that these cell proteins may act as RNA chaperones and maintain the structure of the IRES in a configuration that allows direct binding to the translational machinery [51].

Picornavirus proteins are produced by the translation of the single open reading frame encoded by the viral positive-stranded RNA genome, followed by cleavage of the polyprotein by virus-encoded proteinases. The polyprotein is processed cotranslationally by intramolecular reactions (in *cis*), followed by secondary processing in *cis* or in *trans* (intermolecular). The genomes of enteroviruses encode two proteinases: $2A^{pro}$, and $3C^{pro}/3CD^{pro}$, which carry out cleavage of the polyprotein (Figure 1.2). The cardiovirus, parechovirus, and teschovirus genome encodes only one proteinase, $3C^{pro}/3CD^{pro}$.

RNA synthesis

Picornavirus RNA synthesis is carried out by the virus-encoded RNA-dependent RNA polymerase, $3D^{pol}$, a primer- and template-dependent enzyme that specifically copies picornavirus RNA and not cellular RNAs. The RNA polymerase $3D^{pol}$ is produced by cleavage of a precursor protein, $3CD^{pro}$, which is active as a proteinase but has no RNA polymerase activity. The primer for viral RNA synthesis is VPg, the small protein linked to the 5′ end of viral RNA. VPg is first uridylylated to form VPg-U-U, a reaction that is carried out by $3D^{pol}$ using as a template a short RNA hairpin structure (50–100 nt), the *cis*-acting replication element, *cre*, located in the coding region of the picornavirus genome [52,53,54].

The first step in genome replication is copying the positive-stranded RNA to form a negative-stranded intermediate. The template for this reaction appears to be a circular molecule formed by interaction of a 5′-cloverleaf structure in the viral RNA with the 3′-poly(A) tail. Circularization of the viral RNA is mediated by the interaction of $3CD^{pro}$ with cellular poly(A)-binding protein (PABP). These proteins also interact with the viral RNA: $3CD^{pro}$ with the 5′-cloverleaf structure and PABP with the 3′ poly(A) of the viral genome [55]. The viral polymerase, $3D^{pol}$, initiates RNA synthesis at the 3′-poly(A) tail and produces a complete (−) strand copy of the viral genome. The product is a double-stranded RNA intermediate, which is believed to serve as a template for synthesis of (+) strand viral RNA. Synthesis of (+) strand viral RNA also requires uridylylated VPg [56,57].

Picornavirus RNA synthesis takes place on the cytoplasmic surfaces of membranous vesicles that are induced by viral infection [58,59,60]. It is thought that the replication complex is recruited to these vesicles by the interaction of 3AB, which is inserted into the membrane via a hydrophobic domain, with $3D^{pol}$ and $3CD^{pro}$. Membrane remodeling is induced by several virus proteins, including 2BC, 2C, and 3A. During viral replication, the endoplasmic reticulum (ER) and Golgi are destroyed, and the cytoplasm fills with double-membraned vesicles [61,62]. The vesicles induced during poliovirus and rhinovirus infection appear to be derived from the cellular autophagosomal pathway; they bear several hallmarks of autophagosomes, including a double-membraned structure, the presence of cytoplasmic content within the vesicles [62], and colocalization with autophagosomal markers [63].

Once the pool of capsid proteins is sufficiently large, encapsidation of the viral RNA begins. Coat protein precursor P1 is cleaved to produce an immature protomer, which then assembles into

pentamers. Newly synthesized, positive-stranded RNA associates with pentamers, which then form the provirion, a particle that contains the viral genome, VP1, VP3, and VP0. Cleavage of VP0 to VP4 + VP2 stabilizes the capsid and creates the infectious virion [64]. VP0 is probably cleaved by an autocatalytic mechanism mediated by the viral RNA [65].

The time required for a single replication cycle ranges from 5 to 10 hours, depending on many variables, including the particular picornavirus, temperature, pH, host cell, and multiplicity of infection. Many picornaviruses are released as the cell loses its integrity and lyses, although there is evidence that some viruses, such as poliovirus, might be released from cells by nonlytic mechanisms [63].

Pathogenesis

Poliomyelitis

Near the beginning of the twentieth century epidemics of poliomyelitis, a previously rare disease, began to occur in the United States and Europe. The etiologic agent of this disease, poliomyelitis virus (derived from polios and myelos, Greek for gray and matter) was isolated in 1908 [66]. Research on the virus over the next 40 years led to the development of two effective vaccines. Global eradication of polio now seems within grasp, although lingering pockets of disease confound this goal [67].

Infection with poliovirus begins when the virus is ingested and multiplies in the oropharyngeal and intestinal mucosa (Figure 1.3) [68,69]. Virus shed in the feces of infected individuals is largely responsible for transmission of infection. From the primary sites of multiplication in the mucosa, virus drains into cervical and mesenteric lymph nodes and then to the blood, causing a transient viremia [68]. Most natural infections of humans end at this stage with a minor disease consisting of symptoms such as sore throat, fever, and malaise. Replication at extraneural sites is believed to maintain viremia beyond the first stage and increase the likelihood of virus entry into the central nervous system. These extraneural sites might include brown fat, reticuloendothelial tissues, and muscle [70,71,72]. In 1–2% of infected individuals, the virus enters the central nervous system and replicates in motor neurons within the spinal cord, brain stem, or motor cortex. Viral replication in motor neurons within the spinal cord leads to the characteristic muscle paralysis.

Because only 1–2% of poliovirus infections lead to poliomyelitis, the neurological phase of infection can be viewed as an accidental diversion of the enteric stage. Spread of poliovirus within the population and survival of the virus depend only on viral multiplication in the alimentary tract. It is not known why poliovirus only rarely invades the central nervous system. One hypothesis comes from the observation of a genetic bottleneck in poliovirus spread from peripheral sites to the brain in mice [73]. When a mixture of four genetically marked viruses is inoculated peripherally into mice, only a subset can be detected in the brain. Two mechanisms were proposed to explain the bottleneck. The pathway the virus must travel from the periphery to the brain might be difficult, and each virus has a low probability of reaching the brain. Alternatively, those viruses that initially reach the brain might induce an innate antiviral state that prevents entry and spread of other viruses (see Chapter 14, Innate immunity in the CNS). Such a bottleneck could explain the stochastic nature of poliomyelitis during outbreaks of the disease.

Host range

Humans are the only known natural hosts of poliovirus. Chimpanzees and old world monkeys such as rhesus, cynomolgous, and African green monkeys can be experimentally infected. The resistance of mice and other species to infection by poliovirus is likely due to the absence of a suitable cell receptor. Cultured mouse cells are not susceptible to poliovirus infection, but they are permissive, i.e., they produce infectious virus after transfection with viral RNA [74,75]. The synthesis of CD155 in mouse L cells or in transgenic mice confers susceptibility to infection [15,76,77]. Orthologs of the *CD155* gene are present in the genomes of a number of

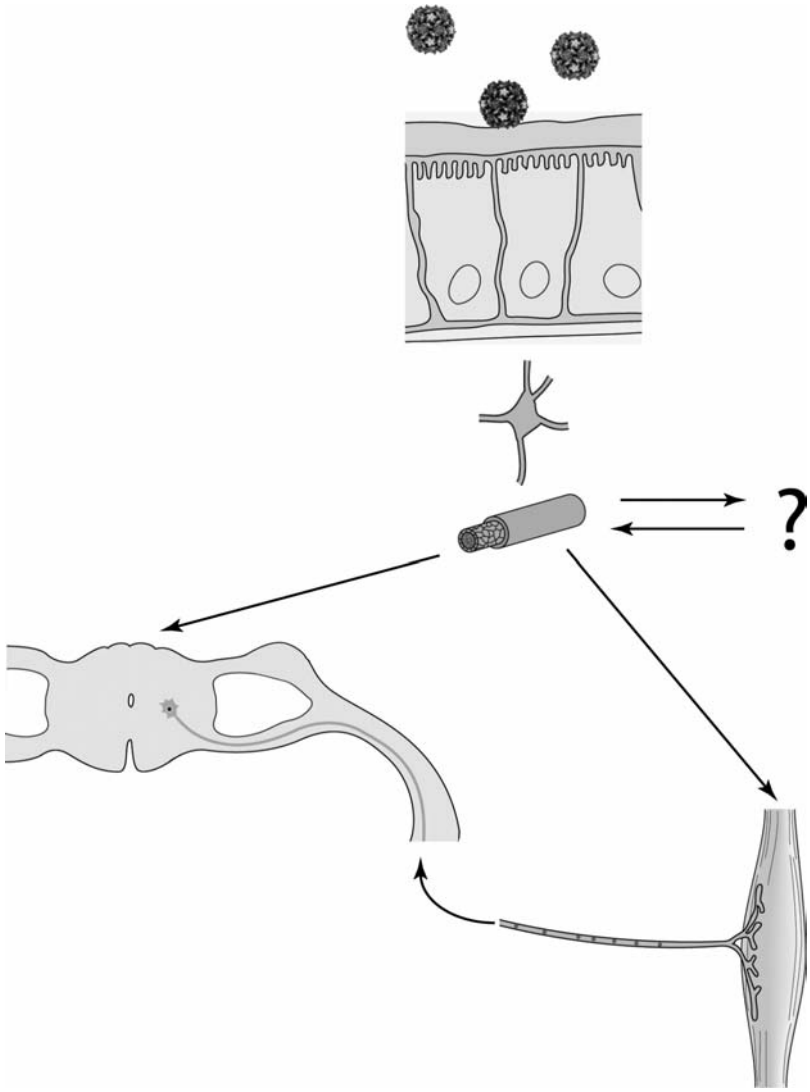

Figure 1.3. Pathways of poliovirus spread in humans. Virus enters at the oropharyngeal and intestinal mucosa, replicates, and spreads to the blood through the lymph nodes, resulting in viremia. Entry of virus into the central nervous system may occur either directly from the blood or by retrograde axonal transport when virus is brought to the muscle via the bloodstream. Invasion of the brain or spinal cord is preceded by viral multiplication in extraneural tissues (possibly skeletal muscle and brown fat), which produces a sustained viremia. Virus replication in the alimentary tract mucosa leads to virus shedding in feces and transmission of infection to other human hosts.

mammals, including those that are not susceptible to poliovirus infection [78]. The amino acid sequence of domain 1 of CD155, which contains the binding site for poliovirus, varies extensively among the nonsusceptible mammals, especially in the regions known to contact poliovirus. The absence of a poliovirus binding site on these CD155 molecules explains why poliovirus infection is restricted to simians.

Some strains of poliovirus can infect mice that do not produce human CD155. The poliovirus strains P2/Lansing, P1/Lsb, and a variant of P3/Leon, were selected for replication in mice by serial passage of viruses in nonprimate hosts [79,80]. Other poliovirus strains are naturally virulent in mice [81]. When mice are inoculated intracerebrally with P2/Lansing, they develop a disease with clinical, histopathological, and age-dependent features that resembles human poliomyelitis [82,83]. The murine cell receptor that allows these strains to enter mouse cells has not been identified. Substitution of a six amino acid sequence of the P1/Mahoney strain with the corresponding sequence from P2/Lansing confers mouse neurovirulence to the recombinant virus [84]. This six amino acid sequence is part of capsid protein VP1 on the surface of the virion at the five-fold axis of symmetry [85] near the binding site for CD155 [3,4,86]. These observations suggest that these six amino acids in the P2/Lansing capsid regulate the interaction with a mouse cell receptor, possibly by direct contact.

Entry into the host

Whether epithelial or lymphoid cells are the primary sites of poliovirus replication in the oropharyngeal and intestinal mucosa has been a matter of debate for many years. Virus has been detected in tonsillopharyngeal tissue and Peyer's patches of chimpanzees that had been orally infected with poliovirus [68]. Poliovirus has been isolated from human tonsillopharyngeal tissue, the wall of the ileum, and mesenteric lymph nodes [87]. However, removal of tonsils or adenoids does not reduce the level of poliovirus multiplication in the throats of humans [69]. Consequently it is not known if poliovirus replicates in lymphoid tissues or if it is absorbed into lymph nodes after replication in epithelial cells.

Examination of CD155 expression in cells of the alimentary tract has provided information on which cell types might be susceptible to infection. Human epithelial cells produce high levels of CD155 RNA, suggesting that these cells might be primary sites of poliovirus replication [88]. In humans, CD155 protein is present on the intestinal epithelium, M cells of Peyer's patches, and in germinal centers within the Peyer's patches [89]. In rhesus macaques, which are not susceptible to oral poliovirus infection, CD155 levels are reduced in follicle-associated epithelium and the protein is not present in germinal centers. These results suggest that poliovirus replication in the gut depends on the presence of CD155 in follicle-associated epithelium, including M cells, and on cells of the Peyer's patches [89].

CD155 transgenic mice are not susceptible to oral infection with poliovirus [76,77]. CD155 protein is present at very low levels in the intestinal epithelium of these mice, and it is absent in the Peyer's patches [89,90]. Overproduction of CD155 in the intestinal epithelium of transgenic mice by the use of a fatty acid-binding protein promoter did not lead to oral susceptibility to poliovirus [90]. These findings suggest that the expression of CD155 in Peyer's patches and in M cells is important for oral susceptibility to poliovirus infection. However, these data do not exclude the possibility that poliovirus might also replicate in the intestinal epithelium. In the mouse intestine, these cells might not be permissive for poliovirus infection due to the absence of an intracellular protein required for viral replication. The production of CD155 transgenic mice that express the poliovirus receptor in Peyer's patches and M cells might resolve these questions. A line of transgenic mice (cPvr mice) has been isolated in which production of CD155 mRNA is under the control of the β-actin promoter [91]. The use of this promoter should result in CD155 mRNA expression in all cells, although CD155 synthesis in individual cell types in the intestine was not examined. However, cPvr mice do not develop paralytic disease after oral inoculation with poliovirus. After intraperitoneal

inoculation, low levels of poliovirus replication were detected in the intestine, but the cells harboring poliovirus were not identified. Additional work is needed to determine the block to poliovirus replication in different CD155 transgenic mouse lines.

Although CD155 transgenic mice cannot be orally infected with poliovirus, inoculation by the intranasal route leads to paralysis accompanied by virus replication in the nasal epithelium and olfactory bulb, cerebrum, brain stem, and spinal cord [91,92]. Early hypotheses on the pathogenesis of poliomyelitis suggested that the virus entered the nose, replicated in the nasal epithelium, and spread to the brain through the olfactory pathway [93]. Experimental findings in humans and monkeys subsequently proved that poliovirus does not invade the central nervous system by the olfactory route [87]. In some cases, viral replication in the nasal mucosa may lead to a viremia that allows virus spread and entry into the central nervous system. Although poliovirus is believed to be transmitted by fecal-oral contamination, virus may be transmitted by the respiratory route in countries with high standards of hygiene. The tonsils and pharynx are the sources of virus for this transmission mode. Replication at these sites usually occurs after virus replication in the intestine and spreads by viremia. It is not known if virus spread by the respiratory route replicates in the nasopharynx or is ingested and replicates in the intestine. The study of nasal infection of CD155 transgenic mice with poliovirus may address some of these questions.

Spread in the host

Two routes of poliovirus entry into the central nervous system have been proposed: the virus enters the central nervous system from the blood or enters a peripheral nerve and is carried to the central nervous system by axonal transport (Figure 1.3). There is evidence to support both routes of entry. It has been established that viremia preceding paralytic infection is necessary for virus entry into the central nervous system. In addition, the presence of antiviral antibodies in the blood prevent invasion of the brain and spinal cord [68]. The results of experiments in CD155 transgenic mice provide additional support for the hypothesis that virus enters the brain and spinal cord from the blood. In one study of the fate of poliovirus inoculated into the tail vein of mice, it was observed that poliovirus is delivered to the brain at higher levels than expected based on the vascular volume of the organ [94]. Furthermore, the distribution of poliovirus in the brain of transgenic and nontransgenic mice is similar, indicating that CD155 does not play a role in delivering circulating poliovirus to the central nervous system. The authors conclude that in mice, polioviruses permeate the blood-brain barrier at a high rate, independent of CD155 or virus strain. The molecular mechanism of poliovirus entry by this route remains to be determined.

Poliovirus infections in humans and monkeys provide evidence for neural pathways of poliovirus dissemination. When poliovirus is inoculated into the sciatic nerve of monkeys, virus spreads along nerve fibers in both peripheral nerves and the spinal cord [95]. After intramuscular inoculation of monkeys with poliovirus, the inoculated limb is usually the first to become paralyzed, and freezing the sciatic nerve blocks virus spread to the spinal cord [96]. Children who received incompletely inactivated poliovaccine in 1954 (the Cutter incident) developed a high frequency of initial paralysis in the inoculated limb [97]. Evidence for neuronal spread of poliovirus has also been obtained from experiments in CD155 transgenic mice. After intramuscular inoculation, the first limb paralyzed is the inoculated limb; poliovirus is first detected in the lower spinal cord, and sciatic nerve transection blocks infection of the spinal cord [98,99]. The rate of poliovirus transport along the sciatic nerve was determined to be >12 cm per day, independent of virus replication [99]. This rate is consistent with fast retrograde axonal transport of the virus.

Skeletal muscle injury is known to be a predisposing factor for poliomyelitis, a phenomenon known as “provocation poliomyelitis.” In Oman, intramuscular injections have been linked to cases of vaccine-associated poliomyelitis [100]. Provocation

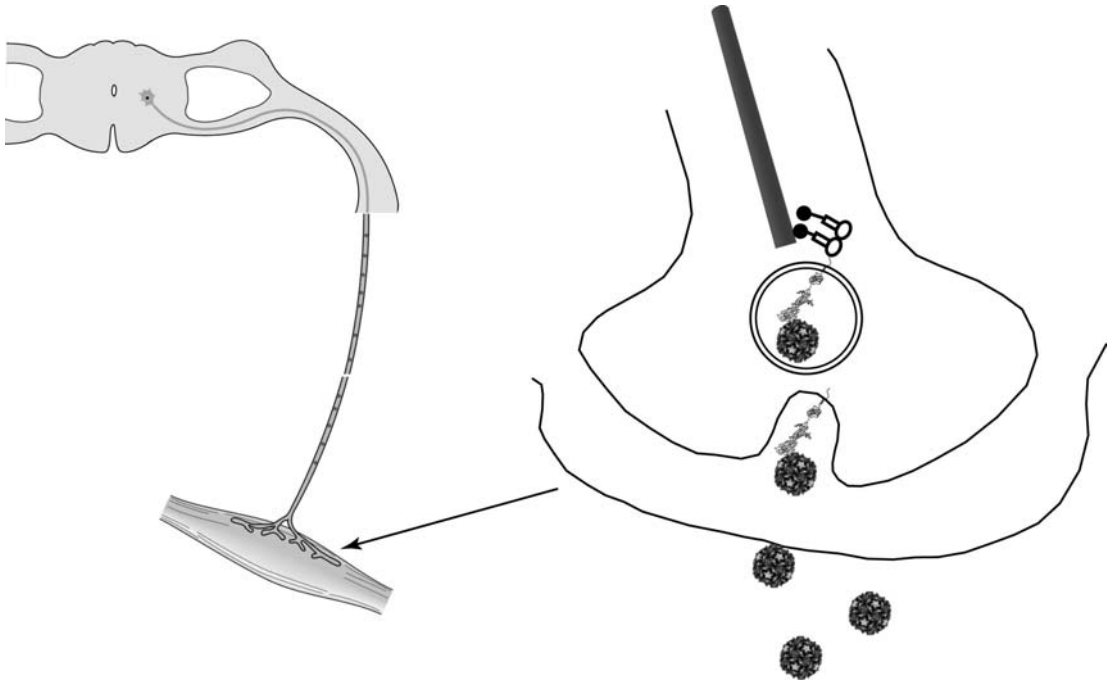

Figure 1.4. Hypothetical mechanism of poliovirus axonal transport. Virus particles are transported to the muscle via the blood. At the neuromuscular junction, virus binds to its receptor, CD155, at the presynaptic membrane and is taken into the cell by endocytosis. The cytoplasmic domain of CD155 interacts with Tctex-1, a component of the dynein motor, allowing transport of the endocytic vesicle containing poliovirus to the cell body of the neuron. Viral RNA is released in the cytoplasm of the neuron cell body, initiating the viral replication cycle.

poliomyelitis has been reproduced in CD155 transgenic mice [101]. It is not yet understood how skeletal muscle injury stimulates retrograde axonal transport of poliovirus to the spinal cord.

The observation that the cytoplasmic domain of CD155 interacts with Tctex-1, the light chain of the retrograde motor complex dynein [102,103], suggests a hypothesis for the mechanism of axonal transport of poliovirus (Figure 1.4). At the muscle and motor neuron interface, the neuromuscular junction, poliovirus binds CD155 and enters the neuron by endocytosis. The endocytic vesicles containing poliovirus are linked to Tctex-1 by the cytoplasmic domain of CD155, which remains on the exterior of the endocytic vesicle. Virus-containing vesicles are transported to the motor neuron cell body, where the viral RNA is released into the cytoplasm and virus replication begins. In support of this hypothesis, CD155 has been detected at the neuromuscular junction of human muscle [104], and it has been shown that poliovirus-containing vesicles are brought to the spinal cord by axonal transport dependent upon Tctex-1 [103]. Poliovirus appears to be transported in axonal endosomes as an infectious, 160S particle. This hypothetical scheme contrasts with virus entry in HeLa cells, where interaction of poliovirus with CD155 leads to conversion of the virus to A particles, which are believed to be intermediates in uncoating [105]. Viral uncoating in axons may be inhibited to avoid degradation of viral RNA before it reaches the neuron cell body. The proposed uptake of poliovirus at the neuromuscular junction would also differ from the process in HeLa cells, where infection does not require dynamin and is

unlikely to involve the clathrin-mediated endocytic pathway [106].

Tropism

Poliovirus replicates only in specific cells and tissues in primates, even though the virus reaches many organs during the viremic phase [69,70]. The proposition that poliovirus tropism is determined by the cellular receptor was supported by the finding that virus-binding activity in tissue homogenates correlated with susceptibility to poliovirus infection [107]. Identification of the poliovirus receptor allowed more extensive study of the role of this molecule in tropism. In humans, CD155 RNA and protein are expressed in many tissues, but not all are sites of poliovirus infection [15,24,108]. CD155 RNA and protein expression are also observed in many tissues of CD155 transgenic mice, including those where poliovirus does not replicate [72,109]. These findings indicate that CD155 is required for susceptibility to poliovirus infection, but tropism is determined at a later stage of infection.

It has also been suggested that poliovirus tropism is controlled by cell-type specific differences in IRES-mediated translation [110,112,113]. Organ-specific synthesis, localization, or modification of cell proteins needed for IRES-mediated translation could control viral replication. When recombinant adenoviruses were used to express bicistronic mRNAs in murine organs, the IRES of poliovirus was found to mediate translation in many organs, including those that are not sites of poliovirus replication [114]. These results indicate that poliovirus tropism is not determined by internal ribosome entry but at a later stage in replication.

The interferon (IFN) response appears to be an important determinant of poliovirus tissue tropism. IFN is part of the innate immune system, which can respond to the presence of virus within hours and has a major influence on the outcome of infection. The tropism of diverse viruses is regulated by alpha/beta IFN (IFNα/β) [115,116]. Poliovirus infection of CD155 transgenic mice lacking the receptor for IFNα/β leads to viral replication in liver, spleen, and pancreas, in addition to the central nervous system [117]. CD155 is produced in all of these tissues, but poliovirus only replicates in the brain and spinal cord of CD155 transgenic mice that synthesize the IFNα/β receptor. In CD155 transgenic mice, poliovirus infection leads to a rapid and robust expression of IFN-stimulated genes (ISGs) (oligoadenylate synthetase, PKR, IFNβ, RIG-I, MDA-5, and IRF-7) in extraneural tissues that are not normally poliovirus replication sites. In the brain and spinal cord, ISG expression was only moderately increased after infection. These results indicate that IFNα/β functions as an important determinant of poliovirus tissue tropism in CD155 transgenic mice by protecting extraneural organs from infection.

The ability of IFNα/β to determine poliovirus tissue tropism suggests that this cytokine might help determine whether the virus invades the central nervous system. As discussed earlier, poliovirus replication at the entry portal leads to a viremia, which allows virus to reach an unidentified extraneural site. Replication at this site appears to be required for the virus to enter the central nervous system. We speculate that in 99% of infections, the IFNα/β response limits poliovirus replication in extraneural tissues, thereby preventing invasion of the central nervous system. In the 1–2% of individuals in which paralytic disease occurs, the IFN response may be defective, allowing unchecked virus replication in nonneural sites followed by central nervous system invasion.

Other enteroviruses

Most enterovirus infections are asymptomatic, but these viruses can also cause a wide range of clinically distinct syndromes [118]. Neurological complications of enterovirus infection include acute flaccid paralysis (similar to the disease caused by poliovirus), aseptic meningitis, meningoencephalitis, and encephalitis. In the United States, enteroviruses are the most common cause of aseptic meningitis and an important cause of encephalitis [119]. In general, the pathogenesis of enterovirus infection is believed to mimic that of poliovirus,

i.e., spread by fecal-oral contamination, multiplication in the alimentary tract, and spread by the blood and axonal transport to target organs.

Coxsackievirus was first isolated during an outbreak of poliomyelitis in 1948 in the town of Coxsackie, NY, by inoculating suckling mice with fecal extracts from children with paralytic disease [120]. The mice developed myositis with flaccid hind limb paralysis. The isolate was subsequently classified as a group A coxsackievirus (CVA). Group B coxsackieviruses were identified the next year; these viruses produce spastic paralysis in newborn mice and infect many organs, including brain, pancreas, heart, and brown fat. Neurological consequences of coxsackievirus infections in humans include aseptic meningitis and paralysis. Though mouse models for coxsackievirus infection of the central nervous system are available, little research has been done to understand the pathogenesis of this disease. Most research on coxsackieviruses has focused on infection of the heart and pancreas.

Several years after the isolation of coxsackieviruses, new viruses were isolated from healthy children and from the stools of patients with aseptic meningitis. The new viruses were called enteric cytopathogenic human orphan (echo) viruses, because it was initially not clear if they caused disease. Echoviruses are now the leading causes of acute febrile illness in young children and infants. These viruses are the most frequent cause of aseptic meningitis where mumps has been eliminated, perhaps causing as many as 75 000 cases a year in the United States [121]. Echovirus infections may also result in paralysis, encephalitis, neonatal sepsis, exanthema, respiratory disease, myalgia, and myocarditis. There may be more than 2000 deaths from echovirus neonatal sepsis in the United States alone each year. Most echovirus infections are subclinical. When symptoms are present, they are not associated with specific serotypes. Transplacental transmission of virus from an infected mother may lead to a severe disseminated infection including meningitis, meningoencephalitis, myocarditis, and hepatitis. Immunocompromised patients may develop chronic echovirus infections that include meningoencephalitis. Despite the medical importance of echoviruses, nearly nothing is known about how they cause disease. Most echovirus infections are limited to humans, and the lack of an animal model has hindered the study of echovirus pathogenesis. Because echovirus infections have such a heterogeneous clinical picture, it is assumed that many other target organs are involved, but this problem has not been studied. In one of the few studies on echovirus neurovirulence, the sequence of the 5′-noncoding region of seven echovirus serotypes was determined [122]. Five of the serotypes are associated with aseptic meningitis; in the genome of these isolates, the sequence of stem-loop VI was similar to that of wild-type poliovirus. In the poliovirus 5′ UTR, this stem-loop harbors determinants of attenuation. In contrast, the sequence in this region was diverged from that of poliovirus in the genome of two isolates that do not cause neurological disease. The results suggest that neurovirulence of these echovirus serotypes might be controlled by sequences within the 5′-noncoding region.

A mouse model for echovirus type 1 infection has been developed by producing transgenic mice that synthesize the cell receptor for echovirus type 1, the integrin $\alpha 2\beta 1$ [123]. Intracerebral infection of neonatal $\alpha 2\beta 1$ transgenic mice with EV1 leads to spastic hind limb paralysis similar to that observed in mice infected with coxsackievirus B. Consistent with this clinical picture, neuronal damage in the motor cortices is observed in paralyzed transgenic mice. In contrast to infection of neonates, intracerebral inoculation of 6–8-week-old mice did not lead to neuropathology or paralysis. Epidemiological studies have shown that infants and agammaglobulinemic patients are more susceptible to central nervous system disease caused by echoviruses [124]. Immune status and decreased susceptibility of neurons in older mice may determine protection from central nervous system disease. A transgenic mouse model for echovirus pathogenesis allows studies that further our understanding of the central nervous system disease caused by the virus.

Approximately 30 new enterovirus serotypes have recently been identified, bringing the number of

serotypes to more than 100 [125,126,127]. Serological survey results suggest that most of these enteroviruses have probably been circulating in the human population for decades. Very little biological, clinical, or epidemiological information is available for most of these serotypes, but some have been associated with paralytic disease.

Two enteroviruses, enterovirus type 70 (EV70) and coxsackievirus A24 (CAV24), are the etiologic agents of acute hemorrhagic conjunctivitis (AHC), a new type of viral conjunctivitis first reported in Ghana in 1969 [128]. During the first AHC pandemic caused by EV70, from 1969 to 1971, approximately 20 million people were infected. The infection spread as a pandemic across western and central Africa to the Middle East and other parts of Asia [129,130]. In 1981, EV70 spread from Kenya to the Western Hemisphere and was reported in South America, Central America, the Caribbean islands, and the United States. CAV24 was first isolated from cases of AHC in 1970 in Singapore [131,132] and subsequently spread to the Western Hemisphere [133]. AHC epidemics continue throughout the world. EV70 might have originated from an insect or animal picornavirus because anti-EV70 antibodies were detected in sera from animals in Ghana when the disease emerged.

EV70 and CAV24 are unusual enteroviruses because the disease that they cause, AHC, is not initiated through the alimentary tract. AHC begins when virus is introduced directly into the eye by contact with an infected eye or eye secretions. An incubation period of 24 to 48 hours is followed by rapid onset of ocular pain, swelling of the eyelids, excessive tearing, pain, and subconjunctival hemorrhage. The disease usually resolves in three to seven days. The virus preferentially replicates in conjunctival and corneal cells, and in rare cases (1 in 10 000 for EV70 [134]) infection leads to a polio-like paralysis. These symptoms develop several weeks after the onset of AHC and are accompanied by antibodies against EV70 or CAV24 in the cerebrospinal fluid (CSF). Unlike paralysis caused by poliovirus, this complication is transient and involves both acute flaccid paralysis of the lower limbs and cranial nerve palsies. Neither EV70 nor CAV24 has been associated with paralysis in the absence of AHC. How the virus spreads from the eye to the central nervous system is not known. The lack of a suitable animal model has hindered our understanding of this disease.

Enterovirus type 71 (EV71) was first isolated in 1969 from a case of fatal human encephalitis in California. The virus has subsequently caused outbreaks worldwide, including a large epidemic in Taiwan in 1998 with 405 cases and 78 fatalities. The virus is distributed worldwide and is spread by fecal-oral and possibly respiratory droplet transmission. EV71 infection may cause hand-foot-and-mouth disease in children, a febrile syndrome accompanied by flat or raised red spots in the tongue, gums, inside of cheeks, palms, soles of feet, and buttocks. Neurological complications of EV71 infection include aseptic meningitis, encephalitis, and poliomyelitis-like polyneuritis. In some infections neurological symptoms occur without previous rash. A study of the Taiwan epidemic of 1998 revealed an unusual neurologic manifestation, brain stem encephalitis [135], which was also observed in Malaysia in 1997 but not in earlier epidemics. It has been suggested that the changes in EV71 disease might be a consequence of the emergence of a more virulent viral strain.

Infection of cynomolgous monkeys has provided some information on the pathogenesis of EV71 infection [136]. After intracranial or intravenous inoculation of monkeys, the virus is distributed widely in the central nervous system, and in addition to paralysis extrapyramidal signs also occur, such as tremor and ataxia. After intraspinal inoculation, the monkeys develop flaccid paralysis. Mouse-adapted strains of EV71 have been isolated that are susceptible to infection by intramuscular, intracerebral, and oral routes of inoculation. After oral inoculation of mice, EV71 replicates in the intestine, establishes a viremia, then spreads to the spinal cord where it replicates and causes flaccid paralysis [137]. Studies in mice show that invasion of the central nervous system likely occurs both from the blood and via axonal transport [137].

EV71 is emerging in some locations as the most significant neurotropic enterovirus [138]. It has been suggested that the incidence of EV71 might increase

after the eradication of poliovirus, in which case there will be increased efforts to develop vaccines and antivirals against this agent.

The parechovirus genus consists of four viral serotypes, two of which were once classified as echovirus types 22 and 23 [139]. Phylogenetic analysis of the viral genomes revealed that these echoviruses are distinct from other picornaviruses, leading to their reclassification. Parechovirus infections are usually associated with mild gastrointestinal and respiratory symptoms in young children, but more severe disease, such as flaccid paralysis and encephalitis, has been reported [139].

Porcine teschoviruses (PTV) were previously classified in the genus enterovirus and were called porcine enteroviruses (PEVs). Two strains of PEV serotype 1 were subsequently shown to be genetically distinct from other picornaviruses and were reclassified in the teschovirus genus [140]. Although infection with PTV is most frequently asymptomatic, some strains may cause clinical disease. Two strains of PTV, the Teschen and Talfan strains, were isolated during serious outbreaks of polioencephalitis in pigs in Europe in 1929 and 1957 [141,142]. The pathogenesis of porcine polioencephalitis caused by PTV is poorly understood.

Cardiovirus disease

Columbia SK virus, the first member of the encephalomyocarditis group isolated in 1940 from cotton rats, was shown to cause flaccid hind limb paralysis in mice (reviewed in [143]). Other related viruses were subsequently isolated, including EMCV in 1945, mengovirus in 1948 (from the cerebrospinal fluid of a paralyzed rhesus macaque), and Maus-Elberfeld virus in 1949. Infection of laboratory animals with these viruses has served as a model for human diseases of the central nervous system.

Rodents, particularly rats, are believed to be the natural host and reservoir of EMCV [143]. The virus has also been detected in domestic and wild mammals, birds, and humans. In these hosts the virus usually causes persistent infections in the absence of disease. Infection of laboratory mice, however, leads to fatal encephalitis or myocarditis. The E variant of EMCV, which was obtained from a naturally infected domestic pig, is highly neurotropic in mice [144]. EMCV has recently been shown to be a pathogen of domestic animals, particularly swine. In pigs, infection results in either myocarditis or reproductive failure in sows. Clinical outbreaks have been reported in zoological parks involving infection of elephants, artiodactyls, marsupials, mongooses, porcupines, and primates. In these animals, infection may lead to sudden death from acute myocarditis.

Humans appear to be susceptible to EMCV infection, but reports of disease are rare [145]. Serological surveys indicate that infection with EMCV is common in certain parts of the world but frequently asymptomatic. When symptoms are present, they usually consist of fever, neck stiffness, lethargy, delirium, and headache. Human cases typically occur in areas with a high incidence of EMCV in pigs.

When inoculated intraperitoneally or intracerebrally into mice, EMCV and mengovirus cause lethal meningoencephalomyelitis [146,147]. When the poly(C) tract of mengovirus is shortened from 50 to 8 nucleotides, the resulting virus is neuroattenuated in mice, despite normal replication in cell culture [148]. The mechanism by which the poly(C) tract regulates mengovirus neurovirulence is not known. However, the effect depends upon the viral genomic context because EMCV with a shortened poly(C) tract is only slightly less virulent in mice than the wild-type virus and is also as virulent as the parental virus in mice, pigs, and cynomolgous macaques [149].

The second group of cardioviruses includes the Theiler's murine encephalomyelitis viruses (TMEV). This virus was first isolated in 1934 by Theiler from laboratory mice with spontaneous paralysis [150] and is now common in mice that are not housed under barrier conditions. In nature, the virus commonly infects *Mus musculus*; in this species, it is transmitted by the fecal-oral route and usually causes an inapparent infection of the gut. Occasionally, the virus may invade the central nervous

system, where it replicates and causes flaccid hind limb paralysis [151,152]. The Theiler's viruses are divided into two subgroups, GDVII and Theiler's original (TO) based on their neurovirulence in mice after intracerebral inoculation (reviewed in [153]). Members of the GDVII group are highly neurovirulent and induce fatal polioencephalomyelitis. Members of the second group, TO, cause a biphasic disease in which the early phase consists of a milder encephalomyelitis followed by virus persistence, mononuclear cell inflammation, and demyelination. The demyelination observed during multiple sclerosis is similar, and therefore TMEV infection of mice has been used as a model for studying this human disease [154].

When mice are orally inoculated with TMEV, the virus replicates in the pharynx or lower gastrointestinal tract and spreads to the blood via the local and regional lymph nodes. The virus then enters the central nervous system where it replicates in neurons and causes flaccid paralysis. Viral invasion of the central nervous system is likely to occur when the virus traverses cerebral capillaries or by axonal transport from peripheral sites. In experimental infections, virus is usually administered by intracerebral or peripheral routes, which results in more efficient induction of disease. GDVII strains primarily infect neurons in the gray matter of the spinal cord while TO strains infect neurons of the gray matter during the acute phase and glial cells and macrophages during the chronic phase. The basis for these differences is not known. It has been suggested that sialic acid and heparan sulfate coreceptors are major determinants of which cells become infected in the central nervous system [155].

Results of infection of mice with recombinant viruses have shown that viral persistence and demyelination depend upon capsid proteins, in particular VP1 and VP2 [156]. Two proteins encoded at the N-terminus of the viral polyprotein, L and L*, also appear to play a role in the disease outcome of infection [153]. The L protein of TMEV is 76 amino acids long and has been shown to inhibit transcription of IFNα/β [157]. Viruses lacking L replicate poorly in cells that produce IFN but not in cells that do not produce IFN [158,159]. However, the L protein is not sufficient to allow TMEV to evade host defenses because TO strains are cleared from mice when L protein is produced.

The 17–18 kDa L* protein is produced by translation at an AUG codon downstream of the polyprotein initiator, in a different open reading frame. This protein is only produced by TO subgroup strains because GDVII strains have an ACG instead of an AUG at the start of the L* coding region. TO strains replicate in macrophages, but DGVII strains do not, a property that depends upon the L* protein [160]. In mice, TO subgroup strains that do not produce L* cause early polioencephalitis but do not persist in the spinal cord and cause minimal demyelination or inflammation [161]. These observations indicate that L* is a key determinant of TMEV persistence. It has been suggested that the L protein allows the virus to escape host innate defenses early in infection. Viruses that remain can persist in macrophages due to the action of the L* protein. Persistence of the viral genome in macrophages could then lead to immune-mediated demyelination [153]. Precisely how L* regulates the pathogenesis of TMEV infection remains to be determined.

Vaccines and antivirals

Two highly effective vaccines to prevent poliomyelitis were developed in the 1950s. Inactivated poliovaccine (IPV), developed by Jonas Salk, consists of wild-type poliovirus strains treated with formalin to destroy infectivity without altering the antigenic properties of the capsid. This vaccine was licensed in 1955 in the United States and reduced the number of cases of paralytic disease from about 20 000 per year to about 2000. Despite this success, it was debated whether a nonreplicating vaccine such as IPV could eradicate the disease. Consequently, the live, attenuated oral poliovaccine, OPV, developed by Albert Sabin, was licensed in the 1960s [162]. OPV has been shown to interrupt epidemics and break

transmission of the virus, leading to elimination of the virus from entire continents by the 1980s. These vaccine strains were selected by the World Health Organization (WHO) for the campaign to eradicate global poliomyelitis by 2000 [163]. When the eradication initiative began, wild poliovirus was endemic in over 125 countries, and over 350 000 cases of polio were reported annually. In early 2007, polio is endemic in only four countries: Nigeria, India, Pakistan, and Afghanistan. Political instabilities, armed conflicts, and other complex social challenges impede immunization initiatives in these countries. As discussed below, circulating vaccine-derived polioviruses have further confounded the eradication effort.

The Sabin vaccine strains were empirically selected to be able to infect the alimentary tract and produce immunity to infection without inducing poliomyelitis. Genetic analysis has shown that a point mutation within the IRES of each of the three poliovirus vaccine strains is a determinant of the attenuation phenotype [164,165,166]. For example, a mutation from C to U at nucleotide 472 in the IRES of poliovirus type 3 attenuates neurovirulence in primate and murine models [165,167,168]. This mutation has been shown to cause a translation defect in vitro and in cultured cells of neuronal origin [169,170,171]. A hypothesis for how the C472U mutation leads to reduced neurovirulence is that it causes a translation defect that is specific to the brain and spinal cord, leading to lower viral replication in these organs [110,169,172]. This hypothesis was tested by examining IRES-mediated translation in mouse organs and cells. The results show that the C472U mutation leads to translation defects in neuronal and nonneuronal cells and tissues [114] and therefore cannot attenuate neurovirulence by specifically reducing translation in neuronal cells. Furthermore, polioviruses with the C472U mutation are attenuated in adult CD155 transgenic mice but cause paralytic disease in newborn mice [114]. These observations lead to the conclusion that the C472U mutation does not eliminate viral replication in the brain. Alternatively, the C472U mutation could reduce viral replication in the alimentary tract enough to prevent spread to the central nervous system without impairing immunogenicity of the vaccine. Because they replicate more poorly than wild-type virus, the vaccine strains may be more effectively limited by the IFNα/β response.

Immunization with the Sabin vaccine strains is associated with a low rate of vaccine-associated poliomyelitis, either in vaccine recipients or their immediate contacts. The rate of vaccine-associated paralysis in primary vaccines is approximately 1 per 750 000 recipients [173]. Vaccine-associated poliomyelitis occurs due to reversion of the mutations in the viral genome that confer the attenuation phenotype. For example, a reversion from U to C at nucleotide 472 is observed in virus isolated from cases of vaccine-associated poliomyelitis caused by Sabin type 3 [165]. Because the Sabin strains undergo reversion in the gastrointestinal tract of nearly all recipients [174], it is surprising that the frequency of vaccine-associated paralysis is so low. Perhaps replication of the Sabin strains is sufficiently delayed to allow containment by the immune response. The individuals who contract vaccine-associated poliomyelitis might have a defective IFNα/β response that allows revertant viruses to replicate to high levels in extraneural tissues, invade the central nervous system, and cause paralytic disease.

Circulating vaccine-derived polioviruses (cVDPV) challenge eradication because they can spread from person to person and cause poliomyelitis. The first outbreak caused by cVDPV was in Hispaniola in 2001, where 22 cases of poliomyelitis were identified [175]. The responsible strain was a type 1 cVDPV strain that had been circulating undetected for 2 years. Subsequent outbreaks of poliomyelitis caused by cVDPVs occurred in the Philippines (2001), Madagascar (2002 and 2005), China (2004), and Indonesia (2005) [176,177]. It has also been reported that all cases of poliomyelitis occurring in Egypt between 1988–1993 were caused by vaccine-derived strains [178]. These outbreaks call into question the WHO plan to stop immunization after polio eradication is achieved. In the postimmunization

world, cVDPV strains will continue to circulate, posing a threat to the increasingly nonimmune population.

One reason polio was believed to be eradicable was the belief that there are no chronic human carriers or nonhuman reservoirs of the virus. This notion was dispelled by the discovery of prolonged excretion of VDPV strains from individuals with defects in humoral immunity [179]. To date, 21 such cases have been identified globally with shedding of VDPV for months to years. In one remarkable case, the patient shed virus for over 20 years but remained healthy, although the excreted virus was shown to be neurovirulent in animals [180]. In contrast, individuals with normal immune systems shed vaccine viruses for approximately 4 weeks. The incidence of long-term poliovirus shedding among patients with immunodeficiencies is unknown, but their existence is another obstacle to the eradication program. As long as these individuals shed VDPVs, it will be difficult to stop immunization against poliomyelitis.

Antiviral compounds have not played a role in controlling picornavirus infections; none have been licensed for human use. However, one class of antipicornavirus drugs has helped elucidate mechanisms of virus entry into cells. These are the WIN compounds, originally produced by Sterling-Winthrop [181]. Similar molecules have been produced by Schering-Plough (Kenilworth, NJ) and Janssen Pharmaceuticals (Titusville, NJ) [182,183]. The compounds bind tightly in a hydrophobic tunnel located within the core of VP1 just beneath the canyon floor of many picornaviruses. In poliovirus types 1 and 3, the pocket appears to contain sphingosine [184], a C16 fatty acid has been modeled in the pocket of coxsackievirus B3 [185,186,187], and coxsackievirus A21 may carry myristic acid [188]. These hydrophobic, sausage-shaped compounds displace the lipid by binding tightly in the hydrophobic tunnel. When a WIN compound is bound to the poliovirus capsid, the virus can bind to cells, but the interaction with Pvr does not lead to the production of A particles [189,190]. WIN compounds block poliovirus infectivity by preventing Pvr-mediated conformational alterations needed for uncoating. Poliovirus mutants have been isolated that are not infectious unless WIN compounds are present [191]. Such WIN-dependent mutants spontaneously convert to altered particles at 37°C, probably because there is no lipid in the hydrophobic pocket to stabilize the particles. It is believed that docking of Pvr onto the poliovirus capsid just above the hydrophobic pocket initiates structural changes in the virion that lead to the release of the lipid.

Some of these drugs have been evaluated in clinical trials, such as pleconaril for treatment of common colds caused by rhinoviruses [192]. The problem of resistance has prevented licensing of these compounds: picornaviruses that are not inhibited by the drugs are readily isolated. This problem could in theory be partially addressed by using three antiviral drugs in combination, an approach that has been successful in controlling infections with human immunodeficiency virus [193]. Because picornavirus infections are generally short lived and the virus must be identified by laboratory diagnosis, by the time an appropriate antiviral could be prescribed, it would have little effect on the outcome. Recently a committee appointed by the U.S. National Research Council recommended that antipoliovirus drugs be developed in case of a posteradication outbreak. Assuming multiple drugs could be administered to overcome resistance, antiviral therapy might be effective to prevent spread of the virus during an outbreak.

A novel approach to antiviral therapy is based on the observation that mutations in the region of the poliovirus genome encoding the capsid proteins, VPg, $2A^{pro}$, and the RNA polymerase, result in dominant negative phenotypes [194]. If antiviral drugs that produce dominant negative proteins could be identified, the replication of drug-resistant genomes could be inhibited by the drug-sensitive genomes. For example, when cells were coinfected with wild-type and WIN-resistant polioviruses, the yield of WIN-resistant virus was reduced to 3–7% of the yield of a single infection. The results suggest that inhibition of virus yields occurs because chimeric capsids consisting of subunits from wild-type and

WIN-resistant genomes are sensitive to the drug. Hence, in the presence of WIN compound, the wild-type capsid subunits display a dominant negative phenotype.

Unanswered questions

If poliomyelitis eradication succeeds, shortly afterwards it will be necessary to halt work with virulent strains of poliovirus, a step that will severely curtail research on the pathogenesis of poliomyelitis. There are many unanswered questions about poliomyelitis, and all the necessary experimental tools are available, but it is not clear whether there will be sufficient time to carry out this work. Fundamental problems include the identity of cells infected by poliovirus in the alimentary tract and the route of virus spread from the intestine to the central nervous system. The mechanism of poliovirus axonal transport remains to be elucidated: how is the virus maintained as a 160S particle in the endosome and subsequently uncoated in the neuron cell body? Although it is clear that the IFNα/β response determines poliovirus tropism, it is not known why ISG expression is limited in the central nervous system. Why is viral replication in mice regulated by IFNα/β when the virus inhibits many important cellular processes, including translation, transcription, and protein secretion? How are neurons destroyed during infection – by the virus, by the immune response, or both?

For nearly 100 years, poliovirus has been the best-studied picornavirus, first as the etiologic agent of a significant human disease then as a model for RNA virus infections of the central nervous system. Perhaps once research on poliovirus ceases, attention will turn to understanding how other picornaviruses infect the central nervous system. Because of a lack of research focus, nearly nothing is known about the pathogenesis of neurological disease caused by cardioviruses and enteroviruses – there is no understanding of initial replication sites, mechanisms of transport to the central nervous system, or the role of specific viral proteins in neurotropism. Mouse models of infection are available to unravel many of these fundamental problems. Which picornavirus will emerge to replace poliovirus as a model system?

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2

Subacute sclerosing panencephalitis

James F. Bale, Jr. and Robert S. Fujinami

Introduction

Although vaccinations against the measles virus have nearly eliminated subacute sclerosing panencephalitis (SSPE) and other measles virus-induced neurologic disorders in countries with compulsory immunization programs, SSPE remains a rare, fatal neurodegenerative disorder in many regions of the world. This chapter summarizes current information regarding the epidemiology, virology, clinical manifestations, diagnosis, and management of SSPE.

Epidemiology

Measles

Due to the relatedness (sequence homology) of measles virus with rinderpest virus [1], whose natural hosts are cattle, goats, sheep, antelopes, and other cloven-hoofed animals, some hypothesize that the measles virus originated from the rinderpest virus or a common predecessor. As humans first domesticated such animals in the Fertile Crescent of the Middle East, they would have been in close contact with rinderpest virus [2]. Many morbilliviruses – of which measles and rinderpest viruses are members – can cross species. Recent examples of this phenomenon are the Nipah and Hendra viruses; their natural host is the fruit bat, but both viruses can also infect pigs and humans [3,4].

Although incursions of the measles virus or a measles-like virus into human populations likely occurred with regularity in ancient times, measles did not emerge as a human disease entity until urban areas of several hundred thousand came into existence about 2500 years ago [5]. Measles virus entered human populations quickly; the virus genome stabilized, and measles virus adapted to replicating efficiently in human cells and transmitting among humans [6]. Measles was appreciated as a disease distinct from smallpox around the tenth century [7].

Prior to the measles virus vaccine and the implementation of vaccination programs, measles occurred worldwide, often in major epidemics in the winter and spring every 2 to 4 years [8,9]. These epidemics historically produced considerable mortality among populations, such as the Native Americans, the First Nations of Canada, and Pacific Islanders, who were not previously exposed to the the measles virus. The native Hawaiian population, for example, declined from more than 250 000 to less than 90 000 within 70 years of the arrival of Captain James Cook and the first Europeans, a decline attributable in large measure to introduction of the measles virus [10].

Before 1969, when the first vaccine was licensed for use, approximately 500 000 persons acquired measles annually in the United States; many millions more were affected worldwide. Most of the infected were young children, and by the age of 15 years, 90% of the population had experienced measles virus infection [11]. Measles virus, a highly contagious agent, spreads among humans via respiratory secretions, especially during the catarrhal stage prior to

the onset of the rash. Humans serve as the only current reservoir of the measles virus.

A single dose of the measles virus vaccine reduced the numbers of measles cases in the U.S. substantially. By the mid-1980s, the Centers for Disease Control and Prevention (CDC) was receiving reports of <4000 cases annually. In 1989–1991, however, the United States experienced a major measles outbreak, causing nearly 60 000 cases and approximately 125 measles-related deaths [9]. This resurgence was attributed, in part, to major outbreaks of measles among unvaccinated Hispanic and black children in Milwaukee, Dallas, Houston, Los Angeles, and New York [12].

These cases, as well as cases of measles among previously vaccinated children, led to the current recommendation that a second vaccination be provided in late childhood or early adolescence. This strategy brought further declines in the incidence of measles in the United States. By the late 1990s, the U.S. had achieved a >99% reduction in the incidence of measles [9]. In 2005, fewer than 70 cases of measles were reported to the CDC [9,13]; nearly half of these were linked to a single outbreak [13]. Imported cases currently account for a substantial proportion of the small numbers of U.S. measles cases [13].

By contrast, measles remains a major source of morbidity and mortality in many parts of the world, especially in South Asia and sub-Saharan Africa [14]. Consequently, the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) have implemented a massive campaign toward >90% vaccination coverage in all districts by 2010 [15]. In 1999, measles accounted for an estimated >800 000 deaths worldwide; by 2004, this number had fallen to approximately 500 000 [14].

Subacute sclerosing panencephalitis

The epidemiology of SSPE parallels that of measles virus infections. SSPE has virtually disappeared in countries, such as the United States, with compulsory measles vaccination [16,17,18]. Prior to the vaccination programs, SSPE affected approximately 3.5 of every 10 million persons under the age of 20 living in the United States [16]; between 1960 and 1976, 453 cases of SSPE were reported to the U.S. SSPE registry [17]. During this period, SSPE was more common among children living on farms, among Caucasian children, and in children living in the States of Kentucky, Arkansas, Georgia, Indiana, South Carolina, and Tennessee [16].

SSPE affects children between the ages of 5 and 15 years (median of 7 to 9 years in most studies), but onset in adulthood has been reported [6,19]. In virtually all reports, cases among boys outnumber those among girls by approximately 2.5:1. A history of wild measles virus infection is nearly always elicited, and approximately 50% of patients with SSPE experience measles before the age of 2 years [20]. The interval between measles virus infection and the onset of SSPE averages 6 to 10 years [8,17,18,20,21], although the interval can exceed 20 years in adult SSPE cases. Currently, most SSPE cases are being reported from Asia; fewer than 80 cases were reported in the U.S. between 1989 and 2004, reflecting the success of widespread vaccination of young children [21,22,23].

Although the role of wild measles virus infection in the pathogenesis of SSPE is undisputed, some controversy remains regarding the relationship between measles vaccine, an attenuated, live virus, and SSPE. The virtual disappearance of SSPE in the United States, for example, argues against a substantial role for measles vaccination in the occurrence of SSPE [24]. However, many studies, including some from the United States, suggest that the vaccination was the only known exposure to measles virus in some patients with SSPE [17,18]. The risk of SSPE after wild measles virus infection is approximately 1 per million cases of measles; the risk after vaccination is *at least* an order of magnitude less [18,24].

Virology

Although neuropathological specimens containing inclusion bodies long suggested a viral etiology for the disorder, the relationship of SSPE to paramyxovirus infection was not established until the 1960s. A causal role for measles virus in SSPE was supported

by the detection of high titers of measles virus antibody in cerebral spinal fluid (CSF) and by the identification of measles virus particles or antigens in brain tissue. Measles virus was eventually isolated from the brain tissues of SSPE patients [25,26,27], confirming that measles is the cause of SSPE.

Measles virus, an enveloped negative single-stranded RNA virus, was first isolated by Enders and Peebles in 1954 [28]. Measles belongs to the paramyxovirus family and the morbillivirus genus. Embedded within the measles virus envelope are two surface glycoproteins. The first, the 80 000-kD hemagglutinin, facilitates viral attachment to susceptible cells. In some instances. the virus uses CD46, a plasma complement inhibitor, as a receptor for binding to cells. CD46 is expressed on most human cells with the exception of red blood cells. Most wild-type measles viruses use signaling lymphocyte activation molecule (SLAM CD150) as a cellular receptor [29]. CD150 is found on activated lymphocytes, macrophages, and dendritic cells. The second glycoprotein, the fusion protein, is used by the virus to fuse the viral envelope with the host plasma membrane once attachment is achieved. Both the H and F proteins are necessary for fusion to occur. Cleavage of the fusion protein into F1 and F2 portions is required for fusion activity.

Besides these two glycoproteins, the virus encodes six other proteins. The M or membrane protein is associated with the inside of the viral and infected cell membrane. M protein is thought to help align nucleocapsids with the plasma membrane viral glycoproteins necessary for virus budding. The NP or nucleocapsid protein, the most abundant viral protein, surrounds the viral RNA. The NP and P, or phosphoprotein, and L, or polymerase protein, are complexed to the viral RNA to form the viral replication complex. Two minor proteins, the V and C proteins, are thought to be involved in inhibiting interferons, potent antiviral cytokines.

Measles virus replicates first in the respiratory tract, where draining lymph nodes soon become infected. As the virus replicates to higher and higher titers, it spills into the blood, initiating viremia. Measles virus then spreads hematogenously to other lymphoid tissues and organs, including the brain. Most of the circulating measles virus is associated with mononuclear cells. T cell immune responses are required for viral clearance. Once the virus is cleared, host immunity is generally lifelong.

On rare occasions (about 1 per 10^6 infected persons), measles virus persists in the central nervous system (CNS) and causes SSPE [20]. Measles virus in the CNS of patients with SSPE has mutations in various genes, particularly those encoding the M, H, and F proteins [30,31,32,33,34,35,36,37,38,39]. Viruses with mutations in the M protein gene can be found clonally expanded throughout the brain [40]. Such mutations lead to defective viruses that travel through the CNS as ribonucleoprotein complexes. It has been shown that these complexes can transit between neurons via synapse jumping [41].

Although the precise pathogenesis of SSPE has not been established, defective viruses and altered host responses play important roles. Due to viral persistence, high titers of antibodies are found in the circulation and CSF of individuals with SSPE. There have been reports that antibodies to M protein are decreased in some individuals with SSPE [42,43]. This could be due to the lack of or decreased amounts of M protein expressed in the CNS of SSPE patients. Measles virus-specific oligoclonal antibody bands can be found in the CSF of SSPE patients [44,45] due to intrathecal antibody production. Cytotoxic T lymphocyte (CTL) killing of measles virus-infected target cells was reported to be decreased in SSPE patients [46], suggesting a reduction in antimeasles virus CTL activity. Most reports suggest that SSPE patients have decreased skin test reactivity to common antigens, decreased proliferation to mitogens, and a lower reactivity to measles virus antigens [47,48, 49,50,51,52,53]. These defects could be the result of measles virus persistence in lymphocytes [54].

Clinical manifestations

After an incubation period that averages 10 days, measles begins with low-grade fever, cough, coryza and conjunctivitis [9,55]. Within 3 to 5 days of the

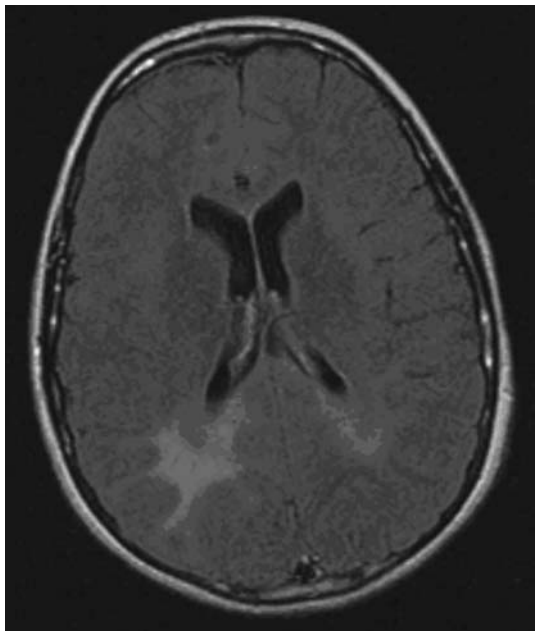

Figure 2.1. Axial FLAIR MRI obtained in the early stages (Stage IB) of SSPE. MRI shows T2 prolongation of the posterior white matter.

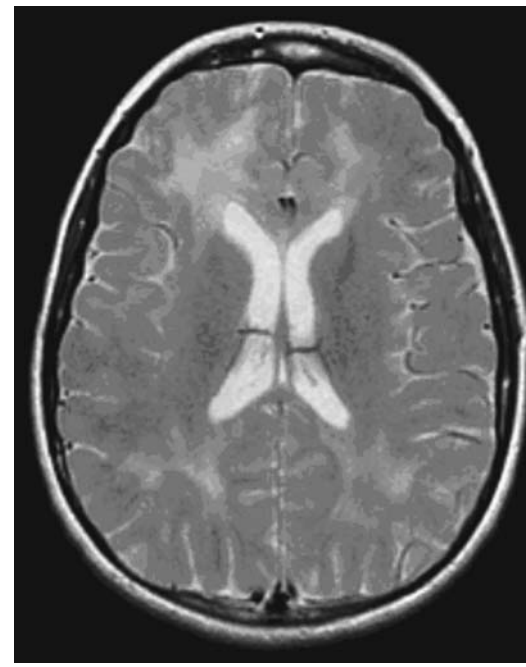

Figure 2.2. Subsequent T2-weighted MRI obtained during Stage IIB. MRI shows more extensive T2 prolongation within white matter, especially frontally, and subtle cortical atrophy.

onset of symptoms, Koplik's spots, grayish-white, sand-like dots, appear on the buccal mucosal adjacent to the lower molars, a pathognomonic feature of the infection. During the next day or two, the measles exanthem appears, first on the face, neck, and upper torso, then spreading over the entire body (Figures 2.1 and 2.2). The rash is commonly accompanied by high fever up to 40° C. The rash consists of erythematous, maculopapular lesions (hence the term "morbilliform") but can be petechial or purpuric, mimicking meningococcemia or Rocky Mountain spotted fever [55].

Severe cases of measles can be complicated by pneumonia, myocarditis, or acute encephalomyelitis [55,56]. Encephalomyelitis, a disorder affecting approximately 1 child per 1000 cases of measles, usually begins 2 to 5 days after the rash appears and within 8 to 10 days of disease onset. Clinical features of measles encephalomyelitis include headache, irritability, seizures, somnolence, or coma [56]; some children have ataxia, choreoathetosis, Guillain-Barré-like paralysis, or bladder or bowel dysfunction. Most patients with measles encephalomyelitis improve after 3 to 4 days, but others develop severe encephalomyelitis with increased intracranial pressure (ICP), focal neurologic signs, progressive seizures, and occasionally, death. The severity of the initial measles virus infection does not, however, predict a higher likelihood of experiencing SSPE.

Patients with disorders of cell-mediated immunity, either congenital or induced by immunosuppressive therapy [55,56,57,58,59], are at risk for a progressive, usually fatal neurodegenerative condition after measles infection or immunization. Because of the temporal profile, usually beginning 1 to 6 months after exposure to the measles virus, this disorder is distinct from acute encephalomyelitis and SSPE [57,58] and has been designated subacute measles

encephalopathy. Rarely, cases have occurred without a recent history of measles or measles virus vaccination [59]. Subacute measles encephalopathy begins gradually with incoordination, dementia, or seizures and progresses in typical cases to debility and coma. Death usually ensues, although patients may improve transiently during therapy with ribavirin [58].

SSPE, a disorder that arises from reactivated, latent measles virus infection, begins insidiously with behavioral alterations or intellectual declines. These features include irritability, emotional lability, or attention-deficit disorder-like behaviors that can be confused initially with psychiatric or behavioral conditions [60]. Cases can begin with visual symptoms, especially in the rare cases of adult onset [60,61,62,63,64]. Myoclonus ensues, although this can be subtle during early stages, consisting only of eye blinking or head nodding [24,60]. Later, myoclonus intensifies, involving the extremities, head, or trunk, and can be provoked by excitement or sensory stimuli. Generalized tonic-clonic or absence seizures can occur during this stage.

As SSPE progresses, myoclonus worsens, affecting gait and other motor activities, and speech, coordination, and intellectual function deteriorate. The myoclonus usually assumes a periodic character [24]. Choreoathetosis, bradykinesia, or rigidity may appear during this stage. Patients worsen and ultimately become completely debilitated with profound dementia, paralysis, and autonomic instability. Approximately 50% of patients with SSPE have chorioretinitis, optic atrophy, cortical blindness, nystagmus, or visual field deficits [65]. Occasionally, some patients have atypical SSPE with acute neurologic deterioration, seizures, focal deficits, or increased ICP [66].

The predictable course of SSPE allows patients to be assigned to reasonably well-defined clinical stages [24,60,64,67]. According to one schema, Stage I denotes the early behavioral manifestations of the disorder, whereas Stages II and III correspond to the phases of neurological deterioration. Stage IV denotes the preterminal vegetative state [24,67]. Other investigators have modified these stages, using subcategories to denote specific clinical manifestations [64,68,69] as follows:

- IA – behavioral, cognitive, and personality changes
- IB – myoclonic spasms
- IIA – further mental deterioration with frequent, generalized myoclonus; independent ambulation still possible
- IIB – apraxia, agnosia, and motor signs present; ambulation still possible
- IIIA – no independent ambulation, frequent and long myoclonic spasms; seizures
- IIIB – no spontaneous speech; may be blind or bedridden; movement disorders may appear
- IV – myoclonic spasms cease; neurovegetative state.

Such clinical staging schema facilitate comparisons of patients during therapeutic trials, as discussed below.

Diagnosis

Measles infection can be confirmed by 1) detecting measles-specific Immunoglobulin M (IgM) or rising titers of measles-virus specific Immunoglobulin G (IgG) in serum, and 2) isolating measles virus from urine, blood, saliva, or nasopharyngeal secretions [9]. Measles virus-specific IgM persists for up to 1 month after measles [9]. Molecular studies can differentiate wild measles strains from the vaccine strain [9].

Routine laboratory studies on patients with SSPE are unrevealing. The CSF typically contains no cells and has normal glucose and protein content. SSPE can be confirmed by detecting high titers of measles virus-specific IgG in serum and CSF. The CSF immunoglobulin levels are usually elevated, reflecting synthesis of measles virus-specific immunoglobulin, and oligoclonal IgG bands can be detected [24]. Measles virus RNA can be detected in the brain tissue of SSPE patients by using reverse-transcription (RT) polymerase chain reaction (PCR) [61]; RT-PCR can also detect measles virus RNA in the CSF of persons with SSPE [62].

The electroencephalogram (EEG), often the initial clue to SSPE, shows bilaterally synchronous high

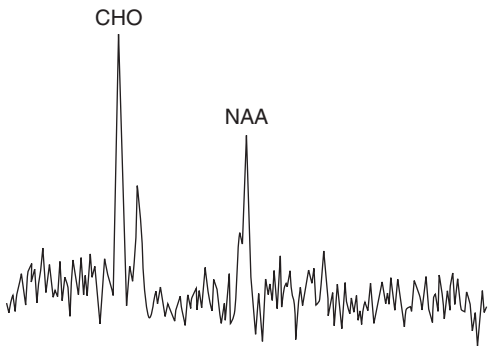

Figure 2.3. MR spectroscopy obtained during Stage IB. Shown are elevation of the choline peak (CHO) and reductions in the n-acetylaspartate (NAA) peak.

amplitude spike or slow wave bursts that often correlate with clinical myoclonus. As SSPE progresses, the background activity of the EEG becomes diffusely suppressed and a burst-suppression pattern appears. Neuroimaging studies demonstrate nonspecific abnormalities or diffuse atrophy, although T2 prolongation can be detected by magnetic resonance imaging (MRI) symmetrically in the cerebral white matter or multifocally in subcortical white matter or cortex (Figures 2.1 and 2.2) [70,71,72]. In the late stages of SSPE, the CAT scan or MRI shows severe, diffuse atrophy. Magnetic resonance spectroscopy may show reductions in N-acetylaspartate and elevations of myoinositol, choline, and lactate (Figure 2.3) [73].

An international consortium established specific criteria to standardize the diagnosis of SSPE [24]. These consist of the following:

1. Typical or atypical clinical history. Typical clinical courses include:
   a. acute progressive
   b. subacute progressive
   c. subacute remitting and relapsing
   d. chronic progressive
   e. chronic remitting and relapsing

Atypical features include early, prominent seizures and unusual age (e.g., adult onset).

2. Increased measles antibody titers in CSF
3. Increased serum and CSF measles-specific γ-globulin (increased IgG synthesis rate)
4. Typical EEG showing periodic slow wave complexes in stage II
5. Brain biopsy or postmortem showing typical pathology and/or culturing of the altered measles virus
6. Molecular diagnostic techniques identifying mutations of wild-type measles virus.

Criteria 1 and 2 are necessary and specific for the diagnosis of SSPE [24]. Criteria 3 to 5 become increasingly important in atypical cases.

Treatment and prognosis

SSPE remains a fatal neurodegenerative condition with variability in its clinical course. Approximately 60 to 80% of patients with SSPE have relentless neurological deterioration that leads to death within 2 years [24]. However, 10% can have a rapid, fulminant course, with death occurring within several weeks or a few months. Another 10% display a chronic course with exacerbations and spontaneous remissions that can persist for several years [24,69]. The variability of SSPE's clinical course has made evaluating therapeutic interventions difficult [69].

During the past 40 years, several antiviral and immunomodulating regimens have been used to treat patients with SSPE; early therapies were summarized by Dyken [60]. Beginning with Huttenlocher and Mattson's observations in the 1970s [74], several studies suggest that patients with SSPE may benefit from therapy with isoprinosine (Inosiplex; Newport Synthesis, LTD, Dublin, Ireland). In this initial open-label treatment trial of 15 patients, Huttenlocher and Mattson observed that one-third experienced remissions and improvements in neurologic function that persisted for 2 years. Isoprinosine (composed of inosine and the p-acetamidobenzoic acid salt of N, N-dimethylamido-2-propanol) has modest antiviral and immunomodulating effects [75]; the latter may account for its potential benefit in SSPE.

A subsequent, larger study by Huttenlocher and colleagues also suggested that patients with SSPE

benefited from isoprinosine [76]. In this study, 98 patients with SSPE living in the United States and Canada were treated with isoprinosine 100 mg/kg/day, given in equally divided doses every 4 hours. Probability of survival at 2, 4, 6, and 8 years was 78%, 69%, 65%, and 61%, substantially better than historical controls. The authors concluded that isoprinosine prolonged life in patients with SSPE. Fukuyama and colleagues reached similar conclusions in a study of 89 isoprinosine-treated patients [77]. However, methodological flaws in both studies (nonrandomized or retrospective design and utilization of historical controls) limit the ability to confirm the efficacy of isoprinosine.

Subsequent studies or case reports have described the use of isoprinosine alone or in combination with β or α interferon and other antiviral agents, including ribavirin and lamivudine [78,79,80]. With few exceptions, these studies represent anecdotal case reports or lack concurrent controls that allow objective measures of efficacy. Although there are no reports of cures, stabilization or improvement have been described with some drug regimens [78,79,80].

Gascon and coworkers conducted the most detailed and well-designed therapeutic trial to date and reported their results in 2003 [81]. In this multicenter trial, 122 patients with SSPE were randomized to either oral isoprinosine 100 mg/kg/day (maximum of 3 grams) alone or oral isoprinosine in combination with intraventricular interferon-α2b (100 000 U/m^2/day initially, escalated to 1 000 000 U/m^2 twice per week). A placebo control group was deemed unethical, given results of previous, uncontrolled studies [76,77]. Neurological status was rated by blinded observers using the Neurological Disability Index. Of 122 patients, 67 had analyzable data. The investigators observed no differences between the two therapeutic regimens. Overall, 35% of the subjects in each group stabilized or improved during the study, a rate substantially higher than the historical remission rates of 5% to 10%. Several "escape" medication regimens, including natural interferon, interferon, corticosteroids, intravenous immunoglobulin, and amantadine, were utilized in nonresponders; none modified the course of SSPE [81].

Based on available data, patients with proven or probable SSPE can be treated with isoprinosine 100/mg/kg/day (maximum of 3 grams/day) in three equally divided doses. Potential side effects include hyperuricemia and nephrolithiasis [81]. The study of Gascon and colleagues indicates that intraventricular therapy with interferon-α2b provides no additional therapeutic benefit. Clearly, additional studies of antiviral or immunomodulating therapies are needed.

Additional resources

National Institute of Neurological Disorders and Stroke (NINDS)
www.ninds.nih.gov/disorders/subacute_panencephalitis/subacute_panencephalitis.htm

Isoprinosine
Newport Pharmaceuticals Ltd
Frans Maas House
Swords Business Park
Swords
Co Dublin, Ireland
Tel: + 353 1 890 3011
Fax: + 353 1 890 3016
info@newport-pharma.com

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3

Rabies

Monique Lafon

Introduction

Rabies virus is a pathogen well-adapted to the mammalian nervous system, where it infects the neurons. It causes rabies, an acute myeloencephalitis that is fatal in most mammalian species and humans in particular. Rabies virus is transmitted by saliva of an infected animal through bites or scratches or by unfortunate transplantation of organs from unsuspected rabid donors. Rabies virus enters the nervous system via a motor neuron through the neuromuscular junction or via a sensory nerve through nerve spindles. It then travels from one neuron to the next along the spinal cord to the brain. It causes behavior changes such as a furious state in dogs, loss of natural shyness in wild animals, or spectacular hydrophobia in humans. After brain invasion, the virus reaches the salivary glands where virions are excreted in the saliva. In the meantime, virus spreads to several peripheral organs of digestive, pulmonary, and urinary systems. Once the virus enters the central nervous system, no therapeutic treatment can battle the infection, and rabies is almost invariably fatal. Successful invasion of the nervous system by rabies virus seems to be the result of a subversive strategy based on the survival of infected neurons. However, rabies can be prevented by prompt postexposure treatment with injection of killed rabies vaccine along with rabies-specific immunoglobulins. Postexposure treatment of rabies requires public information, access to medical facilities, and availability of efficient postexposure rabies vaccine, which are lacking in most parts of the world. Combined with poor control of rabies in animal vectors (dogs, bats), rabies still causes more than 70 000 deaths a year, half of them in children, and remains a severe threat for humans.

Human rabies

Rabies virus causes fatal encephalomyelitis in most mammals [1]. Humans are infected mostly after bites or scratches from rabid animals. Aerosol contamination is rare [2,3]. The incubation period ranges from a few weeks to 1 year or more [4,5]. This variation could be related to the site of exposure or to the viral load associated with the trauma.

Clinical presentations of rabies in humans can be categorized as furious (75% of cases) and paralytic (25% of cases) [6]. Local prodromal signs such as aching pain near the bite site and parasthesia of the infected limb, sometimes associated with fever, are the first signs of admitted patients. Cardinal features of furious rabies are fluctuating consciousness, severe agitation, hydrophobia, inspiratory spasms, and autonomic dysfunctions. Consciousness is preserved until preterminal phases. Death occurs from circulatory insufficiency, cardiac arrest, and respiratory failure [7,8].

In paralytic rabies, only one or two classical signs of furious rabies develop. Weakness of all limbs and the respiratory muscles and an absence of deep tendon reflexes are the initial manifestations of paralytic rabies. Paralytic rabies can be confused with Guillain-Barré syndrome and related, treatable autoimmune diseases of peripheral nerves.

Misdiagnosis of rabies has led to human-to-human transmissions through corneal, liver, and kidney transplants. Analysis of regional distribution of rabies virus antigen (mainly in spinal cord, brain stem, thalamus, and basal ganglia) revealed similar infection patterns in the two clinical forms of rabies [9]. However, the distinct symptoms of paralytic and furious rabies suggest that dysfunction of the anterior horn motor neurons and peripheral nerves occurs in the case of paralytic form, whereas cerebellar and limbic functions are altered in furious rabies. Indeed, clinical, electrophysiological, and postmortem immunohistological studies showed that nerve demyelination is associated with limb weakness in paralytic rabies, whereas demyelination is rarely observed in furious rabies. In contrast, electrophysiological analysis reveals that denervation associated with central chromatolysis (flattening and displacement of nucleus, loss of Nissl bodies, and cellular edema) of cellular bodies in the anterior horn of spinal cord occurs in furious rabies [10]. In both paralytic and furious rabies, the dorsal root ganglia are infiltrated by large numbers of T cells (mainly CD3). Nevertheless, inflammation seems to be more severe in paralytic than in furious forms [9,10]. Origin of neuropain at the bite sites could be related to this dorsal root ganglionopathy.

After its establishment in the central nervous system, the rabies virus reaches peripheral organs by centrifugal spread. Rabies virus can be detected in the nerve plexus in multiple organs, including salivary glands, heart, in several cell types of the gastrointestinal system, in adrenal medulla, and in skin hair follicles [11,12]. The infection of extraneural organs was sometimes, but not always, associated with an inflammatory reaction. In contrast to transmission by organ transplantation, human-to-human transmission by bites has never been reported.

Rabies has the highest case-fatality rate of any human infectious disease and is considered to be virtually 100% fatal once symptoms have developed. A few exceptions have been noted in case reports, including one spectacular recovery after intensive treatment [13]. It is still unclear which therapeutic measures were critical for survival [14,15,16].

Zoonotic rabies

The vast majority of animals infected by rabies are dogs. Dogs transmit classical rabies and develop furious rabies. Less often, paralytic rabies has been observed in dogs as in humans. Behavioral changes such as the furious state (in dogs, cats, bears) or loss of natural shyness in wild life animals (foxes) are common features of rabies.

Bats are an important reservoir for several zoonotic viruses including rabies viruses [17] (see also Chapter 21). Most members of the *Rhabdovirus* family have been found in bats. This is the case of classical rabies, with variants of rabies viruses harbored by vampire [18] or insectivorious bats (silver-haired bats and eastern pipistrelle) [19] in the Americas. This is also the case for various rabies-related viruses: Lagos bat and Duvenhage viruses in Africa [20,21,22], or Australian bat [23,24] and European bat lyssaviruses [3,25,26,27,28,29,30,31,32,33], as well as Aravan, Khujand, Irkut, and West Caucasian [34] bat viruses on other continents. Transmission of rabies virus bat variants to humans is commonly reported [35,36,37,38] but origin remains cryptic because the bite or exposure to bats is often unrecognized due to the negligible size of the lesion and the possible infection by aerosol [3,39,40]. Spillover of bat rabies can occur by cross-species transmission involving infection of terrestrial nonflying animals (skunk, fox, marten) [29,41,42]. It has been proposed that all rabies variants that infect terrestrial mammals originated from cross-species transmission of bat-associated variants [43].

Rabies is usually fatal in bats as in other mammals. However, healthy bat rabies carriers have been described [44,45]. Neurotropism of bat rabies variants is possibly not as stringent as for rabies variants transmitted by dogs, since bat rabies variants replicate better in dermal cells and at lower temperatures than canine virus variants [46].

While wild rodents are not natural hosts for rabies virus, it has been adapted to laboratory rabbits, mice,

and rats by serial intracranial injections of brain tissues. Most pathogenicity and immunological studies have been performed in the mouse with mouse neuroadapted rabies virus (the fixed rabies virus strain). Some investigations have been performed in monkeys, mostly experiments using rabies for neurotrack tracing, exploiting the rabies virus capacity to be transported by retrograde pathways [47,48,49,50]. Rare immunopathological studies have been done in bats [2,44,51,52].

Several strains of rabies virus with different levels of pathogenicity in mice have been selected. After intramuscular or intraplantar routes, encephalitic rabies virus strains invade the spinal cord and brain regions and cause fatal encephalitis [53,54,55]. In contrast, injected by the same routes, the attenuated strains of rabies virus result in a nonfatal abortive disease characterized by a transient and restricted infection of the central nervous system (CNS) followed by irreversible paralysis of the inoculated limbs [54,56,57,58,59].

Rabies virus

Rabies virus structure

Rabies virus is an enveloped bullet-shaped virus belonging to the *Rhabdoviridae* family, genus *Lyssavirus*. It is a nonsegmented negative-strand monoviridae encoding five proteins. The viral particle, 180–200 nm in length with a diameter of 75–80 nm, consists of a membrane composed of host lipids and two viral proteins, glycoprotein (G) and matrix protein (M), surrounding a helical nucleocapsid (NC). The G protein is assembled in trimers and forms 6–10-nM-long spikes at the surface of the virion. NC is composed of a single nonsegmented negative-strand RNA molecule protected by the nucleocapsid (N) and phosphoprotein (P) and the RNA-dependent RNA polymerase, large (L) protein.

The NC, and not the free RNA, is a template for viral gene expression and replication. The genome of rabies virus weighs 4.6 10^6 daltons and is comprised of 11 932 nucleotides. A 47-nucleotide sequence encodes a leader RNA at the 3′ end. RNA synthesis occurs exclusively in the cytoplasm. Transcription is initiated by the polymerase complex (L and P protein) at the 3′ end of the genomic RNA; genes are ordered: N-P-M-G-L. Transcription results in monocistronic mRNAs production. Transcription of individual genes is differentially regulated by nontranscribed spacer regions (intergenic regions [IGR] or pseudogenes) located between transcriptional start and stop signals of consecutive genes [60,61]. Lengths of the IGR are variable: 2, 5, 5, and more than 24 nucleotides at the N/P, P/M, M/G, and G/L junctions, respectively. By playing a role in attenuation of downstream transduction [62], IGR could contribute to rabies virus pathogenicity [63]. Replication of full-length genomes is performed at the 3′ ends of genomic and antigenomic NCs by the viral polymerase complex (L and P proteins). Structural proteins G and M are required for assembly and budding of new viral particles. The balance of replication and mRNA synthesis is tightly regulated by the M protein, which contributes to ensure the production of appropriate amounts of viral proteins and viral genomes.

Properties of the rabies virus proteins

The nucleoprotein

Rabies virus N protein is a 450 amino acid protein of 57 000 daltons. It is the major constituent of the virus and of the NC and the most conserved antigen among genotypes [61,64,65,66]. This sequence stability could be the result of the vital functions N encodes: protection of RNA template from ribonuclease activity and encapsidation of genome RNA. Phosphorylation of N protein at serine 389 [67,68] may control transcription and replication efficiency [69]. N protein forms complexes with P and binds RNA [70,71]. N protein and NC function as an exogenous superantigen [72,73,74]. This could explain its potent activation of peripheral blood lymphocytes in human vaccinees [75] and its ability to increase and potentiate immune response to vaccination [76,77,78,79,80,81,82].

The phosphoprotein

The P protein (formally M1 for membrane protein 1 or NS for nonstructural) is a multifunctional 297 amino acid (40 kDa) phosphorylated protein. Phosphorylation is obtained by two types of protein kinases – the rabies virus protein kinase and the gamma protein kinase C [83]. The P protein is an essential cofactor of the L polymerase. The P functions also as a chaperone for the newly produced N protein in the infected cells, preventing their nonspecific and irreversible binding to cellular RNA [71]. P is required for RNA encapsidation. P protein binds to N protein, L protein, and STAT-1 [84,85,86].

P protein mediates inhibition of the interferon (IFN) system by different pathways: it inhibits IFN production by impairing the phosphorylation of IFN regulatory factor 3 (IRF3) and IFN signaling by blocking nuclear import of STAT-1 and finally alters promyelocytic leukemia (PML) nuclear bodies by retaining PML in the cytoplasm [84,87,88,89].

P binds to cellular dynein light chain-1 protein light chain-8 (LC8) [90,91]. Interaction of the NC to the dynein motor complex could play a role in the retrograde transport. Through its binding site to LC8, P could also be involved in the transcriptional activity of viral polymerase [92]. P plays an important role in controlling rabies virus pathogenesis, as demonstrated by the decreased pathogenicity of a recombinant virus expressing low level of P protein [93].

The matrix protein

The 202 amino acid M protein (20 kDa) is located at the inner part of the viral envelope. It is a multifunctional protein that interacts with G and N protein as well as with membrane lipids. The M protein binds and condenses the NC into a tightly coiled NC-M complex which initiates virus budding from the cell membrane-expressing G protein [94]. M downregulates transcription [95,96] and, by suppressing maximal viral gene expression, could promote cell survival. However, M could play a role in controlling apoptosis in a TRAIL-dependent pathway, at least in non-pathogenic strains of lyssavirus [97].

The glycoprotein

Rabies virus G protein is a 505 amino acid (65 kDa) type I membrane glycoprotein with three potential N-glycosylation sites composed with an ectodomain, a transmembrane domain and a short cytoplasmic domain of 44 amino acids. It adopts a trimeric form in the endoplasmic reticulum. The G is responsible for the attachment of rabies virus to target cells and transport to the CNS via the retrograde pathway [98,99].

The glycoprotein (G) of rabies virus induces virus-neutralizing antibodies and T cell responses [100–102]. Its ability to mount a protective immune response depends on its structure. Soluble G, a glycoprotein lacking the 58 carboxy-terminal amino acids, elicited 15 times less neutralizing antibody than the intact full-length G and failed to protect mice [103]. IgG antiglycoprotein antibody, but not IgM, confers passive protection against rabies [104].

G protein, among other factors, contributes to pathogenicity of rabies virus [63,105,106,107, 108,109]. Mutations in position 333 (antigenic site) slow virus uptake by the cell [110] and in adult mice reduce neuroinvasiveness [111]. G protein is a key player in the balance of apoptosis/survival in neurons [112,113,114]. The cytoplasmic tail of G could play a role in the assembly of virions since production of recombinant viruses expressing G lacking the C-terminal tail was reduced compared to recombinant virus expressing full-length G cytoplasmic domain [115,116].

The polymerase L

The L protein with 2142 amino acid is the largest rabies virus protein [60,117]. L is the catalytic component of the RNA-dependent virus associated polymerase complex. Along with the non-catalytic cofactor P it controls viral replication and transcription.

Rabies virus strains

Rabies virus may be cultivated *in vitro* in most types of mammalian cells including neuroblastomas and chick fibroblasts. Virus directly isolated from animals

requires several passages to be adapted to cell culture. Strains adapted to laboratory animals by prolonged serial passage leading to death of the animal following a defined incubation period are designated as "fixed" strains. The prototype of fixed rabies virus strain is the Pasteur virus. Pasteur virus strain was isolated from a rabid dog by intracerebral inoculation of spinal cords of rabbit. Nishigahara, challenge virus standard (CVS), and Pittman Moore (PM) were derived from Pasteur virus with further passage history in mouse or guinea pigs. The strain Flurry was isolated from a human case. Fuenzalida, SAD (Street Alabama Dufferin), and Kelev were also isolated from dogs in Chile, the United States, and Israel, respectively. SHBRV-18 (Silver-haired bat rabies virus, strain 18) has been isolated from a silverhaired bat and adapted to neuroblastoma. Attenuation of pathogenicity has been obtained by further passages in nonneuronal cells. Flurry was passaged in chick embryo at low passage (low egg passage, LEP) or high passages (high egg passage, HEP) resulting in attenuation of HEP. Further passages of SAD in hamster or pig kidney cells gave rise to the vaccine ERA (Evelyn Rokitniki Abelseth) or SADB19 strains. The vaccine Ni-CE and RC-HL strains were obtained by propagating the Nishigahara strain in chick embryo or fibroblast. CVS-N2C and CVS-B2C derived from CVS-24 by passages on neuroblastoma (N2C) or fibroblast (B2C) [107,118,119]. Other strains are escape mutants resulting from a single mutation in the G sequence, such as CVS-F3 (also named RV-194–2) [106] or AVO-1 with an Arg-Gln mutation at position 333 affecting the antigenic site [105,120,121] and also ts mutant [122]. Primary sequences of ERA, PV and CVS, CVS-B2C and N2C are different by only a few amino acids [60,117,123,124].

Recombinant viruses can also be obtained by reverse genetics. Recovery of infectious virus has been achieved for three attenuated rabies virus strains, SADB19, HEP Flurry, and RC-HL [125,126, 127] and for the bat virus strain SHRBV-18 [63]. These viruses with different pathogenicity, especially those engineered by reverse genetics, are powerful tools to understand the molecular basis of rabies virus pathogenicity [63,107,109,113,128,129,130].

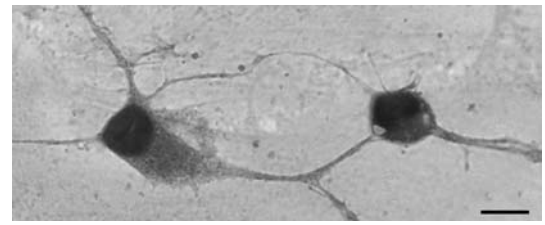

Figure 3.1. Rabies virus infection in human postmitotic neurons (NT2-N). Human neurons, infected for 24 hours with rabies virus (strain CVS), were stained with FITC-labeled antibodies directed against nucleocapsid. Viral nucleocapsid proteins accumulate in inclusion bodies near the cell's nucleus. Bars represent 10μm. (For figure in color, please see color plate section.)

Rabies virus replication cycle

Rabies virus neurotropism and neuroinvasiveness

Virus particles from the saliva of infected animals or progeny virus particles produced by muscle infection enter the nervous system via a sensory nerve through nerve spindles or via the neuromuscular junctions (NMJs), where motor axons bifurcate in invaginations of the muscle surface [50,131]. Rabies virus enters motor nerves through NMJs [132,133,134,135]. After a primary wave of replication within the motor neuron cell bodies (Figure 3.1), rabies virus travels from one neuron to another one, along the spinal cord to the brain before spreading to the salivary glands. Virions are then excreted in the saliva and transmitted to another host by bite.

After crossing the NMJs, rabies virus is seen both in neutral vesicles and in some acidic vesicles, which may trigger the fusion of the virus envelope and the release of nucleocapsids into the axoplasm [135,136]. In cultures of rat hippocampal neurons and in cocultures of rat myotubes and nerves, rabies virus was found in endosomal vesicles and in synaptic vesicles (synapsin 1-positive vesicles) shortly after uptake [135]. Electron microscopy suggested that in the infected central nervous system, rabies virus transport occurs primarily at synaptic junctions [135,137,138]. Rabies virus is transported

in the brain to anatomically connected sites exclusively by the retrograde pathway [50,139]. The transport, estimated at 50 to 100 mm per day [140], is blocked by colchicine, which induces microtubule depolymerization [141].

The G protein trimer is responsible for the attachment of the virus to target cells. Rabies virus G protein enables the virus to be transported to the CNS via the retrograde pathway as demonstrated by comparing the neuroinvasiveness of lentivirus vectors pseudotyped with envelope proteins from different viruses after peripheral intramuscular injection [98]. Conversely, unlike G-expressing viruses, G-deficient rabies virus is not transmitted transsynaptically after being stereotaxically inoculated into the rat striatum [99]. The leading role of G in the virus entry and propagation is consistent with the observations that the nature of G is a key element of rabies virus neuroinvasiveness and pathogenesis [112,124,126,142,143].

Rabies virus receptors

There is convincing *in vitro* evidence that the muscular form of the nicotinic acetylcholine receptor (nAChR), the neuronal cell adhesion molecule (NCAM), and the p75 neurotrophin receptor (p75NTR) bind rabies virus and/or facilitate rabies virus entry into cells [144,145,146,147,148,149,150,151]. Other components of the cell membrane, such as gangliosides, may also participate in the entry of rabies virus [152]. So far, it is unknown when and where nAChR, NCAM, and p75NTR participate in virus propagation *in vivo*. Analysis of the *in vivo* repartition of nAChR, NCAM, and p75NTR in the nervous system and at the synaptic junction may give some clues of the function of these molecules in rabies virus biology.

At first, it is puzzling to note that nAChRs are located at the postsynaptic muscle membrane and not at the presynaptic nerve membrane. This may indicate that nAChR is a receptor for muscle and not for the nerve. According to this hypothesis, nAChR may allow the amplification of the virus inoculum in the muscle before entry into the nervous system. Alternatively, nAChR may improve the probability of virus particles being taken up by the nerve terminal by concentrating the virus particles at the NMJ [133,135]. In contrast to nAChR, NCAM molecules accumulate at the presynaptic membrane deep within the junctional folds of the NMJs [153]. The location of NCAM at the presynaptic membrane makes it a serious candidate for the passage of NMJs and synapses by rabies virus. The absence of p75NTR at the NMJ suggests that p75NTR is not involved in the passage of the rabies virus across the NMJ. In contrast, the fact that p75NTR is mainly found in the dorsal horn of the spinal cord implies that it might participate in the virus trafficking by a sensory route that is not the main entry of rabies virus [50,55]. It has been proposed that in the uninfected nervous system, p75NTR plays a role not only in the internalization and trafficking of neurotrophins but also in the internalization and trafficking without degradation of proteins that bind p75NTR such as wheat germ, β-amyloid and prion proteins as well as tetanus toxoid indirectly through its binding to the ganglioside GT1b [154]. Similarly, it is possible that the binding of rabies virus to p75NTR [148], may allow retrograde axonal trafficking of rabies virus.

Thus, the model for virus entry and trafficking into the nervous system could be the following: Saliva from rabid animals contains rabies virus particles that are transferred by bites to the vicinity of NMJs and sensory terminations. At the NMJs, free rabies virus particles bind to nAChR located on the top of junction folds, in areas where nerves and muscles are in close contact. This concentrates virus particles at the NMJs and improves the probability of rabies virus being taken up by the nerve terminal. Rabies virus particles bind to NCAM present at the presynaptic membrane (Figure 3.2). The presence of gangliosides in this membrane concentrates NCAM into "lipid raft" microdomains, thereby allowing the simultaneous binding of G proteins and improving the membrane fusion process or allowing the detachment of rabies virus from nAChR. After crossing the NMJ, rabies virus is internalized by neutral and acidic vesicles, which may trigger the fusion of the virus envelope and the release of nucleocapsids. Alternatively, intact rabies virus remains in vesicles and

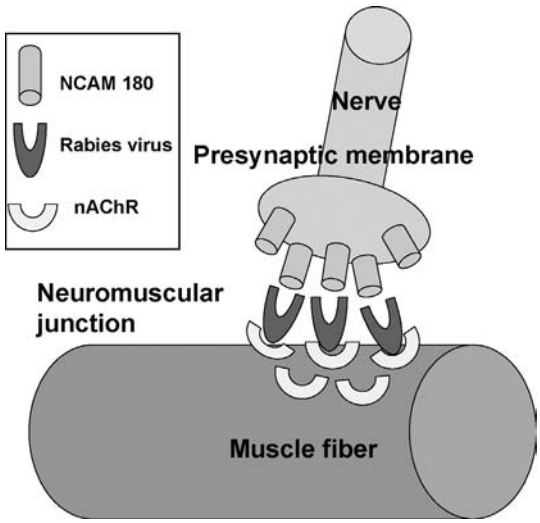

Figure 3.2. NCAM is the main receptor for rabies virus entry at the neuromuscular junction (NMJ). At the NMJ, free rabies virus particles bind to nAChR, located on the apex of junction folds. This concentrates virus particles at the NMJs. Rabies virus particles then enter the neuron at the peripheral end termination through their binding to NCAM molecules present at the presynaptic membrane.

travels along the nerve endings to the soma of the neurons where replication can occur. Possibly in sensory endings or at subsequent steps of the rabies virus transport, but probably not at the NMJs where p75NTR is not detected, the binding of G to p75NTR may enable rabies virus to follow caveolae transcytosis, allowing the retrograde transport of viral particles along the axons.

Rabies virus evasive strategies

In the absence of postexposure treatment, rabies is one of the very few human infections with a near 100% mortality rate [1]. Intriguingly, once the rabies virus has entered the CNS, its progression is interrupted neither by destruction of the infected neuron nor by the immune response, two classical strategies developed by the host to usually battle infection. It seems that rabies virus has developed strategies to enter the nervous system without causing host immune responses and to preserve the integrity of neurons and of the neuronal network. This adaptation could be crucial for rabies virus to be transmitted successfully to a new host.

Evasion from apoptosis

Apoptosis of infected neurons is not a prominent feature of natural rabies in humans [155] nor in animal models infected peripherally with pathogenic strains of rabies virus [142,156,157]. This feature is also observed *in vitro*, for example in human neuroblastoma cells infected by neuronotropic virus strains [113,114]. In contrast, attenuated rabies virus strains such as vaccine strains are strong inductors of apoptosis [113,157,158,159]. Induction of apoptosis by a vaccine strain of rabies virus and release of immunogenic apoptotic bodies could contribute to the strong immunogenicity of live attenuated rabies virus vaccine [160]. Rabies virus-induced apoptosis is inversely correlated with pathogenicity [97,112,143,157,161,162].

Protection against apoptosis is largely determined by the level of expression of rabies virus glycoprotein (G protein) [118,163]. The more G expression, the more apoptosis; conversely, the minimal G expression, the less apoptosis. Submaximal expression of virus G protein might delay apoptosis. However, the nature of the G protein is also an important factor in controlling apoptosis since in a system of maximal expression of viral protein the replacement of a pro-apoptotic G gene by a nonapoptotic G gene was sufficient to prevent destruction of the infected cells by apoptosis [113]. Some cellular factors could also control apoptosis induction upon rabies virus infection, since neurons from a suckling mouse brain or hippocampal neurons are fully susceptible to rabies virus-induced apoptosis even after infection with pathogenic virus strains, whereas in the same conditions of infection, spinal cord neurons or neurons of other parts of the brain were resistant to apoptosis [156,164,165,166,167]. Late in the infection, neuronal destruction not connected to the active process of apoptosis, such as degeneration and demyelination

of processes of infected neurons, can be observed [10,168].

Evasion from host immune responses

After injection of rabies virus in the hind limbs, progressive infection within the spinal cord and the brain is accompanied by a robust innate immune response characterized by interferon response of type 1 (IFN-β exclusively and no IFN-α) as well as chemoattractive and inflammatory responses [53,169,170,171]. Infected neurons play an active part in this early immune response [172,173]. This early innate immune response seems to be modulated according to the pathogenicity of the virus [171]. The more pathogenic, the more limited the innate immune response.

It has been shown *in vitro* that the P protein of rabies virus interferes with type 1 IFN production. P protein mediates inhibition of the IFN system by impairing the phosphorylation of IFN regulatory factor 3 (IRF3) and IFN signaling by blocking nuclear import of STAT-1 and finally alters promyelocytic leukemia (PML) nuclear bodies by retaining PML in the cytoplasm [84,87,88,174]. Thus, the P protein could be an important marker of pathogenicity [175].

Nevertheless, *in vivo*, escape of innate immune responses by the infected neurons may not be as strict as described *in vitro*, since sustained production of IFN-β mRNA could still be detected in the spinal cord of mice infected with a pathogenic strain of rabies virus up to 5 days postinfection and in the brain up to the death of the animal [176].

In the periphery, injection of rabies virus in the hind limbs of mice induces local (in lymph nodes of the hind limb) and systemic (in spleen) proliferative and cytotoxic T cell responses. The immune response in the periphery is similar in mice infected either with acute or attenuated strain of viruses [53,177,178,179]. Rabies virus infection triggers the appearance of activated lymphocytes ($CD69^+$) expressing Collapsin Response Mediator Protein 2 (CRMP2), a marker of cell polarization and migration [180].

Attracted by the gradient of chemokines and inflammatory triggered by the infection of the nervous system [128], lymphocytes migrate into the infected nervous system [53,181,182]. Migration of T cells is observed in mouse model and also in human rabies [10].

These cells after entering the nervous system encounter a dramatic decline. The severity of rabies virus infection was inversely correlated with the number of $CD3^+$ and $CD8^+$ T cells in the nervous system. Migratory T cells expressing the death receptor Fas were triggered to death by apoptosis in a Fas/FasL pathway [170,181] [182,183]. It was shown that neurons infected with acute rabies upregulated the expression of FasL [181] and that migratory T cells in mice lacking functional FasL were resistant to apoptosis and consequently mice were more resistant to rabies virus.

In addition to FasL, rabies virus infection upregulates the expression on the surface of the neurons of two other immunosubversive molecules HLA-G and B7-H1 [176,184,185]. These molecules are involved in a process leading to the inactivation of T cell and natural killer cell attack, which protect the infected neurons from the control by the host immune response.

These findings support a sequential model of events after infection of the nervous system by rabies virus, contributing to rabies virus immunoevasion as illustrated in Figure 3.3. Injection of virus particles in the periphery (muscle) generates an immune response. Immune cells enter the nervous system by crossing the blood-brain barrier. Early in the nervous system infection by rabies virus, neuronal cells mount an innate immune response including TLR3 signaling, chemokines, inflammatory cytokines, and IFN-β. Chemokines attract immune cells, and IFN-β production leads to B7-H1, HLA-G, and FasL expression. Infected neurons which express TLR3 can directly contribute to the IFN-β production. B7-H1, HLA-G, and FasL proteins subsequently reach the cell surface of the infected neurons, where they can interact with their corresponding receptor (Fas for FasL, PD-1 for B7-H1, or CD8 for HLA-G) expressed by T cells. Interaction of the immunosubversive molecule FasL, HLA-G, or B7-H1, with their respective ligand, would then trigger the exhaustion of

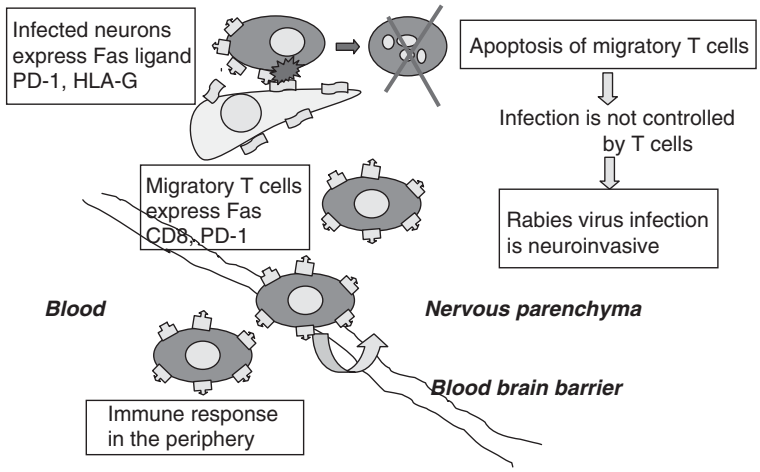

Figure 3.3. Immunoevasive strategy of rabies virus. Injection of virus particles in the muscle generates an immune response in the periphery. Immune cells attracted by chemokines and inflammatory cytokine gradient enter the nervous system by crossing the blood-brain barrier. Upon infection, neurons express B7-H1, HLA-G, and FasL. B7-H1, HLA-G, and FasL proteins subsequently reach the cell surface of the infected neurons, where they can interact with their corresponding receptor expressed on the surface of T cells (Fas for FasL, PD-1 for B7-H1, or CD8 for HLA-G). Interaction of the immunosubversive molecule FasL, HLA-G, or B7-H1, with their respective ligand, would then trigger the exhaustion and/or death of $CD3^+/CD8^+$ T cells, thus favoring the viral invasion of the nervous system.

$CD3/CD8^+$ T cells (e.g., reducing cell expansion or promoting active elimination) and thus favor the viral invasion of the nervous system. This pathway appears of crucial importance to ensure the progression of the disease in the nervous system since mice eliminate much more efficiently the invading virus when it is abrogated (mice lacking FasL or B7-H1) [176,181]. As a result, there is a global subversion of the host immune defenses by rabies virus. This can be seen as a successful well-tailored adaptation of rabies virus to the host. One would expect that the host 's natural capacity to fight such a well-adapted virus is greatly limited.

Control and prevention of rabies

Control of rabies in animals

Vaccination of pets is the most efficient human protection against rabies in Western countries. Vaccination campaigns, sometimes combined with sterilization of feral and pet dogs, have been successful in countries with enzootic canine rabies [186]. To be efficient, such preventive measures have to be constantly applied and repeated due to the poor anamestic response of animal rabies vaccines and the turnover of stray dog populations. Vaccines used in these campaigns are inactivated rabies vaccines injected by intramuscular or intradermal routes. Vaccines for oral administration have been engineered as an alternative to vaccine injection [187,188,189,190].

Wide-scale rabies vaccination campaigns of wild animals have been undertaken in Western Europe (Germany, Switzerland, France, Belgium, Italy) in the 1980s [189,191,192,193,194]. These campaigns consist of orally baiting vectors, in Europe, the red foxes, Vulpes vulpes. The vaccines were either recombinant vaccinia virus-expressing rabies virus G protein [187] or attenuated rabies virus variants such as SADB19 [61] or SAG-2 [190]. The European rabies vaccination campaigns encountered tremendous success and constitute a flagship for wildlife vaccination programs [193,194,195,196,

197,198,199,200,201]. France was declared free of rabies in 2002.

In North America, the success was less dramatic, due to the prevalence of several species able to transmit rabies. Some of which, such as skunks (Mephitis sp.), were resistant to recombinant vaccines [202,203,204]. Attempts in dogs have so far been disappointing [205,206,207,208]; however, a new formula of baits adapted to dogs and skunks is currently being tested [209,210,211].

Post-exposure vaccination

Rabies is still responsible for more than 70 000 human deaths per year worldwide. Treatment of rabies consists of a series of injections of vaccine combined with rabies immunoglobulin (RIG) given after the person has been exposed. The post-exposure treatment (PET) validated by a WHO expert committee uses four or five vaccine injections given either intramuscularly or intradermally with instillation of RIG at the wound sites in case of severe exposure or when an animal is not captured for disease confirmation (Category exposure, WHO, 2005). PET against rabies could be considered a race between the virus replication and the patient's immunity against rabies. PET failures may occur when PET is begun late after the exposure. Rabies vaccination triggers not only neutralizing antibodies but also long-lived plasma cells, memory B and T cells.

Comparison of the immunopathological events that participate in virus clearance from the nervous system in animal models has identified the production of antibodies [57,212,213] associated with a $CD4^+$ T cell response [214,215] as major factors for immune protection against rabies. A dual role is assigned for the $CD8^+$ T cells: they participate in the CNS clearance by controlling infection together with antibodies and, in contrast, they induce neuronal apoptosis [59,215] and thus can initiate an immunopathological reaction. So far assays measuring rabies virus-specific antibodies (either by immunoassay [216] or by neutralization test with the rapid fluorescent focus inhibition test (RFFIT) technique [217,218]) are the clinical standard to assess immunoconversion after rabies vaccination. Tests of cellular immunity are under development. By measuring the T cell response, these tests could provide a more accurate knowledge of the immune status of the vaccinees [219].

Conclusions

Rabies remains an important public health problem in the world as a result of uncontrolled enzootic rabies and lack of vaccines and information. Since the 1990s, rabies is becoming a re-emerging disease in several parts of the world, including China, where the number of rabies cases is currently exploding. Dogs are the main vector of rabies, and efforts are currently under way to make oral baiting vaccination of stray dogs. Efficacy of PET requires population information, prompt vaccination, and availability of RIG. Half of the victims being children, pre-exposure vaccination of young individuals should be considered in an attempt to improve the global health of mankind.

Acknowledgments

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4

Neurotropic coronavirus infections

Stanley Perlman and Noah Butler

Introduction/classification

Mouse hepatitis virus (MHV) is a member of the *Coronaviridae* family in the order *Nidovirales*. Coronaviruses are classified into one of three antigenic groups, with MHV classified as a member of group 2 [1]. Members of the *Coronaviridae* family infect a wide range of species including humans, cows, pigs, chickens, dogs, cats, bats, and mice. In addition to causing clinically relevant disease in humans ranging from mild upper respiratory infection (e.g., HCoV [human coronavirus]-OC43 and HCoV-229E responsible for a large fraction of common colds) to severe acute respiratory syndrome (SARS) [2,3], coronavirus infections in cows, chickens, and pigs exact a significant annual economic toll on the livestock industry.

MHV is a natural pathogen of mice that generally is restricted to replication within the gastrointestinal tract [4,5]. However, there exist several laboratory strains of MHV that have adapted to replicate efficiently in the central nervous system (CNS) of mice and other rodents. Depending on the strain of MHV, virulence and pathology ranges from mild encephalitis with subsequent clearance of the virus and the development of demyelination to rapidly fatal encephalitis. Thus, the neurotropic strains of MHV have proved to be useful systems in which to study processes of virus- and immune-mediated demyelination, virus clearance and/or persistence in the CNS, and mechanisms of virus evasion from the immune system.

Neurotropism and neuroinvasiveness have also has been described for two other members of the *Coronaviridae* family, HCoV-OC43 and SARS-coronavirus (CoV) (Table 4.1). Replication of these coronaviruses is generally restricted to the upper and lower airways of humans; however, several lines of evidence suggest that these viruses exhibit an inherent predilection for invading and replicating in the CNS of experimentally infected mice (discussed later). Coronavirus-like particles have been identified in the brains of patients with multiple sclerosis (MS) [6,7], and HCoV-OC43 and 229E-like RNA sequences have been detected in MS-associated brain lesions using a highly sensitive reverse-transcriptase polymerase chain reaction (RT-PCR) assay [8,9]. However, a causal relationship between coronavirus infection of the human CNS and the development of MS is lacking. Similarly, SARS-CoV RNA has been detected in the brains of patients that succumbed to respiratory disease [10,11]. The clinical and pathological relevance of this finding is unknown because signs and symptoms of CNS disease were not commonly reported in patients with SARS. However, patients who survived the acute infection appear to have an unusually large number of neurological and psychiatric sequelae [11,12], indicating that the CNS may be infected to a greater extent than is commonly believed.

Virus structure

Coronaviruses are large (80–120 nM) pseudospherical particles that contain a long, helical nucleocapsid surrounded by an envelope bearing both

Table 4.1. Neurotropic coronaviruses

Virus	*In vivo* host range	CNS cell-type tropism	Disease
MHV	Mouse, rat, monkey	Astrocytes, microglia, oligodendrocytes, neurons, macrophages	Acute and chronic encephalitis, with or without demyelination
HCoV-OC43	Human airway, mouse CNS, human CNS?	Neurons	Acute encephalitis in mice
SARS-CoV	Human airway, mouse CNS, human CNS?	Neurons	Acute encephalitis in mice

virus- and host-derived glycoproteins (reviewed in [13]). The largest among known RNA viruses, the genome of coronaviruses consists of a single-strand, positive-sense, 5′-capped, and polyadenylated RNA of 27–31 kilobases. Because coronavirus RNA genomes are 5′ capped and polyadenylated, they are infectious. Several virus-encoded proteins are packaged into the virion, including the nucleocapsid (N), the spike (S) glycoprotein, the envelope (E) protein, and the transmembrane (M) glycoprotein (Figure 4.1). In some strains of MHV and several other group 2 coronaviruses, the envelope also contains a hemagglutinin-esterase (HE) protein. The S protein mediates attachment and fusion with the host cell, is the target for neutralizing antibody and often the cellular immune response, and has been shown to play a pivotal role in pathogenesis (discussed later). The N protein is intimately associated with the viral RNA genome, forms the basic structure of the helical nucleocapsid, and has been shown to be involved in several aspects of genome replication [13,14]. The M protein, the most abundant of all structural proteins in the virion, is known to play a key role in assembly and particle formation through specific interaction with S [15], N [16], and possibly [17] E proteins. The E protein is also believed to play a role in virus assembly, even though it is relatively underrepresented in the mature virus particle [18] and is not absolutely required for this process [19]. The function of the HE is not fully understood, and this protein, while it possesses esterase activity [20], is not required for virus replication in tissue culture cells [21,22]. However, recent evidence suggests that it may enhance infectivity and spread of coronaviruses within certain tissues, perhaps by serving as a second receptor-binding protein or by modulating virus release [23].

Genome organization

The 5′ two-thirds of the RNA genome of coronaviruses encodes the replicase-transcriptase machinery and is expressed as two very large open reading frames (ORF), ORF1a and ORF1b. The remainder of the genome encodes the structural proteins HE, S, E, M, and N, as well as additional group-specific, accessory ORFs (reviewed in [13]). For MHV, the three ORFs interspersed within the structural genes include ORF2a, ORF4, and ORF5a [24] (Figure 4.1B). The functions of all of these proteins are unknown and they are not required for growth in tissue culture cells [21]. With regard to pathogenesis, some accessory ORFs appear dispensable while others (alone or in combination) are critical for replication in the intact animal. For example, MHV-JHM, in which the ORF4 gene is deleted, is as lethal as parental virus [25], while mutation or deletion of the ORF2a protein did not affect growth in tissue culture cells but attenuated replication in mice [22,26]. Deletion of all accessory genes from MHV attenuates the virus *in vitro* and *in vivo* [21]. These general features of genomic organization are shared among all members of the *Coronaviridae* family; however, substantial variability exists in the number and type of ORFs expressed in the 3′ region of the genome. Remarkably, there is

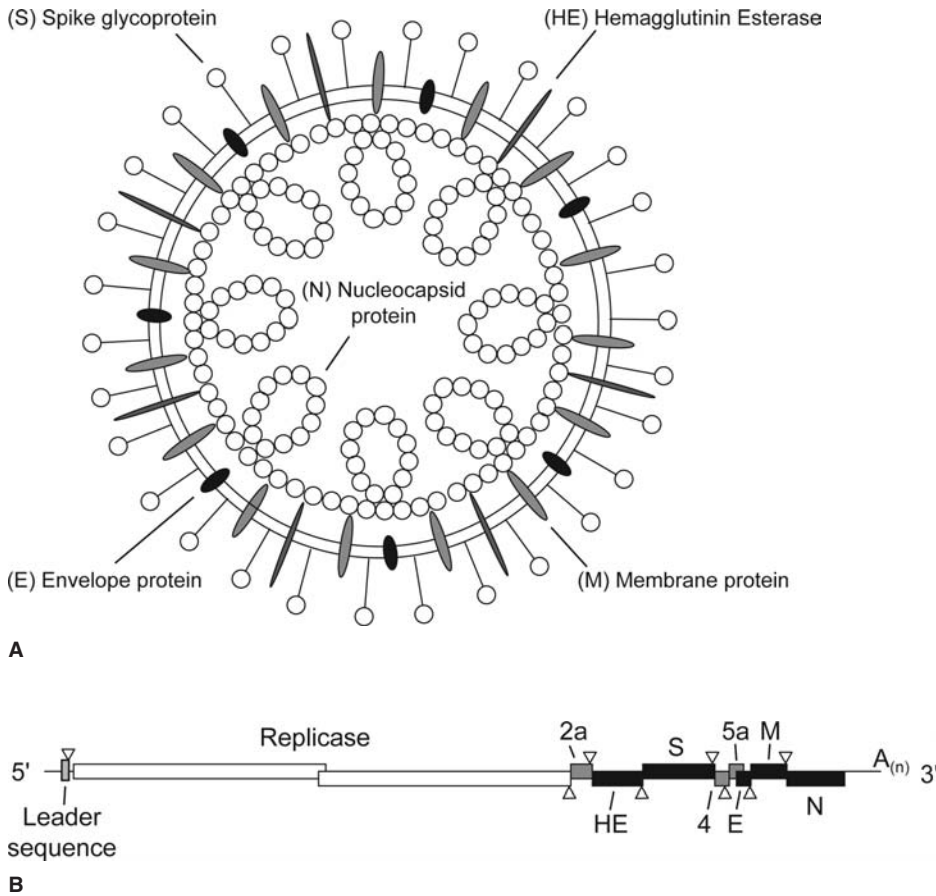

Figure 4.1. (A) Structure of the MHV coronavirus virion depicting structural proteins. (B) Schematic representation of the genomic organization of mouse hepatitis virus. Replicase genes, accessory genes, and structural genes are denoted by open, shaded, and closed rectangles, respectively. Open triangles depict the location of transcription-regulating sequences (TRS).

no obvious homology between the group-specific proteins encoded by different coronaviruses. In fact, in the case of the SARS-CoV, several of these "nonstructural" proteins, including the ORF 3a, 7a, and 7b proteins, have turned out to be structural [27,28,29]. Although deletion of many 3′ ORFs has little effect on virus replication in tissue culture, their conservation within species suggests that they play important roles in modulating the host immune response or general host cellular processes *in vivo*.

Life cycle

Cell entry

Neurotropic members of the *Coronaviridae* family utilize both host cell proteins and host cell carbohydrates as receptors for binding and entry (summarized in Table 4.2) (reviewed in [13]). For MHV, infection of host cells involves specific interaction of the S glycoprotein with a proteinacious host cell receptor, carcinoembryonic antigen cell

Table 4.2. Receptors utilized by neurotropic coronaviruses

Virus	Host	Receptor
MHV	Mouse	CEACAM-1a, PSG, isoforms of CEACAM
HCoV-OC43	Human, mouse	*N*-acetyl-neuraminic acid
SARS-CoV	Human, mouse	ACE2

adhesion molecule (CEACAM-1a) [30]; however, the pregnancy-specific glycoprotein (PSG) and other isoforms of CEACAM have also been shown to serve as a receptor for some strains of MHV. While PSG is expressed at high levels in the CNS [31], only CEACAM-1a has been definitively proven to be the receptor used in mice [32]. The S protein consists of two functional domains. In many strains of MHV, cleavage of S into S1 and S2 domains is mediated by a furin-like enzyme and occurs during virus egress [33]. However, for some coronaviruses, including MHV-2, virion S protein is not cleaved. Infection by MHV-2 requires acidification or treatment with a protease, which cleaves the S protein. Recently, these results have been reconciled by the demonstration that MHV-2 (like SARS-CoV) is cleaved by a protease, cathepsin, which is present in low pH endosomes [34]. Thus, in these viruses, acidification is necessary for S protein cleavage and not for virus-host cell fusion. The S1 domain is responsible for host cell receptor binding and is prone to mutation, while the S2 domain mediates fusion with the host cell membrane and is more conserved between MHV strains. The receptor-binding domain of the MHV S protein is present within residues 1–330 of the protein [35,36,37]. The ligation of CEACAM-1a induces conformational changes between the S1 and S2 domains, which ultimately triggers fusion of the viral and host cell membranes. The precise location of the fusion domain within the S protein remains controversial. Virus entry can occur through one of two mechanisms. The viral envelope can fuse at neutral pH with the plasma membrane of the host cell resulting in the uncoating and release of the viral genomic RNA into the cytoplasm or, alternatively, virus can be taken up into endocytic vesicles, followed by fusion of the viral envelope and host vesicle membranes with subsequent release of the genomic RNA into the cytoplasm. The latter process occurs at acidic pH and is inhibited by lysosomotropic agents such as chloroquine [38,39]. The replication life cycle of MHV, like all coronaviruses, is believed to take place entirely within the host cell cytoplasm (reviewed in [13]).

For HCoV-OC43, cellular binding and entry involves ligation of the S protein to sialidated carbohydrate moieties in the surface of cells [40], while for SARS-CoV, entry requires binding to the angiotensin-converting enzyme (ACE2) [41]. As with MHV, entry of SARS-CoV or HCoV-OC43 involves conformational changes in the S protein and functional activation of the fusogenic S2 domain.

Genome replication

Because the genomes of coronaviruses are 5′ capped and polyadenylated RNA, replication begins immediately after virus entry via direct translation of the genome by host cell machinery (Figure 4.2). The translation of the viral RNA genome results in the generation of two large polyproteins (pp), pp1a (450–500 kDa) and pp1ab (750–800 kDa): the translation of the second is a result of a (−1) ribosomal frame shift at a pseudoknot structure during translation of ORF1a [42]. The polyprotein is processed into component proteins by at least two different viral proteases, a papain-like proteinase and a second proteinase with some properties similar to those of the picornavirus, 3C protease (M^{pro}). In addition to a viral RdRp and helicase, coronaviruses encode several novel proteins including a uridylate-specific endoribonuclease (NendoU), a 3′ to 5′ exoribonuclease (ExoN), and a 2′-*O*-ribose methyltransferase, which are likely critical for viral RNA synthesis. The 3C- and papain-like proteinases auto-process the large polyproteins either during or after translation [43]. Sixteen total proteins are generated from the two large polyproteins (nsp1–16), eight of which are predicted to have enzymatic activity [44]. Interestingly, while many of the described functions of

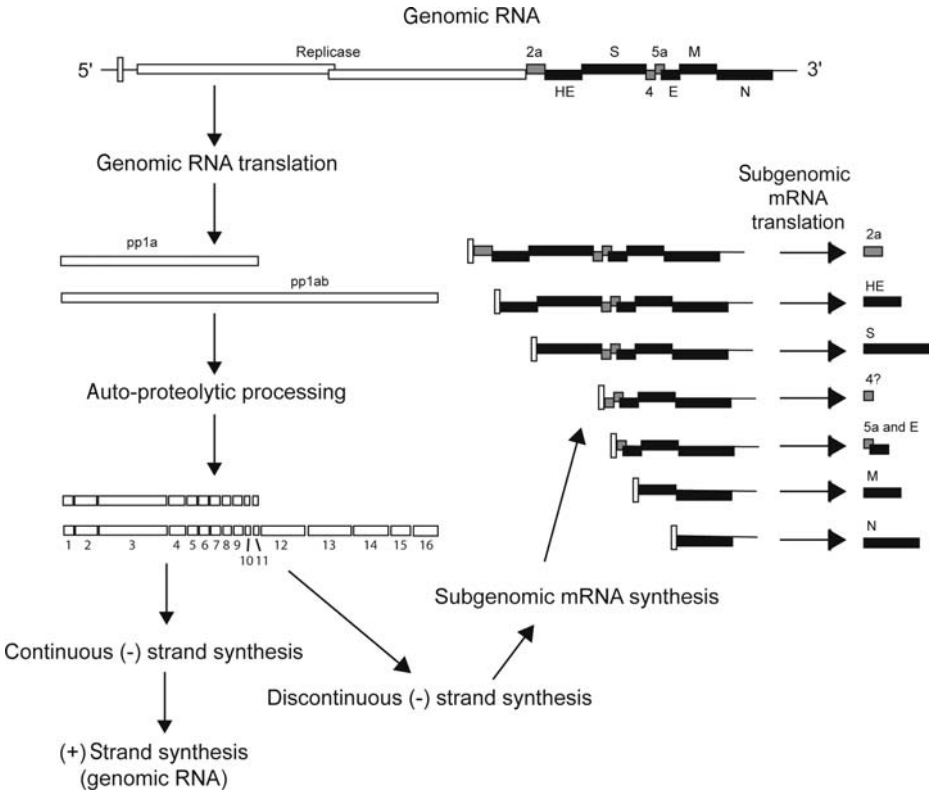

Figure 4.2. Overview of coronavirus replication. Upon uncoating, virus RNA is directly translated via host cell machinery into two large polyproteins, pp1a and pp1ab, the latter a result of a ribosomal frameshift during translation of pp1a. Both polyproteins undergo autoproteolytic processing to generate nonstructural proteins (nsp) of the replicase gene complex (nsp1–nsp16). Proteins with defined function or predicted activity include; nsp1, involved in cell cycle arrest; nsp3, the papain-like protease; nsp5, main protease (M^{pro}); nsp9, RNA-binding protein; nsp12, RNA-dependent RNA polymerase (RdRp); nsp13, helicase/NTPase/RNA 5′ triphosphatase; nsp14, 3′-5′ exoribonuclease (ExoN); nsp15, endoribonuclease (NendoU); and nsp16, 2′-*O*-ribose-methyltransferase. The replicase proteins mediate continuous or discontinuous replication of negative-strand RNA templates. Genome-length negative strands serve as template for the replication of genomic, positive-strand RNA that is packaged into virions. Discontinuous replication results in subgenomic-length negative strands that serve as template for the nested set of subgenomic messenger RNAs (mRNA). mRNA is translated by host cell machinery into structural and accessory proteins, including; gene 2a, hemagglutinin-esterase (HE), spike glycoprotein (S), gene 4, gene 5a, envelope protein (E), matrix protein (M), and the nucleocapsid protein (N). E, M, and S assemble on intracellular membranes, along with newly synthesized full-length, positive-strand RNA that has been encapsidated by the N protein. Virus assembly occurs in the endoplasmic reticulum Golgi intermediate complex (ERGIC), and eventual release of virus particles occurs through host cell secretory pathways.

nsp1–16 are common to RNA viruses and are clearly important for virus replication or transcription, several others are wholly unique to coronaviruses and may play important roles in modulating cellular processes [44].

The replication of viral RNA is critically dependent on key *cis*-acting sequence elements present at both the 5′ and 3′ ends of the genome, as well as within the genome [13,24,45]. The viral RdRp initiates negative strand synthesis via recognition of

signals at the 3′ end of the RNA genome. Interestingly, this process can be continuous, resulting in genome-length negative strand molecules, or discontinuous, resulting in the generation of a nested set of subgenomic negative strand templates (transcription, Figure 4.2). Genome-length negative strands serve as template for RdRp-mediated synthesis of positive strand, genome-length RNA that eventually is packaged into new virions.

Transcription

In addition to the elements required for replication, c*is*-acting elements within the genomic sequence, termed transcription-regulating sequences (TRS), are required for transcription. TRS elements are located in the 5′ leader sequence and in front of each ORF (Figure 4.1B). As described above, negative strand synthesis can be discontinuous, resulting in subgenomic-length RNA molecules. It is generally believed that subgenomic RNA is produced during negative RNA synthesis [45]. During negative strand synthesis, elongation by viral RdRp proceeds from the 3′ end of the positive strand genome until the first functional TRS sequence. At this point, via mechanisms that are unclear, the RdRp either continues to elongate (to generate genome-length negative strand RNA) or dissociates from the positive strand, relocates to the 5′ end of the positive strand, and reinitiates elongation of the nascent negative strand with subsequent incorporation of the 5′ antileader sequence. The newly synthesized negative strand RNA, with 5′ leader incorporated, then serves as template for sub-genomic-length mRNA synthesis. The subgenomic mRNAs are subsequently translated via host cell machinery into structural and non-structural proteins.

Virus assembly and egress

After translation by host cell machinery, key structural proteins including E and M traffic to and assemble on intracellular membranes located in the endoplasmic reticulum and Golgi regions [46]. The S protein has a more disperse distribution throughout the cell and also co-localizes at these sites of E and M accumulation. Full-length genomic RNA is encapsidated by the N protein via specific binding between N or M and a site present on viral genomic, but not subgenomic RNA, located in gene 1 [47,48]. Virus assembly, which occurs in the ERGIC (endoplasmic reticulum Golgi intermediate complex), is believed to be driven by both host- and virus-specific factors, but the details are not fully understood. Virus egress occurs when the particles are released from the cell, probably, at least in part, through host cell secretory processes similar to exopinocytosis.

Coronavirus reverse genetics

The exceedingly large size of coronavirus genomes, as well as the occurrence of regions of genomic instability, has hindered the development of coronavirus infectious cDNA clones. Two general strategies have been utilized to generate infectious coronavirus genomes: cloning full-length cDNA into bacterial artificial chromosomes (BAC) or vaccinia virus (VV) constructs, or the *in vitro* ligation of a series of overlapping subclones [49,50,51,52,53]. For the second approach, the infectious clone is generated from a series of six (or more) plasmids that encode overlapping fragments that span the entire sequence of the virus. Using any of these methods to generate infectious RNA, mutations can be introduced at virtually any given nucleotide, foreign genes can be inserted, or virus-encoded genes can be deleted with relative ease.

Prior to the development of infectious cDNA clones, the method of targeted recombination was used to introduce mutations into the genome in order to dissect the essential and non-essential gene products of coronaviruses [54]. This approach takes advantage of the high rate of RNA recombination in coronavirus-infected cells. The most widely used version of this approach relies on the strict species-specific infectivity of most coronaviruses, which is mediated by the S protein [55]. For example, the feline coronavirus (feline infectious peritonitis virus [FIPV]) only infects feline cells, and mouse coronaviruses, such as MHV, are generally limited

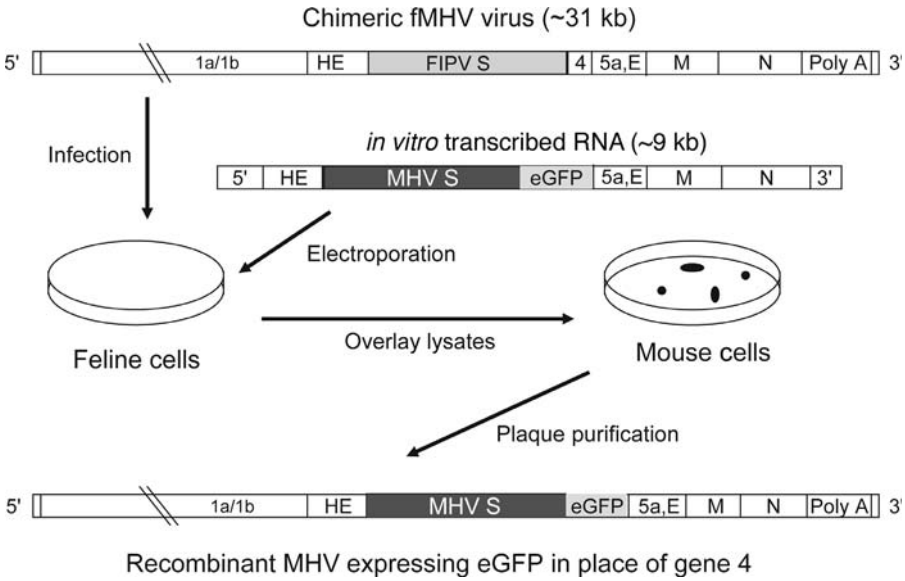

Figure 4.3. Strategy of targeted recombination for generating recombinant MHV variants. Feline tissue culture cells are first infected with a recombinant, chimeric MHV expressing the feline S gene (fMHV). Four hours after inoculation, these cells are electroporated/transfected with a synthetic (*in vitro* transcribed) RNA encoding the 3′ end of the MHV genome, including the genetic alteration of interest. In this example, the synthetic donor RNA has been engineered to encode the enhanced green fluorescence protein (eGFP) in place of gene 4. After transfection, cells are overlayed onto mouse tissue culture cells. Only recombinant viruses that have incorporated the MHV S gene will grow on the mouse cells. In this manner, recombinant MHV expressing eGFP can be plaque purified and subsequently propagated on mouse tissue culture cells. The chimeric fMHV used in the first step is generated using similar methods.

to infection of mouse cells. Exchange of S genes allows for selection of recombinant viruses using cell lines from different animal species. In specific, mouse cells infected with MHV are transfected with synthetic RNA engineered to encode MHV-specific genes flanking the S gene from feline coronavirus. Recombinant viruses, which can infect feline but not murine cells, consist of an MHV genome engineered to express the feline spike gene. This recombinant virus (termed fMHV, Figure 4.3) can then be selected and propagated on feline cells. Infection of feline cells with fMHV, followed by transfection of synthetic RNA encoding the mouse S gene (in combination with the genetic alteration of interest) results in the generation of recombinant MHV. In the example depicted in Figure 4.3, recombinant MHV virus expressing eGFP is then selected by passage onto mouse cells.

Each approach has advantages and disadvantages. The generation of infectious cDNA clones has enabled modification or deletion of coronavirus replicase proteins at the 5′ end of the genome. Further, this approach may more efficiently determine whether specific alterations are lethal to the virus. However, given the size of the MHV genome and the relative instability of certain genomic regions, targeted recombination remains the method of choice for manipulating the 3′ end of the MHV genome.

Transmission and epidemiology

Intraspecies transmission

Mechanisms of transmission vary among the coronaviruses. For naturally occurring enteric strains of MHV, virus is transmitted via the fecal-oral route

[4]. Enteric strains of MHV are highly contagious and, once introduced into a mouse colony, virus spreads rapidly, eventually infecting all mice. Eradication of the virus from a colony is essentially impossible, and generally requires the destruction of the colony. For the neurotropic strains of MHV, such as MHV-JHM and MHV-A59 (described later), virus can be inoculated into mice via intranasal route, and although these strains are highly virulent, these viruses do not spread to uninfected animals, even animals in the same cage (S. Perlman, unpublished observations). For respiratory coronaviruses, including HCoV-OC43, HCoV-229E, and SARS-CoV, virus is spread via large droplets and respiratory tract secretions (see also Chapter 21). Additionally, SARS-CoV is detected in the feces and may have spread via this route in the 2002/2003 epidemic [56]. The relative transmissibility of human respiratory/enteric coronaviruses is not precisely known, but epidemiologic studies of the SARS outbreak of 2002/2003 suggest that aerosol transmission of SARS-CoV is not very efficient, generally spreading only from patients after they developed clinical signs [57,58].

Interspecies transmission

Like all RNA viruses, the coronavirus RNA-dependent RNA polymerases lack proofreading activity; therefore, these viruses exist as a quasispecies, with several variants present in the population at any given time. For some coronaviruses, the result of this rapid ability to evolve is manifested by the ability to cross species, with rapid adaptation to growth within the new host. This has been illustrated in *in vitro* studies, in which MHV was shown to readily adapt to the use of alternate receptors [59,60,61,62,63]. In addition, SARS-CoV crossed species from Chinese horseshoe bats to infect animals such as the Himalayan palm civet and Chinese ferret badger, which in turn led to infection of humans [64,65,66] (see also Chapter 21, on "The role of bats as reservoir hosts of emerging neurological viruses.") Lastly, bovine coronavirus (BCoV) and HCoV-OC43 are very closely related and careful genetic analyses suggested that the virus crossed species about 100 years ago [67]. Thus, at least for some coronaviruses, there is a substantial body of evidence that suggests interspecies transmission can occur, both in the laboratory and in natural infections.

Pathogenesis of MHV-induced disease

While several coronaviruses infect and replicate in the CNS, the pathogenesis and host response in mice infected with neurovirulent strains of MHV has been most intensively studied. Thus, this section of the chapter will focus on results from classic studies and recent advances that have contributed to our understanding of coronavirus pathogenesis in the CNS. The central theme of MHV-induced pathology is that the host immune response contributes in large part to host morbidity and mortality.

The neurovirulence and severity of MHV-induced CNS disease, as well as the nature of the host immune response, is dependent on the strain of MHV, the route of inoculation, and the age and genetic strain of the murine host. Two well-characterized laboratory strains of MHV are the John Howard Mueller (JHM) and the A59 strains. MHV strain JHM (MHV-JHM) was originally isolated from a single mouse with hind limb paralysis [68,69], and serial passage through suckling mouse brains resulted in the selection of a virus that caused rapid and fatal encephalitis in adult mice [70,71]. MHV strain A59 (A59) is a naturally occurring variant of MHV that was isolated from a mouse with severe hepatitis [72]. MHV-JHM and A59 are very distinct from one another in their relative infectivity, spread, cell tropism, and neurovirulence. While A59 is generally hepatotropic, intracerebral or intranasal inoculation of mice with an appropriate amount of virus can result in a persistent infection of the CNS characterized by chronic demyelination and minimal parenchymal inflammation [73,74]. On the other hand, intracerebral or intranasal inoculation of mice with MHV-JHM generally results in rapid and fatal encephalitis. Several attenuated variants of MHV-JHM have also been isolated and are commonly used to study mechanisms of virus persistence and virus- and

immune-mediated demyelination. Attenuated variants have been selected after chemical mutagenesis, by exposure to neutralizing antibodies or by plaque size [75]. One of the most commonly studied attenuated variants, termed 2.2-V-1, was selected after treatment of viral stocks with the anti-S protein neutralizing monoclonal antibody (MAb), J2.2 [76]. Unlike the parental strain of MHV-JHM, this virus minimally infects neurons but preferentially infects oligodendrocytes. Because this variant is relatively neuroattenuated, infected mice uniformly survive the acute infection but remain persistently infected. The disease course, as well as the nature of the host immune response (described later), make infection with 2.2-V-1 very useful for examining the host response to persistent virus infection of the CNS, as well as studying virus-induced immune-mediated pathology.

Initial studies with MHV-JHM suggested that demyelination was largely virus-mediated [70,77]. However, in subsequent studies it was determined that irradiated mice or congenitally immunodeficient mice (mice with severe combined immunodeficiency [SCID] or deficient in recombination activation gene activity [$RAG^{-/-}$]) do not develop demyelination [78,79,80]. Moreover, demyelination occurs in immunocompetent mice, or SCID or $RAG^{-/-}$ mice reconstituted with immune cells, during the course of virus clearance (discussed later). Thus, the host immune-effector cells that enter the CNS to protect from the acute phase of the infection can ultimately cause immunopathology during the persistent phase, leading to tissue damage and clinical evidence of demyelinating disease. Because infection with MHV can result in persistent infection with subsequent demyelinating disease, MHV is widely used as a model of the human disease multiple sclerosis (MS).

CNS cell tropism and virus spread

Interestingly, not all cells that express CEACAM-1a support productive infection and replication of MHV, and cells that support replication may have very low levels of receptor on their surface. The best example of the former phenomenon is the inability of MHV to productively infect B cells, despite very high levels of CEACAM-1a expression on the cell surface [81]. In addition, MHV replicates efficiently in the CNS of mice despite extremely low levels of CEACAM-1a mRNA and protein expression in this tissue [82,83,84]. While these observations suggest that virus or host cellular factors other than CEACAM-1a also contribute to productive infection, other data indicates that MHV can spread in CNS-derived cells independent of CEACAM-1a expression [85,86]. This phenomenon occurs only with highly fusogenic strains of MHV-JHM and only when the S protein is expressed on the surface of cells. It is postulated that S1 is released from the S protein when expressed on the surface, exposing the fusogenic S2 fragment. If an uninfected cell is in close proximity, virus may spread, even in the absence of specific receptor.

Resident CNS cell types that support MHV-A59 and MHV-JHM replication include neurons, microglia, astrocytes, and oligodendrocytes. Importantly, the relatively more neurovirulent strains, such as MHV-JHM, exhibit an enhanced ability to infect and replicate in neurons [76]. As discussed below, the infection of neurons and astrocytes may directly contribute to virus persistence in the CNS, as these cell types do not generally express measurable levels of major histocompatibility complex (MHC) class I or class II antigen [87,88,89].

Spread of the virus within the CNS has been studied extensively. In models of intracranial inoculation, virus appears to first infect ependymal cells in the brain and spinal cord [90]. Here the virus replicates rapidly and then migrates into the brain and spinal cord parenchyma. In the parenchyma, several cell types support replication of MHV, including astrocytes, macrophages, microglia, and oligodendrocytes. In contrast to intracranial inoculation, after intranasal inoculation MHV first infects and replicates in the olfactory nerve and bulb, and then spreads transneuronally to infect distal parts of the brain that are linked through neuroanatomic connections of the main olfactory bulb (MOB) [91,92] (Figure 4.4). The virus disseminates via retrograde (not anterograde) spread along axonal

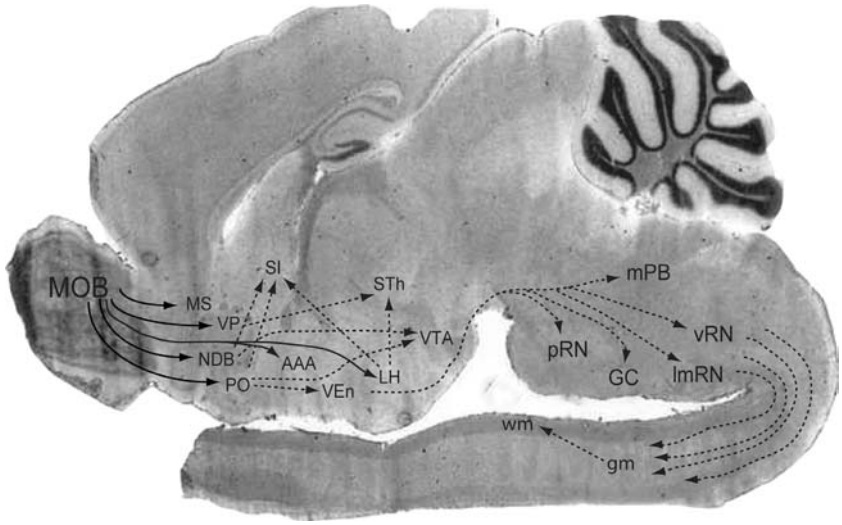

Figure 4.4. Schematic representation of MHV spread in the CNS. Upon intranasal inoculation, MHV initially replicates in the main olfactory bulb (MOB) and nerve. Dissemination to distal parts of the brain and spinal cord occurs via retrograde spread along neuronal tracts that comprise the primary (solid lines), secondary, and tertiary (dashed lines) neuroanatomic connections of the MOB. In the spinal cord, virus spread first replicates in neurons within the gray matter (gm) before spreading to oligodendrocytes and neurons that comprise the white matter (wm). Virus spread to the white matter likely involves infection of astrocytes, a cell type that associates with both neurons in the gray matter and neurons and oligodendrocytes in the white matter. Additional abbreviations; MS, medial septal nucleus; VP, ventral pallidum; NDB, nuclei of the diagonal band; PO, primary olfactory nucleus; SI, substantia annominata; AAA, anterior amygdaloid area; VEn, ventral endopiriform nucleus; STh, subthalamic nucleus; LH, lateral hypothalamic area; VTA, ventral tegmental area; mPB, medial parabrachial nucleus; pRN, pontine reticular nucleus; GC, gigantocellularis; lmRN, lateral medullary reticular nucleus; vRN, ventral reticular nucleus.

tracts to the spinal cord [91]. Eventual spread of the virus to the white matter and infection of oligodendrocytes in the spinal cord likely involves infection of astrocytes, a cell type readily infected *in vitro* and *in vivo*. Astrocytes are intimately associated with neurons in the gray matter and with oligodendrocytes and neurons in the white matter [93]. Demyelination occurs when the host immune response attempts to clear virus from this site of infection.

Acute encephalitis mediated by MHV-JHM

Infection with virulent MHV-JHM results in acute encephalitis, with extensive neuronal infection [70,77]. This disease is similar to acute encephalitis caused by several other virulent viruses and has not been extensively characterized. While the precise mechanisms by which MHV-JHM causes death in acutely infected hosts remain unclear, it is likely that rapid replication and broad cell-type tropism of the more virulent strains of MHV contribute to general neurologic dysfunction. However, the extent to which direct virus destruction of infected cells contributes to the death of the infected mouse is unknown, and recent data suggests that this disease, like the chronic demyelinating disease, may also be partly immune-mediated (discussed later). Widespread apoptosis in CNS-resident cells is not generally observed after acute MHV-JHM-induced encephalitis [79,94].

Persistent CNS infection by MHV-JHM

Infection of the CNS by virulent MHV-JHM results in rapidly lethal encephalitis in the majority of mice. However, in mice protected by antivirus antibody or

T cells, or in mice infected with less virulent variant 2.2-V-1, a variable percentage of mice survive the acute phase of infection and exhibit chronic disease characterized by hind limb paralysis and demyelination of the spinal cord [76]. The virus replicates to high titers during the acute phase and replication peaks at approximately day 5 postinfection (p.i.). In mice that survive the acute disease, the virus is not cleared from the CNS, effectively resulting in persistent infection. While infectious virus cannot be recovered from mice beyond approximately 2 weeks p.i., virus antigen and RNA can be identified in the CNS out to 1 year p.i. [95,96,97]. As virus replication increases in the CNS, the integrity of the blood-brain barrier (BBB) is disturbed such that host inflammatory cells are now able to enter the CNS [98]. Interestingly, the infiltration of inflammatory cells coincides with the onset of clinical disease. Ongoing clinical disease and the progression of demyelinating disease likely result from chronic inflammatory changes in the spinal cord of mice.

Several factors likely contribute to the ability of MHV to persist in the CNS of experimentally infected mice. First, several target cells of MHV infection in the mouse CNS (e.g., astrocytes and neurons) do not generally express MHC class I or MHC class II antigen [87,88,89]. Thus, by virtue of the cellular tropism of the virus, persistently infected cells may not serve as targets for virus-specific cytotoxic CD8 T cells (CTL) that enter the CNS. On the other hand, virus-specific CD8 T cells do become activated and traffic to the CNS of infected mice, and the ability of CTL to recognize and eliminate infected target cells is inferred from analyses of MHV-infected, antibody-protected suckling mice (discussed below). Second, the brain is a tissue subject to minimal immune surveillance [89,99], so virus could replicate for longer periods of time and to higher titers while remaining undetected. Third, as both macrophages and microglia can be infected by MHV, and both are critical antigen-presenting cells in the CNS, direct infection of these cells might influence the overall presentation of virus-specific antigens in the CNS. In support of this possibility, MHV infects both macrophages and dendritic cells *in vitro*, and infection results in diminished ability to activate virus-specific CD8 T cells [100,101]. Interestingly, CNS infection results in downregulation of CEACAM-1a receptor expression on macrophages and microglia [102]. CEACAM-1a downregulation was specifically linked to the infiltration of CD4 T cells. It is not known whether this phenomenon is strictly MHV-specific or whether this also occurs during infection of the CNS with other neurotropic viruses; however, it is postulated that this phenomenon may contribute to MHV persistence via retargeting of the virus to other cell types or by limiting T cell activation in the CNS. Finally, prolonged infection of the CNS results in a loss of effector function by CD8 T cells. MHV-JHM-specific CD8 T cells isolated from the persistently infected CNS still express cytokines such as interferon-gamma (IFN-γ) on exposure to antigen directly *ex vivo* but no longer are able to lyse infected targets [103].

Other experimental models of MHV infection

In addition to mice, MHV is also capable of infecting and replicating in the CNS of rats [104,105,106,107], hamsters [69], and nonhuman primates [108]. While infection of monkeys can result in MHV-induced demyelinating disease, the mechanisms underlying this phenomenon have not been systematically examined. In contrast, much more is known about MHV-induced disease that occurs in rats. Infection generally results in fatal encephalitis in both suckling Lewis rats and suckling outbred animals; however, a percentage of mice do survive the acute disease. Infection of weanling rats results in variable disease, but infectious MHV can be recovered from all symptomatic animals. Disease in symptomatic animals is characterized by demyelination of the optic nerve, brain stem, and spinal cord, manifesting clinically as hind limb paralysis. In rats that remain asymptomatic, virus is neither recovered nor is there evidence of demyelination out to 60 days p.i. In one study, the adoptive transfer of myelin-reactive T cells from MHV-infected rats to naïve rats resulted in widespread CNS inflammation in the absence of demyelination [109]. This is the only example suggesting that an autoimmune process contributes

to demyelination in MHV-infected animals. Brown Norway rats are also susceptible to MHV infection, but these rats remain asymptomatic with evidence of subclinical levels of demyelination [110] and little evidence for virus persistence or continued replication [106]. The lack of clinical disease in Brown Norway rats is believed to be due to an effective antiviral neutralizing antibody response. The role of antiviral antibody responses in acute and chronic encephalitis is discussed in detail below.

Role of the MHV S protein in pathogenesis

It is well-established that the S protein of coronaviruses dictates species specificity and cell tropism. However, a large body of evidence also suggests that the S protein influences pathogenesis and neurovirulence of MHV, presumably by altering cellular tropism [76,94,111] or efficiency of spread [25,94] within the CNS. Studies indicate that alterations in the S protein can also influence the nature and magnitude of the host innate and adaptive immune responses [94,112,113]. The direct link between sequence changes in the S protein and altered neurovirulence stems from several analyses. Initial studies with viruses such as 2.2-V-1 showed that diminished disease severity correlated with mutations in the S glycoprotein [76]. The role of the S protein was shown more directly using targeted recombination. A recombinant variant of MHV-A59 was engineered to express the MHV-JHM S glycoprotein [114,115]. This recombinant virus was nearly as virulent as parental MHV-JHM and did not exhibit the hepatotropism of MHV-A59.

Innate immune response to MHV infection

Intracerebral or intranasal inoculation of mice with MHV-JHM results in a rapid and massive infiltration of host immune cells (reviewed in [116]). Soon after infection, infected and uninfected astrocytes elaborate chemokines and tissue remodeling factors that facilitate disruption of the blood-brain barrier (BBB) as well as recruit additional effectors of both the innate and adaptive arms of the host immune system [117,118,119]. Several key factors that are detected early in the infected CNS are the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α [113], and the chemokines MIP-2 [113], CCL2, CCL3, CCL4, and CXCL10 [117,118]. Although IL-1α, IL-1β, IL-6 may directly and indirectly alter the permeability of the BBB and increase the expression of adhesion molecules on endothelial cells, the role of TNF-α in modulating infection remains uncertain [120,121]. Depletion of TNF-α with neutralizing antibody does not change the inflammatory response, diminish virus clearance, or affect the demyelinating process [121]. Similarly, the type I interferons, IFN-alpha (α) and IFN-beta (β), are known to be critically important for establishing an antiviral state in virus infected tissues, and IFN-α/β has been shown to modestly inhibit MHV replication and infectivity *in vitro* [120,122]. However, several studies demonstrate that MHV infection does not trigger production of IFN/β from most infected cells [123,124,125,126] with the exception of plasmacytoid dendritic cells (pDC) [127]. IFN-α is induced at high levels in these cells after infection with MHV-A59. Of note, high levels of IFN-β mRNA do not necessarily correlate with a favorable outcome. Mice infected with virulent MHV-JHM express high levels of IFN-β mRNA in the CNS for prolonged periods of time, low levels of IFN-γ, and mount a minimal CD8 T cell immune response. On the other hand, infection with MHV-A59 results in much lower levels of IFN-β mRNA and an effective antiviral CD8 T cell response [94,113,128]. Early release of the chemokines MIP-2, CCL2, CCL3, CCL4, and CXCL10 at the site of virus replication likely plays a critical role in recruiting inflammatory cells from the blood, as well as recruiting of microglia and triggering the proliferation of astrocytes within the brain parenchyma. CXCL10 is particularly important for recruiting T cells to the MHV-infected CNS and studies have shown that mice genetically deficient in CXCL10 have a much reduced T cell response and worsened outcome after acute MHV infection [129,130]. Moreover, infection of $RAG1^{-/-}$ mice (which lack B and T cells) with a recombinant MHV engineered to express CXCL10

(termed Alb274) resulted in reduced virus titers, enhanced infiltration of NK cells, and protection from acute disease, suggesting that CXCL10 can also recruit natural killer (NK) cells, which may contribute to virus clearance in the absence of T cells [131]. In contrast to a protective role during acute infection, CXCL10 may play a pathogenic role during chronic MHV infection, as *in vivo* neutralization of CXCL10 in chronically infected mice resulted in both reduced demyelination and clinical signs of neurologic dysfunction [129]. In addition to promoting protective antiviral responses in the CNS, the aforementioned cytokines and chemokines may also be pathogenic, as prolonged exposure of brain parenchyma cells to these factors could lead directly or indirectly to apoptosis or necrosis.

In response to deterioration of the BBB and upregulation of adhesion molecules on vascular endothelium, blood-derived inflammatory cells soon begin to infiltrate the infected CNS. By 3 to 5 days p.i., there is a massive infiltration of macrophages, neutrophils, and NK cells [98,103]. Depletion of neutrophils with anti-Ly6C/G (GR-1) antibody results in diminished BBB breakdown and enhanced virus replication [98]. These results are not completely straightforward, since GR-1 also depletes macrophages and some lymphocytes. However, they do indicate that inflammatory cell infiltrates are critical for BBB breakdown and inflammatory cell infiltration. Furthermore, macrophage depletion with liposome-encapsulated clodronate results in enhanced lethality, demonstrating an important role for macrophages in the initial response to infection [132]. In addition to playing a critical role in protection from acute disease, macrophages also serve as critical effectors of the demyelinating process during chronic disease (discussed below) (Figure 4.5). NK cells are detected at early times after infection as part of the initial response [133,134]. While NK cells are known to secrete significant amounts of IFN-γ in response to virus infection [135], there is little evidence that their presence is important in the host response to MHV in immunocompetent mice [103,136,137,138]. The possible exception to this may be the protective role of NK cells in Alb274-infected $RAG^{-/-}$ mice, described above.

The initial MHV-induced inflammatory response in the CNS also includes the expression and secretion of tissue remodeling factors such as matrix metalloproteinases (MMP). MMPs are secreted by both inflammatory cells, such as neutrophils, and CNS resident cells. MMPs are thought to play a role in disrupting the BBB, recruiting inflammatory cells, and activating CNS-resident and blood-borne cells for secretion of cytokines [139,140]. Interestingly, only two MMPs have been shown to be consistently upregulated in response to MHV infection; MMP3, expressed primarily by astrocytes, and MMP12, expressed in large part by oligodendrocytes [119,141]. This is similar to the array of MMPs that are expressed during autoimmune and autoinflammatory processes such as experimental autoimmune encephalomyelitis (EAE) [140]. Among the blood-borne inflammatory cells, neutrophils are known to secrete high levels of MMP9 upon entry and activation within the MHV-infected CNS. The role of neutrophil-derived MMP9 has been linked to upregulation of adhesion molecules on endothelial cells, thereby directly facilitating the continued entry of blood-derived inflammatory cells into the CNS. The complexity of the initial inflammatory response is underscored by the observation that a tissue-specific inhibitor of MMPs (TIMP-1) is also rapidly upregulated in the CNS in response to MHV infection [141]. TIMP-1 is known to negatively regulate the activation and function of MMPs. Thus, the upregulation and expression of TIMP-1 may serve to protect the CNS from over-exuberant inflammation. Future studies are required to precisely define the roles of these pro- and anti-inflammatory mediators in the MHV-infected CNS.

Adaptive immune response to MHV infection

Despite the robust innate immune response described above, MHV-JHM continues to replicate and spread. Declines in virus replication are only observed after the appearance of antiviral T cells in the

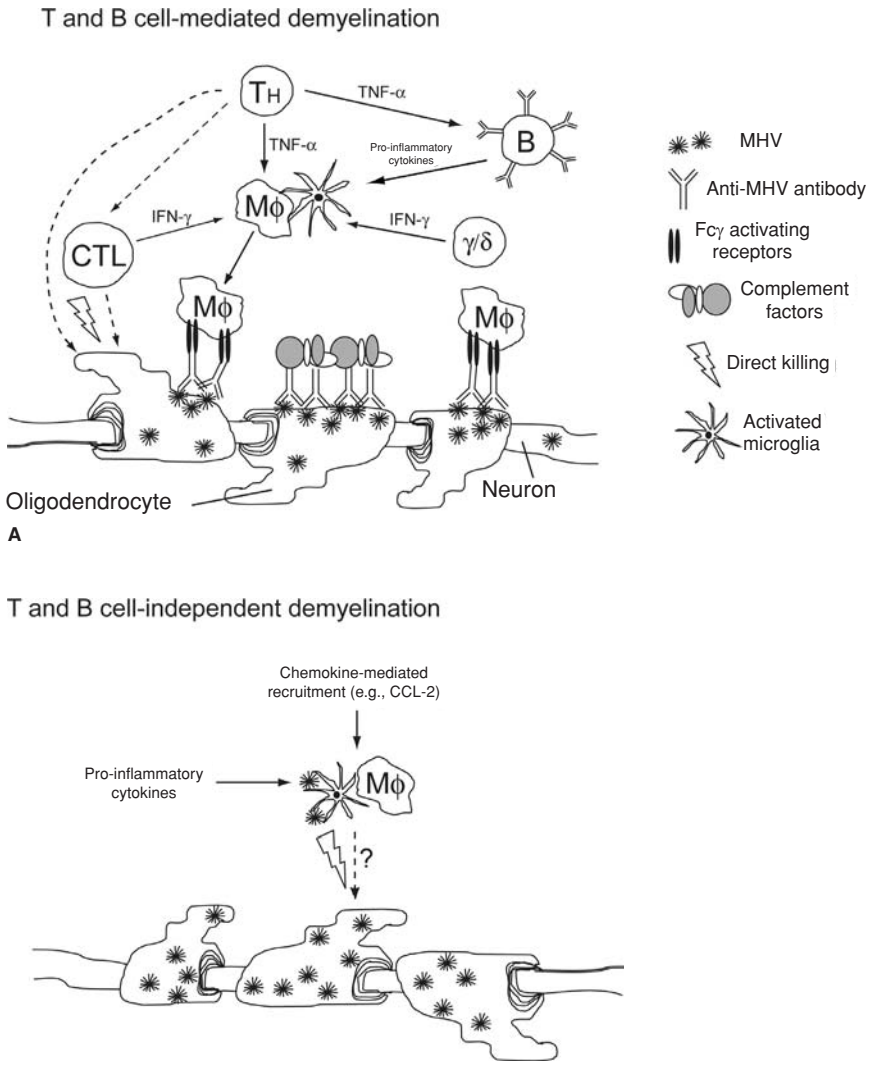

Figure 4.5. Schematic representation of the host-specific factors and cell types that contribute to demyelination in the infected CNS. (A) Intranasal or intracerebral MHV infection initiates an inflammatory cascade that results in the recruitment of CD8 (CTL) and CD4 (T_H) T cells, B cells, γ/δ T cell (γ/δ), and macrophages (Mϕ) to CNS. CTL and T_H cells may kill infected oligodendrocytes directly (dashed lines), but it is more likely that they secrete proinflammatory cytokines that activate macrophages/microglia and damage oligodendrocytes (solid lines). T_H cells also activate virus-specific B cells, which in turn secrete antiviral antibody. Antivirus antibody and activated macrophages/microglia are sufficient for destruction of oligodendrocytes and demyelination of spinal cords in MHV-infected mice; however, these processes are dependent on complement factors and Fcγ-activating receptors. (B) In the absence of T and B cells (SCID or $RAG1^{-/-}$ mice), virus-encoded chemokine- (e.g., MCP-1/CCL2) mediated recruitment and activation of macrophages is sufficient to trigger demyelination in one model [189]. The common feature of macrophage/microglia activation in each scenario underscores the critical role of these two cell types in MHV-induced demyelination.

CNS, which begins by day 5 p.i. and peaks at approximately day 7 and day 9 p.i. for CD8 and CD4 T cells, respectively [134,142]. CD8 T cells exert antiviral activity via direct and indirect mechanisms, whereas CD4 T cells are primarily responsible for augmenting the magnitude and quality of CD8 T cell and B cell responses. Antiviral B cells do not infiltrate the inflamed CNS until approximately 2–3 weeks p.i., but several lines of evidence suggest that this arm of the adaptive response is critical in suppressing virus replication and spread and preventing virus recrudescence during the persistent phase of disease [143,144]. As virus replication is controlled and infectious virus titers decrease, so does the number of innate and adaptive immune cells. However, virus-specific T and B cells are retained at low levels in the CNS of persistently infected mice [145].

Infiltrating T cells are largely MHV-JHM-specific, but it is now clear that infection also results in the recruitment and activation of virus-non-specific, bystander T cells [146,147,148]. Several lines of evidence suggest that efficient virus clearance is critically dependent on both CD8 and CD4 T cells as depletion of CD4 or CD8 cells prior to infection with MHV-JHM or infection of mice deficient in CD4 or CD8 T cells results in incomplete virus clearance and increased morbidity and mortality [80,134]. While clearly important for virus clearance, infiltrating CD8 and CD4 T cells also appears to play a pathogenic role. CD4 and CD8 T cells can be detected in the CNS of acutely encephalitic mice 1–2 days prior to the death of the animal, concomitant with the onset of virus clearance, consistent with, but not proving a role in both virus clearance and immunopathological disease.

CD8 T cell responses

In BALB/c mice, one dominant CD8 T cell epitope has been identified and is located in a conserved region of the N protein, N318 ($N_{318–326}$, H-2L^d-restricted) (Table 4.3). In C57BL/6 (B6) mice, at least two immunodominant CD8 T cell epitopes are recognized (Table 4.3). Approximately 30–50% of CD8 T cells that infiltrate the B6 CNS at the peak of the adaptive response specifically recognize the dominant epitope S510 ($S_{510–518}$, H-2D^b-restricted) when measured by staining with MHC class I/peptide tetramer. A second population of infiltrating CD8 T cells is specific for a subdominant epitope, S598 ($S_{598–605}$, H-2K^b-restricted). Both epitopes are derived from the hypervariable region of the S protein. This region tolerates both deletions and mutations, although deleted virus is usually attenuated [111,149,150,151,152].

Table 4.3. CD8 T cell epitopes of MHV recognized in MHV-infected mice

Mouse strain	MHV protein	Amino acids	Reference(s)
C57BL/6	S	510–518	[199,200]
C57BL/6	S	598–605	[200]
BALB/c	N	318–326	[201]

The precise mechanisms by which CD8 T cells mediate virus clearance and antiviral activity in the CNS are largely cell-type dependent. Clearance of MHV from macrophages, microglia, and astrocytes is largely dependent on perforin-mediated cytolysis, whereas clearance of virus from oligodendrocytes is primarily dependent upon IFN-γ expression [153,154]. CD8 T cells are also capable of eliminating virus-infected cells via FasL/Fas pathway, but this mode of clearance does not play a prominent role in clearance of MHV *in vivo* [155]. While direct cytolytic activity is a hallmark of CD8 T cell effector function, this activity must be carefully controlled in the CNS to avoid destruction of neurons, which are not generally replaceable. As described above, cytolytic activity is rapidly turned off in the infected CNS, possibly facilitating virus persistence [103,136,145].

The critical role for anti-MHV CD8 T cells in virus clearance is illustrated by results obtained from analyses of infected suckling mice. As described above, infection of naïve mice with highly neurovirulent MHV-JHM is rapidly fatal. However, in mice protected by antivirus antibody, MHV-JHM is initially cleared but virus persists [156]. In one such example, suckling mice are infected at 10 days postnatal and are nursed by dams that were previously

immunized with MHV-JHM [157]. Maternal antibodies protect the mice from acute encephalitis; however, a variable percentage of survivors develop clinical signs of chronic disease (hind limb paralysis) by 3 to 8 weeks p.i. In each symptomatic mouse, virus recovered from the brain and spinal cord is mutated in the immunodominant S510 CD8 T cell epitope (CTL escape variant virus). Thus, immune pressure exerted by CD8 T cells on MHV-JHM-infected cells results in the selection of variant viruses that have undergone mutation in the immunodominant CD8 T cell epitope, which is known to be targeted by a vigorous CTL response [158]. Generally, a single mutant is isolated from each animal, with mutations detected in positions 2 to 7 of the epitope (original sequence CSLWNGPHL) that abrogate either binding to the MHC class I molecule or T cell receptor (TCR) binding. The biological relevance of CTL escape in MHV-JHM was demonstrated by showing that infection with the mutant viruses resulted in increased morbidity and mortality, as compared to naïve mice infected with wild-type virus [159]. These results further underscore the notion that virus-specific CD8 T cells are critical for controlling virus replication and that at least one CNS-resident cell type required for virus maintenance or replication expresses MHC class I. That CTL escape variant viruses can be recovered from MHV-JHM-infected, antibody-protected mice is of particular importance, as CTL escape variants are generally only identified in humans infected with HIV or HCV or nonhuman primates infected with simian immunodeficiency virus (reviewed in [160]). Therefore, this mode of establishing a persistent MHV-JHM infection has begun to provide key insight into the virus- and host-specific factors that influence the selection of CTL escape variant viruses, including the relative contribution of antivirus antibody [161], epitope immunodominance [162], and virus fitness and T cell functional avidity (N. Butler and S. Perlman, unpublished observations). For example, the anti-MHV antibody response at the site of infection (the CNS) is critical for preventing the development of CTL escape variants. CTL escape is rarely detected in BALB/b mice, even though epitope S510 is recognized in this mouse strain, because, unlike B6 mice, a large number of virus-specific antibody-secreting plasma cells (ASC) are detected in the infected CNS [161].

During persistent infection, MHV-specific CD8 T cells are retained in the CNS at low levels and can be detected out to greater than 45 days p.i. [103,136,145]. As described above, CTL that are retained in the CNS during persistent infection progressively lose cytolytic activity [103] but remain competent to secrete IFN-γ in response to stimulation, showing that antiviral CTL do not entirely lose effector function. In addition to dramatically influencing the clearance of MHV early after infection, CD8 T cells also play an important and varied role in mediating demyelination, as described below.

CD4 T cell responses

Several MHV-derived CD4 T cell epitopes are recognized in B6 and BALB/c mice (Table 4.4). B6 mice recognize at least three MHC class II-restricted epitopes derived from the MHV M protein (M133) or the S protein (S358 and S333) [163]. M133 is immunodominant in B6 mice, with up to 25% of infiltrating CD4 T cells exhibiting specificity for this epitope during the initial effector response [142]. Similarly, MHV-derived MHC class II-restricted epitopes have been identified in BALB/c mice in both the S protein (S333) and the N protein (N266).

Virus-specific CD4 T cells are important for MHV clearance. In the absence of CD4 T cells, either by antibody-mediated depletion or through the use of mice genetically deficient in CD4 T cells, there is a marked delay in clearance of MHV from the CNS

Table 4.4. CD4 T cell epitopes of MHV recognized in MHV-infected mice

Mouse strain	MHV protein	Amino acids	Reference(s)
C57BL/6	M	133–147	[142,163]
C57BL/6	S	333–347	[163]
C57BL/6	S	358–372	[163]
BALB/c	S	333–347	[202]
BALB/c	N	266–279	[203]

[80,134,164,165]. Also, adoptive transfer of MHV-specific CD4 T cell lines into infected mice or rats revealed that protection could be conferred by CD4 T cells of multiple virus specificities. While a reduction in clinical signs of acute encephalitis was uniformly observed, each virus-specific CD4 T cell line exhibited variable effects on virus titers, demyelination, and CNS inflammation [166,167,168,169,170]. While not experimentally examined, these observations likely reflect differential production of cytokines, altered trafficking to the CNS, or altered expansion by each unique CD4 T cell clone upon activation. In addition, several studies reveal that CD4 T cells are important mediators of MHV-induced demyelination during persistent infection (described below).

The mechanisms by which CD4 T cells contribute to virus clearance are not completely understood but likely involve release of proinflammatory cytokines, most importantly IFN-γ, which may promote antigen presentation by blood-borne and CNS-resident cells [116]. Furthermore, evidence suggests that CD8 T cells do not persist in the brain parenchyma in the absence of CD4 T cells. In these studies, depletion of CD4 T cells correlated with decreased numbers of virus-specific CD8 T cells infiltrating the brain parenchyma [171]. Thus, secretion of cytokines that serve as survival factors for CD8 T cells may also be a key effector function of virus-specific CD4 T cells that infiltrate the MHV-infected CNS. Although evidence for direct cytolytic activity of CD4 T cells *in vivo* is lacking, Heemskerk *et al.* [172] demonstrated that virus-specific CD4 T cells were able to lyse MHV-infected target cells *in vitro*. Moreover, the adoptive transfer of these cells to MHV infected mice protected them from fatal encephalitis [172,173]. Further analyses of the effect of CD4 depletion on MHV-induced CNS disease revealed a role for CD4 T cells in sustaining recruitment of macrophages and lymphocytes to the MHV-infected CNS. These observations correlated with a decrease in release of the chemokine RANTES, which has been shown to be critical for recruitment of leukocytes [174].

Similar to CD8 T cells, recent evidence suggests that virus-specific CD4 T cells also contribute to pathology associated with MHV infection of the CNS, both during acute encephalitis and during persistent infection associated with demyelinating disease. A pathogenic role for CD4 T cells during acute encephalitis was demonstrated by using targeted recombination to generate a virus that lacked the immunodominant CD4 T cell epitope, M133. Infection of mice with this recombinant resulted in 100% survival, in contrast to 100% mortality observed when mice were infected with wild-type virus [175]. Introduction of a novel CD4 T cell epitope into this variant virus reversed the phenotype, resulting in 50% mortality. This showed that the anti-virus CD4 T cell response and not some other factor caused more severe disease. The ratio of MHV-specific effector cells to T regulatory cells may be critical for these different outcomes (D. Anghelina and S. Perlman, unpublished data).

A substantial body of evidence suggests that CD4 T cells also play a critical role in demyelination of the spinal cords of chronically infected mice (discussed below).

Antibody responses

The critical role of anti-viral antibody responses is best illustrated in 2.2V-1-infected mice that lack either functional antibody (μ chain (IgM)-deficient, μMT mice) [176] or in mice that lack mature B cells (Jh locus-deficient, JhD mice) [144]. Initial virus clearance was not significantly impaired in these mice; however, several weeks p.i. virus recrudesces, replicates to high titers, and eventually causes lethal encephalitis. Further experiments demonstrated a direct role for antibody in preventing re-emergence of virus, as passive administration of antivirus antibody to these mice prevented recrudescence. Of note, viruses that re-emerge in adult antibody- and B cell-deficient mice exhibit no evidence of CTL escape, in contrast to MHV-infected, antibody-protected suckling mice.

Analysis of MHV-infected Brown Norway rats also demonstrates a critical role for antibody in protection from acute encephalitis. Brown Norway rats remain asymptomatic after challenge with virulent MHV-JHM. The presence of neutralizing antivirus

antibody can be detected in the spinal fluid of these animals as early as 7 days p.i., which correlates with protection from acute encephalitis. While these antibodies protect Brown Norway rats from acute MHV-JHM-induced disease, subclinical demyelination can be detected as late as 2 months p.i. [106]. The role of antibody in demyelinating disease is discussed below.

Recent evidence suggests that autoantibodies could potentially have a role in MHV-induced CNS pathology. While not detected in wild-type mice, transgenic mice engineered to express a CNS-specific autoantibody develop enhanced disease with more severe encephalitis upon infection with MHV [177]. Whether autoantibody production occurs to a significant extent in non-transgenic mice remains unknown.

Host-specific factors that influence demyelination

Key insight into the host-specific factors that mediate demyelination during acute and chronic infection comes from studies of mice that are genetically manipulated to abrogate some aspect of immune function or in which a key cell or cytokine/chemokine is depleted with neutralizing antibody. These systems have included the use of lethally irradiated mice and SCID or RAG-deficient mice, which lack B and T cells. Inoculation of any of these mice with 2.2-V-1 results in acute and chronic encephalitis in the absence of demyelination of the spinal cord [78,79,80]. However, reconstitution of these mice with splenocytes results in the rapid development of demyelination. Demyelination is most reproducible when cells are transferred from MHV-JHM-immune mice. Houtman and Fleming also showed that when mice lacking CD4 or CD8 T cells were infected, demyelination developed, showing that neither cell type is required for this process [80]. Subsequent work showed that several components of both the innate and adaptive immune system could mediate demyelination in the brains and spinal cords of these immunodeficient recipient mice. While demyelination via immune- or virus-mediated destruction of oligodendrocytes is considered to be primary (not secondary to axonal damage), T cell-mediated damage of axons has been observed concomitant with demyelination. Although not proven, this process is probably cytokine-mediated [178]. Of note, similar findings are observed in the CNS of MS patients and contribute to long-term, irreversible disability [179]. This section will provide an overview of the immune-mediated mechanisms of demyelination in MHV-infected animals, with particular emphasis on the RAG1$^{-/-}$ and SCID adoptive transfer models. The cells and effector molecules that have been identified as playing a critical role in virus-induced demyelination are summarized in Figure 4.5. Activated macrophages/microglia are a common feature of MHV-induced, immune-mediated demyelination (see also Figure 4.6), suggesting that these cells may actually serve as the final effectors of this process.

Adaptive immune cells

As outlined above, MHV-JHM-induced demyelination is in large part immune-mediated, as RAG1$^{-/-}$ and SCID mice do not develop demyelination in spite of high levels of virus replication in the CNS and the presence of elevated levels of several proinflammatory molecules such as TNF-α, MCP-1, CCL2, and IP-10/CXCL10 [78,79,142,180,181,182]. Initial experiments demonstrated that adoptive transfer of MHV-immune splenocytes to MHV-infected lethally irradiated mice results in both clinical and histological evidence of demyelination [78]. Later, similar results were obtained after transfer of splenocytes into infected SCID or RAG1$^{-/-}$ mice: demyelination occurred with only modest reductions in virus titers [79,116,120,161,181,183]. Both primary effector cells [79] and memory T cells [184] are able to mediate demyelination.

Subsequent analyses revealed that both CD4 and CD8 T cells can mediate demyelination after adoptive transfer into MHV-infected immunodeficient mice; however, the mechanisms by which these two cell types mediate demyelination is markedly different, as is the resulting clinical disease. Adoptive

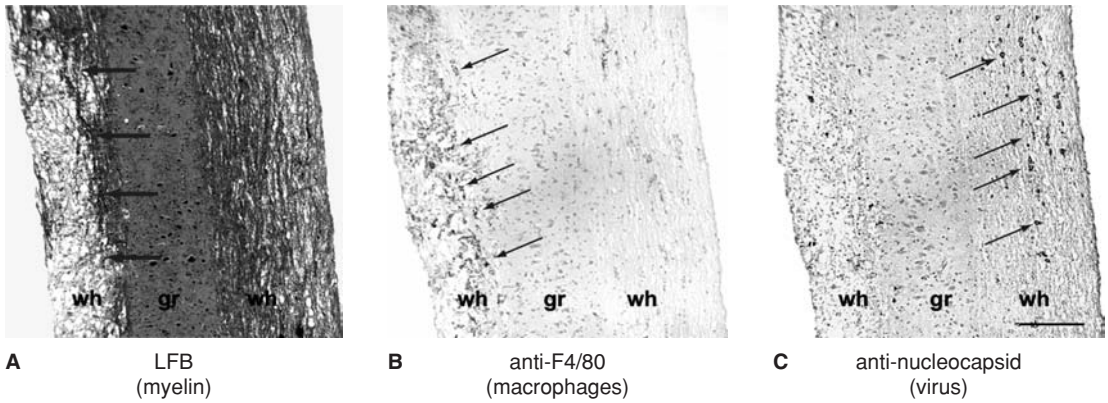

Figure 4.6. Representative serial sections of an MHV-infected spinal cord demonstrating loss of myelin (A), macrophage infiltration (B), and presence of virus antigen (C). Luxol-Fast Blue (LFB) specifically stains myelin that comprises the white matter (wm), and regions of demyelination are denoted by loss of LFB staining. Macrophages/microglia ($F4/80^{+}$) are clearly visible in areas of demyelination, in the absence of virus antigen. Demyelination, macrophage/microglia infiltration, and cells staining positive for virus antigen are denoted by leftward, downward, and upward arrows, respectively.

transfer of CD4 T cell-enriched fractions resulted in severe clinical disease, with mice presenting as moribund by 7 days posttransfer [120], sooner than is observed after transfer of undepleted splenocytes [79]. In contrast, adoptive transfer of CD8 T cell-enriched preparations resulted in widespread demyelination in the marked absence of severe clinical disease and only modest inflammation [183]. In addition, experiments using splenocytes isolated from mice deficient in IFN-γ, TNF-α, or perforin reveal several interesting features [120,183]. Adoptive transfer of unfractionated splenocytes from IFN-$\gamma^{-/-}$, perforin$^{-/-}$, or TNF-$\alpha^{-/-}$ mice resulted in similar amounts of demyelination as observed after transfer of wild-type cells. However, the transfer of CD8 T cell-enriched fractions from IFN-$\gamma^{-/-}$ mice nearly completely abrogated demyelination [116,183], similar to the effect observed in mice with CD8 T cell-mediated EAE [185]. The transfer of IFN-$\gamma^{-/-}$ CD4 T cell-enriched fractions exacerbated demyelination and clinical disease [120]. This enhanced histological and clinical disease paralleled findings in mice with CD4 T cell-mediated EAE, in which more severe disease occurred in the absence of IFN-γ, reflecting an enhanced neutrophil infiltrate into the CNS [186]. In contrast to IFN-γ, there were only modest reductions in demyelination after transfer of perforin$^{-/-}$ or TNF-$\alpha^{-/-}$ CD8 cells [183]. However, transfer of CD4 T cells from TNF-$\alpha^{-/-}$ resulted in milder disease, with prolonged survival and only modest amounts of demyelination (S. Perlman, unpublished observations), suggesting that TNF-α produced by CD4 T cells exacerbated clinical disease, the inflammatory response, and demyelination. These experiments illustrate the complexity of MHV-induced demyelination and show that the same effector molecule may have radically different effects, depending upon whether it is expressed by CD4 or CD8 T cells.

In addition to conventional α/β T cells, γ/δ T cells are also able to mediate demyelination [187]. In mice that lack a thymus (nude mice), conventional α/β T cell development is compromised. However, a subset of T cells expressing the γ/δ TCR develop athymically in these mice. Nude mice infected with 2.2-V-1 develop hind limb paresis/paralysis with histological evidence of demyelination of the spinal cord, and in these animals, myelin destruction is mediated by γ/δ T cells, showing that α/β T cells are not required for this process. γ/δ T cell-mediated demyelination, like that mediated by α/β CD8 T cells, is dependent upon the expression of IFN-γ.

Anti-MHV antibody

Passive administration of antivirus antibody also results in demyelinating disease in 2.2-V-1-infected RAG1$^{-/-}$ mice. Antibody-mediated demyelination is dependent upon both complement and Fcγ-activating receptors since demyelination occurs to a much lesser extent in FcR$\gamma^{-/-}$ mice and after depletion of complement with cobra venom factor [188].

Innate immune factors

One striking feature of demyelination in 2.2-V-1-infected RAG1$^{-/-}$ mice receiving MHV-immune splenocytes or antibody is the massive infiltration of macrophages and widespread activation of microglia in the white matter of the spinal cord [79]. Macrophages/microglia have been identified as the final effector cell in many models of demyelination and in patients with MS. Activation of these cells, in the absence of an adaptive immune response, is sufficient to mediate demyelination. Kim *et al*. [189] used targeted recombination to generate a recombinant version of 2.2-V-1 that expressed the macrophage chemoattractant MCP-1/CCL2 (termed J2.2.CCL2). Virus-derived CCL2, in the absence of any anti-viral T cells or antibody, was sufficient to induce demyelination in the spinal cord.

Collectively, these results suggest that a pro-inflammatory milieu is present in the MHV-infected RAG1$^{-/-}$ or SCID CNS, but activated macrophages do not enter the spinal cord in the absence of an additional intervention (anti-MHV T cells or antibody or over-expression of a macrophage chemoattractant). Once this trigger is provided, the process of demyelination is rapidly initiated, often accompanied by worsened clinical disease. Thus, macrophages serve as the final effectors of demyelination in MHV-infected mice. Demyelination occurs during the process of virus clearance, in areas devoid of virus antigen (Figure 4.6), and a future research goal will be to determine how to maximize virus clearance without also causing myelin/oligodendrocyte destruction.

Murine infection with human coronaviruses

In addition to MHV, at least two other members of the *Coronaviridae* family, HCoV-OC43 and SARS-CoV, can infect the murine CNS. HCoV-OC43 was originally isolated from the upper respiratory tract of a human with bronchiolitis [190], and in an effort to develop a model system to study and characterize the virus it was passed in the CNS of suckling mice. Curiously, the primary HCoV-OC43 isolate rapidly adapted to replicate in the mouse CNS and eventually resulted in the selection of a virus that caused rapidly fatal encephalitis. The neuroinvasive properties of several laboratory isolates of HCoV-OC43 have been examined, each with varying tissue culture passage history and widely varying degrees of pathogenicity in mice [191]. From these analyses it became clear that HCoV-OC43 infects and replicates exclusively in neurons [191,192], spreads via routes of infection that overlap with MHV [191], and directly kills neurons via both apoptotic [193] or necrotic [192] changes. Interestingly, as with MHV, HCoV-OC43 elicits an adaptive immune response that contributes to the morbidity and mortality in HCoV-OC43-infected mice [191]. Whether this virus also infects or causes CNS disease in humans remains questionable (see above). Nevertheless, experimental HCoV-OC43 infection of mice may serve as a useful system for understanding the general features of neuroinvasiveness, spread, and pathological changes upon human coronavirus infection of the CNS.

While most coronavirus infections cause only mild disease in humans, the identification of a coronavirus as the etiologic agent of SARS revealed the potential for coronaviruses to cause significant disease with high mortality. Initial efforts to develop animal models to study the pathogenesis of SARS-CoV and identify potential immunologic or pharmacologic interventions met with problems. While SARS-CoV infects and replicates in a number of animals (mice, hamsters, ferrets, and nonhuman primates) infection was not fatal and did not closely recapitulate the infection and disease observed in humans (reviewed in [194]). The difference was presumed to reflect, in part, the inefficient ability of SARS-CoV

to utilize the host cell receptor (ACE2) from different animal species. The development of strains of transgenic mice engineered to express human ACE2 (hACE2) and of mouse- and rat-adapted strains of SARS-CoV resulted in significant steps toward developing suitable models for studies of pathogenesis and therapy [195,196,197,198]. Notably, SARS-CoV infection of hACE2-transgenic mice resulted in a uniformly lethal disease with high levels of replication and variable pathology in the lung and, unexpectedly, in the brain [197,198]. The relative contribution of the CNS infection to mortality of SARS-CoV-infected mice is still not completely known, but hACE2 transgenic, and not wild-type, mice infected intracranially with SARS-CoV develop rapidly fatal encephalitis in the absence of lung involvement (J. Netland and S. Perlman, unpublished observations). While the pathology observed in the transgenic mice does not completely mimic that observed in humans infected with SARS-CoV, these systems should enable more detailed understanding of the virus- and host-specific factors that contribute to SARS-CoV-mediated disease.

Conclusions and future directions

Due in part to the emergence of SARS in 2002 and the continued potential for SARS-CoV to re-emerge, new emphasis has been placed on understanding both coronavirus-induced pathology and the host immunological response to coronavirus infection. While much is known about the host-specific factors that contribute to demyelinating disease during persistent infection, there still is much to be learned about the pathogenesis of coronavirus infection during acute phase of disease. For example, the relative contribution of antigen presentation within the CNS by resident glial cells is largely unknown, and an understanding of the impact of coronavirus infection of the CNS on innate signaling events that eventually shape the adaptive immune response is incomplete.

Developing ways to combat virus replication during the acute phase of CNS infection, while simultaneously minimizing damage to the CNS, is an important avenue of research. It is clear that the cells of the immune system that work to clear virus also contribute to morbidity of coronavirus-infected mice. Important insight into these processes has been made clear by recent work demonstrating a pathogenic role for effector T cells in the CNS of acutely ill MHV-infected mice [175]. One surprising observation has been that T regulatory cells (Tregs) seem to play an important role in modulating disease outcome during the acute infection (D. Anghelina and S. Perlman, manuscript in preparation). Adoptive transfer of Tregs to MHV-infected mice protects a fraction of mice from acute fatal encephalitis. Thus, understanding the balance between CD4 effector and regulatory T cells and the mechanisms of Treg function in the acutely infected CNS will be of particular interest, as Tregs may also have a protective role in other human and experimental encephalitides.

The recent development of cDNA infectious clones for several coronaviruses are important achievements and will provide direct insight into coronavirus gene function and the virus-specific factors that directly contribute to acute and chronic encephalitis. In combination with reverse genetic approaches, the development of transgenic mouse models for studying SARS-CoV infection will also provide important clues as to how coronaviruses mediate such severe disease, as well as further our understanding of the curious predilection for coronaviruses to infect and replicate in the CNS. In addition, these approaches will also enable the development of therapeutic and prophylactic interventions that will likely provide novel strategies and new tools to modulate virus infection within the acutely infected CNS while minimizing damage to tissue.

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5

Arenavirus infection in the nervous system: uncovering principles of virus–host interaction and viral pathogenesis

Stefan Kunz and Juan-Carlos de la Torre

Discovery and classification of arenaviruses

The prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) was one of the first human pathogenic viruses isolated. In 1933, Armstrong and Lillie obtained a filterable infectious agent from a brain of a patient who died during a St. Louis encephalitis epidemic [1]. In the mid-1930s, Rivers and Scott isolated a virus from cerebrospinal fluid (CSF) of a patient with aseptic meningitis [2], which was later shown to have the same serologic properties as the virus isolated by Armstrong and Lillie and a pathogen causing chronic infections in mouse colonies [3]. By the 1960s, several other viruses had been discovered that shared common morphology with a characteristic sandy (Latin, *arenosus*) appearance of ribosomes seen in thin sections of virions in electron microscopic images, serology, and biochemical features. These findings led to the establishment of the new virus family *Arenaviridae* in 1970 [4].

The *Arenaviridae* are a large group of viruses, which is currently subdivided into two major subgroups, the Old World (OW) arenaviruses and the New World (NW) arenaviruses [5,6,7]. The OW lineage contains LCMV endemic in Europe, the Americas, and likely present also in other geographic regions, and the African viruses Lassa (LASV), Mopeia (MOPV), Mobala (MOBV), and Ippy (IPPY). LCMV infections in humans are common, in some cases severe, and are of considerable concern in human pediatric medicine [8,9,10]. Fatal LCMV infection has also been recently documented in several transplant patients [11]. LASV is the causative agent of a severe viral hemorrhagic fever (VHF) with high morbidity and mortality in humans in its endemic region in Western Africa, and is the most relevant arenavirus human pathogen [12] (also see Chapter 17). Despite being phylogenetically closely related to LASV, the other African arenaviruses MOPV, MOBV, and IPPY have so far not been associated with human disease.

The NW arenaviruses are divided into three clades: A, B, and C [5,6,7]. Clade A includes the North American viruses Whitewater Arroyo, Bear Canyon, and Tamiami and the South American viruses Pichinde, Parana, Pirital, Flexal, and Allapahuayo. Clade B contains the viruses Junin, Machupo, Guanarito, Sabia, Tacaribe, Amapari, and Cupixi. Clade C contains the viruses Oliveros, Latino, and Pampa. All known human pathogenic New World arenaviruses are included in clade B. However, they do not form a distinct phylogenetic group but rather are distributed in sublineages, together with nonpathogenic viruses. The pathogenic Junin and Machupo are associated with Tacaribe virus that has so far caused only mild febrile illness in infected laboratory workers, and the pathogenic Guanarito virus is found together with the non-pathogenic Amapari and Cupixi viruses.

Each arenavirus species has as a natural reservoir one or a limited number of closely related rodent species, with the possible exception of Tacaribe virus, which has been only isolated from the fruit-eating bat species *Artibeus*. The present phylogenetic diversity of arenaviruses is likely the result of long-term coevolution between viruses and their corresponding

host species, involving vertical and horizontal transfer of viruses within and between populations, respectively, and probably occasional genetic recombination events [13,14,15,16].

Structure and life cycle of arenaviruses

Recent excellent reviews have described in detail the genome organization, virion composition, and properties, as well as molecular biology and cell biology of arenaviruses [17,18,19,20]. Here we will provide only a brief summary, with an emphasis of the most recent findings in these areas of the arenavirus field.

Virion and genome organization

Arenaviruses are enveloped negative-strand RNA viruses with a bisegmented genome and a life cycle restricted to the cell cytoplasm (Figure 5.1). Arenavirus particles are pleomorphic, ranging in size from 40 to 200 nm, with a median diameter of 90 to 110 nm. The virion surface is decorated with characteristic spike-like surface structures representing the viral glycoprotein (GP). Recent high-resolution cryo-EM studies revealed a highly organized structure of arenavirus particles, in which the surface GP spikes are aligned with subjacent lattices formed by the bona-fide matrix protein Z and viral ribonucleoproteins packed in a two-dimensional pattern at the inner surface of the viral membrane [21].

Each genomic RNA segment, L (ca 7.3 kb) and S (ca 3.4 kb), uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientation, separated by an intergenic region (IGR) with a predicted folding of a stable hairpin structure (Figure 5.1). The S RNA encodes the viral glycoprotein precursor GPC (ca 75 kDa) and the nucleoprotein NP (ca 63 kDa), whereas the L RNA encodes the viral RNA-dependent RNA polymerase (RdRp, or L polymerase) (ca 200 kDa) and a small RING finger protein Z (ca 11 kDa) (Figure 5.1). The NP and L coding regions are transcribed into a genomic complementary mRNA, whereas the GPC and Z coding regions are translated from genomic sense mRNAs that are transcribed using as templates the corresponding antigenome RNA species. NP is the most abundant viral protein and encapsidates viral genomes and antigenomic replicative intermediates. In virions, the RNA segments are present as helical ribonucleoprotein complexes with L:S ratios estimated to be in the range of 1: 2.

Arenaviruses exhibit high degree of sequence conservation at the genome 3′-termini (17 out of

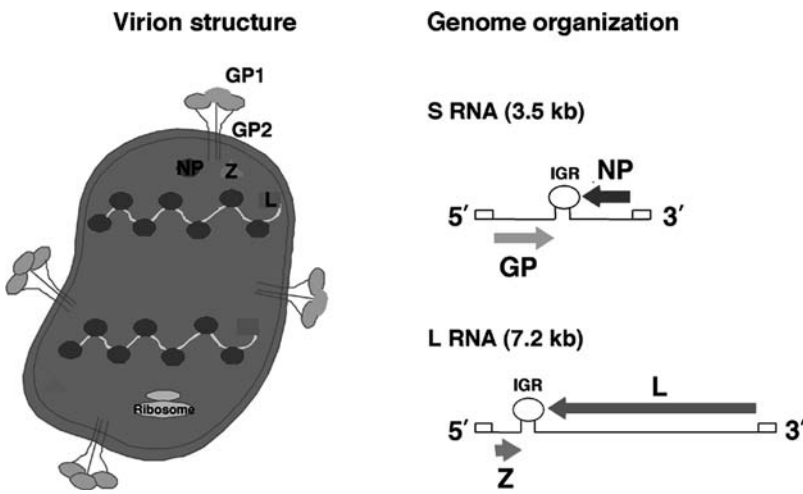

Figure 5.1. Schematic of arenavirus particles and their genome organization. For details, see text. (For figure in color, please see color plate section.)

19 nucleotides are identical) and, as with other negative-strand (NS) RNA viruses, arenaviruses genome termini exhibit terminal complementarity with the 5′ and 3′ ends of both L and S genome segments predicted to form panhandle structures. This terminal complementarity may reflect the presence at the 5′ ends of *cis*-acting signals sequences that provide a nucleation site for RNA encapsidation, required to generate the nucleocapsid (NC) templates recognized by the virus polymerase. Terminal complementarity may be also a consequence of strong similarities between the genome and antigenome promoters used by the virus polymerases. For several arenaviruses, an additional nontemplated G residue has been detected on the 5′ end of their genome RNAs.

The IGRs are predicted to form a stable hairpin structure. Transcription termination of the S-derived NP and GP mRNAs occurs at multiple sites within the predicted stem of the IGR, suggesting that a structural motif promotes the release of the virus polymerase from the template RNA.

Arenavirus proteins

Among the viral proteins, the NP is most abundant in virions (about 1500 molecules per virion) and infected cells. NP is detected a few hours after infection, plays an important role in regulation of transcription and replication (see below), and becomes the major structural element in the viral nucleocapsid.

The arenavirus RdRp L contains several structural features that are conserved among the RdRps of other negative-strand RNA viruses [22]. There is compelling biochemical and genetic evidence for a functional interaction and thus oligomerization between L molecules required for polymerase activity.

The envelope GP of arenaviruses is the only virus-derived surface structure on virus particles and mediates host cell attachment and entry. The GP precursor GPC is synthesized as a single polypeptide chain and post-translationally cleaved by the cellular protease SKI-1/S1P to yield the two mature virion glycoproteins GP1 (40–46 kDa), GP2 (35 kDa) [23,24,25], and a stable signal peptide (SSP) of 58 amino acids [26,27,28,29]. GP1 is the virion attachment protein that mediates virus interaction with host cell surface receptors [30] and is located at the top of the mature GP spike present in the viral envelope. GP1 is associated via ionic interactions with the transmembrane GP2 that forms the stalk of the spike and structurally resembles the fusion-active membrane proximal parts of other viral glycoproteins [31,32]. The SSP of arenaviruses is of unusual length and stability. In addition to targeting nascent GP into the endoplasmic reticulum (ER) lumen, the SSP is thought to play crucial roles in cellular trafficking and biological function of arenavirus GP [33].

The arenavirus small RING finger protein Z is a structural component of virions, but has no obvious homologue among other negative-strand RNA viruses. The recent advent of arenavirus reverse genetic systems has revealed several crucial functions of the Z protein in the regulation of viral RNA synthesis and budding of progeny virions from infected cells [34,35].

Arenavirus life cycle

Cell attachment and entry

As in every virus infection, the first step of the arenavirus life cycle is the attachment of virus particles to receptor molecules at the host cell surface. So far two cellular receptors for arenaviruses have been identified. Alpha-dystroglycan (α-DG), a cell surface receptor for proteins of the extracellular matrix (ECM), serves as cellular receptor for most isolates of LCMV, LASV, the Old World arenaviruses MOPV, MOBV, and IPPY, as well as the clade C New World arenaviruses [36,37]. The human pathogenic clade B New World viruses Junin, Machupo, Guanarito, and Sabia were recently found to use transferrin receptor 1 for infection [38]. Following initial attachment to the target cell, virions of the prototypic LCMV are taken up in smooth-walled vesicles, which are not associated with clathrin [39]. Following internalization, the vesicles enter the endocytic pathway and are acidified as they move through the cell. Fusion of the viral membrane with the vesicle

membrane is triggered as the pH drops to 5.3 to 5.5, corresponding to late endosomes [40,41,42,43]. Low pH is thought to trigger conformational changes in the arenavirus surface glycoprotein that result in exposure of a "fusion peptide," a hydrophobic moiety, which can mediate fusion of the virion and host cell membranes analogous to fusion-active GPs of other enveloped viruses [31,32].

RNA replication and transcription

Upon penetration into the cytoplasm, the viral ribonucleoprotein (RNP) serves as a template for both transcription and replication that is mediated by the arenavirus RdRp L. The L and NP proteins are necessary and sufficient for these initial steps of viral transcription and replication [34,44,45]. The Z protein is not required for these initial steps, but rather shows a dose-dependent inhibitory effect on the biosynthesis of both viral transcription and replication [46,47,48]. Heterologous expression of Z renders cells resistant to infection with LCMV and LASV [48], suggesting a crucial role of Z in the known phenomenon of homotypic viral interference among genetically related arenaviruses [49,50]. The basic mechanisms of the regulation of arenavirus RNA replication and transcription are currently emerging. Recognition of the viral genomic promoter at the 3′ end of the S and L RNA segments requires specific conserved sequences and an intact panhandle structure formed between the complementary sequences at the 3′ and 5′ end [51]. At early times of infection, low levels of NP prevent the RdRp to read through the stem-loop structures present in the IGR, favoring viral gene transcription over replication. Although viral replication and transcription strictly depend on NP, experimental increase in NP-enhanced replication and transcription to a similar degree, excluding a significant role for NP in setting the balance between the two processes [52].

Assembly and budding

In the final stages of the arenavirus life cycle, progeny particles assemble and are released by budding from the plasma membrane. The key factor in the budding process is the small RING-finger Z protein that functions as a bona-fide matrix protein in arenavirus particle assembly. Budding activity is mediated by the Z protein's proline-rich late (L) domains PT/SAP and PPxY [35] and strictly depends on Z myristoylation [53]. As with other bona-fide matrix proteins, arenavirus Z interacts with specific cellular factors of the endosomal/multiple vesicle body pathway including TSG101, Vps4A, and Vps4B [35,54].

For most enveloped NS RNA viruses, production of infectious progeny is assumed to depend on the interaction between the RNP core and the virus-encoded transmembrane GPs, which are mediated by the matrix (M) protein. Accordingly, the generation of infectious arenavirus progeny requires both Z and GP. In addition, the correct processing of GP into GP-1 and GP-2 is strictly required for the formation of infectious arenaviral particles. Consistent with the roles played by Z and GP in the arenavirus life cycle, Z and the GP exhibit subcellular colocalization and associate biochemically.

Arenavirus infection in humans

LCMV infection in humans

LCMV is a prevalent human pathogen and is probably a greatly under-diagnosed cause of human pediatric disease [8,9,10]. In nature, LCMV is maintained by congenital transmission within infected populations of the mouse species *Mus domesticus* and *M. musculus*. Prevalence of LCMV in wild and domestic mice in the United States and Europe varies with geographic location and is between 3–20%. Asymptomatically, LCMV chronically infected mice move freely in their natural habitat and may invade human habitation. Humans are infected through mucosal exposure to aerosols, or by direct contact between infectious materials and abrade skin. Serologic studies for human antibodies to LCMV revealed a prevalence of approximately 5% with a decreasing tendency [55,56,57,58]. Apart from mice, pet hamsters have been identified as sources of human LCMV infections on several occasions [59].

In immunocompetent adults, the majority of cases of human LCMV infection are asymptomatic or, after an incubation period of 1 to 3 weeks, manifest as a self-limited disease characterized by non-specific symptoms including fever, myalgia, headache, and nausea. In a minority of patients with febrile prodrome, illness can progress to aseptic meningitis or meningoencephalitis [60,61,62,63]. Among patients hospitalized with aseptic meningitis in a Washington, DC, hospital between 1941 and 1958, LCMV was associated with 10% of cases [61], but more recent studies indicate a lower frequency of LCMV associated with meningitis [62]. Patients normally fully recover and fatalities are rare. During central nervous system (CNS) involvement pathophysiological abnormalities are largely restricted to cerebral spinal fluid with significant increase in mononuclear cells [64]. Rare neurological complications associated with LCMV infections in adults are ascending and transverse myelitis, paralysis, and sensorineural hearing loss. Hydrocephalus occurs occasionally, likely as a consequence of ependymal inflammation [65]. Rare fatal LCMV encephalitis in man is characterized by both, ependymal and meningeal inflammation with prominent infiltration of mononuclear cells [66], similar to the lesions observed in experimental lethal choriomeningitis in LCMV's natural host, the mouse (discussed later).

In most LCMV infections of adult humans, the patient recovers without sequelae. However, some rare cases show a dramatically different course of disease that resembles the VHF caused by the highly pathogenic LASV. VHF-like pathology was also observed in three lymphoma patients intentionally infected with LCMV in an attempt to induce tumor regression [67]. These cases were remarkably similar to fatal LCMV infections recently documented in a number of immunosuppressed transplant patients [11]. In this incident, organs from two LCMV donors infected eight recipients of solid organ transplants. Seven out of eight recipients died from a severe systemic illness. In these cases, CNS abnormalities occurred but were dominated by the VHF-like fatal systemic disease. The surviving patient received intravenous ribavirin and a release in immunosuppression. Histological examination of the deceased patients showed only limited tissue destruction and no prominent inflammatory infiltrates [11], similar to the situation in fatal human Lassa fever [68]. In contrast to the aseptic meningitis in LCMV infection of humans and experimental animals, the pathogenesis in the VHF-like syndrome in LCMV-infected transplant patients appeared to be the consequence of sustained viremia rather than mediated by the host's immune response. These findings indicate that immunosuppression is not protective in these cases but predisposes the patient to fatal disease.

In contrast to adult infection in which severe disease is rare, prenatal LCMV infection in humans is frequently associated with a severe negative impact on the fetus's health. Due to lack of awareness and difficulties associated with laboratory diagnosis, LCMV is likely greatly under-diagnosed as a cause of congenital CNS defects in humans, and the cases so far described may represent only the tip of the iceberg [8,9,10].

LCMV infection during the first trimester of pregnancy is associated with an increased risk of spontaneous abortion [69]. Infection during the second and third trimesters has been linked to severe CNS abnormalities including hydrocephalus, psychomotor retardation, macrocephaly or microcephaly, visual loss, aqueductal stenosis often associated with periventricular calcifications, and chorioretinitis [70,71,72]. Fetal LCMV infection is thought to occur by transplacental infection during symptomatic or asymptomatic acute infection of the mother in mid- to late pregnancy. Infants congenitally infected with LCMV do not become persistently infected [72]. Out of 54 cases of congenital LCMV infection, 34 have been diagnosed since 1993. Only half of the cases were associated with symptomatic illness of the mother. Hydrocephalus and chorioretinitis were diagnosed in the majority of children with congenital LCMV infection [70,71] and overall mortality was 39% [73]. Survivors suffered from long-term neurological impairments including microcephaly, mental retardation, seizures, and visual impairment. Considering its severe

teratogenic potential, LCMV has to be included in the differential diagnosis of every congenital human infection in which the classical TORCH pathogens (toxoplasmosis, rubella, cytomegalovirus, and herpes virus) have been excluded.

For diagnosis of LCMV infection, virus isolation from blood or CSF during acute febrile illness is preferred. Antibody-based and polymerase chain reaction (PCR)-based tests applied to CSF are also recommended approaches [74,75]. There is currently no specific treatment for congenital LCMV infection. The only licensed drug for arenavirus infections, the nucleoside analogue ribavirin, penetrates poorly into the CSF, and its use during pregnancy is not recommended due to suspected teratogenic potential [76].

Human Lassa virus infection and CNS disease

Among the arenaviruses, LASV affects by far the largest number of people, with over 300 000 infections per year and several thousand deaths [77]. There are no licensed LASV vaccines and current therapies are limited [77]. The natural reservoir of LASV is the rodent *Mastomys*, and Lassa fever (LF) is endemic in Sierra Leone, Liberia, Guinea, and Nigeria, where it represents a major cause of death [78]. The fatality rate of LF in hospitalized patients is >15% [78], rising to more than 50% in some outbreaks [79]. LF continues to be the most often imported VHF into Europe and the United States [77].

After an incubation period of 7 to 18 days LF starts with fever, weakness, and general malaise and patients develop cough, headache, sore throat, and gastrointestinal manifestations. Signs of increased vascular permeability such as facial edema indicate a poor prognosis [12]. Survivors develop an efficient anti-viral immune response during the second week of disease and ultimately clear the virus. In lethal cases, deterioration is rapid, with progressive signs and symptoms of shock, accompanied by bleeding from mucosal surfaces. Fatal human LF is characterized by high viral titers and marked immunosuppression, which is a consequence of the virus's ability to infect antigen-presenting cells (APC) of the immune system and to block their function [80,81]. Despite the widespread viral replication and development of shock in terminal stages of the disease, histological analysis of fatal LF cases revealed surprisingly little cellular damage and only modest infiltration of inflammatory cells [68]. Endothelial cell and platelet function failure precede the onset of shock and death [82,83,84], possibly due to a direct effect of the virus on the endothelium. In addition to the hemorrhagic shock syndrome, important CNS manifestations of disease occur in >40% of hospitalized LF patients, including early acute encephalitis, late or convalent ataxia, and a range of subacute or chronic neuropsychiatric syndromes, such as mania, depression, sleep disorders, dementia, and psychosis [85]. Early encephalitis in LF is a systemic illness that progresses within 4 days to a brief period of generalized seizures, agitation, personality and cognitive alterations, followed by recovery in the second week of disease [85]. Despite the pronounced CNS involvement in some fatal LF cases, postmortem examination did not reveal overt brain pathology, and viral titers in brain tend to be low [68]. Convalescent LF patients suffer frequently from sequelae involving the CNS. A common problem among survivors is postviral fatigue syndrome characterized by disabling fatigue, motivation loss, and poor concentration. A less frequent problem is convalescent cerebellar syndrome, and ataxic illness clinically resembling the cerebellar ataxia observed infections with chicken pox and some cases of measles and mumps. A major complication late in the course of disease or in early convalescence is sensorineural hearing deficit (SNHD) that can affect up to 29% of prospectively studied patients [86]. LF is associated with an incidence of SNHD that exceeds that of any other postnatally acquired infection and is the most important cause of deafness in Western Africa. In most cases, LASV-associated SNHD occurs in convalescence after seroconversion and does not correlate with the severity of the acute disease [86]. This suggests an

immune-mediated mechanism of disease, rather than a direct effect of the virus.

CNS infection of the South American hemorrhagic fevers

Junin virus was recognized as the causative agent of an Argentine hemorrhagic fever (AHF) in the humid Pampas, the major agricultural area of Argentina, in the 1950s [87,88]. The rodent *Calomys musculinus* and several other rodent species represent the natural reservoir of the virus. The exact mechanism of rodent to human transmission is not known, but there is strong experimental evidence that these viruses are infectious as aerosols [89]. While former endemic hot spots are currently cooling off, the disease area increases progressively, placing at present more than 3 million people at risk [90]. AHF is a severe illness with hemorrhagic and neurological manifestations and a case fatality of 15–30% [91,92,93]. After an incubation period of 1 to 2 weeks, AHF begins with fever and malaise, accompanied by mild neurologic symptoms and early signs of vascular damage. Severe cases develop a frequently fatal hemorrhagic and/or neurologic disease. Those recovering from AHF improve during the second week of disease, develop a detectable antibody response after 12 to 17 days, and clear the virus [94].

After entry by inhalation [89], the virus is able to spread into lymphoid organs, the endothelium, and the parenchyma of various organs. Lesions consistently found in fatal cases are present in the lymphatic tissue and bone marrow. Widespread necrosis is found in the splenic white pulp and cortical and paracortical areas of lymph nodes. Postmortem, highest virus titers are detected in spleen, lymph nodes, and lung. The human disease is marked by immunosuppression, lymphopenia, and neutropenia. Severe secondary infections like pneumonia are common. High interferon levels and extensive activation of inflammatory mediators occur in acute AHF and correlate with fatal outcome [95,96,97]. Hemorrhagic manifestations are presumably due to thrombocytopenia and hemostatic alterations and do generally not involve disseminated intravascular coagulation [98,99]. Despite the classification of AHF as a hemorrhagic fever, in many cases neurologic symptoms dominate and patients die of progressive nervous system dysfunction in the absence of significant hemorrhages. Postmortem examination of the brains of these patients revealed no overt pathology, and the pathogenesis of the fatal CNS disease associated with AHF remains unclear.

Machupo virus is also a rodent-borne pathogen that caused serious outbreaks of HF in Bolivia in the 1960s [100,101], but the number of cases has declined afterwards [102]. Human-to-human transmission has been reported [103,104]. Guanarito virus emerged as the cause of Venezuelan HF in the 1990s [105,106]. Recently, the disease incidence has significantly increased, putting larger populations at risk [107]. Based on their close phylogenetic relation to Junin virus, infections with Machupo and Guanarito resemble AHF in their pathology, clinical manifestations, and mortality and frequently involve CNS pathology.

Animal models for the experimental study of arenavirus infection in the CNS

Infection of LCMV in its natural host, the mouse

Arenaviruses readily infect rodents, including mice, rats, guinea pigs, and hamsters. Infection of the prototypic arenavirus LCMV in its natural host, the mouse, is an important model system for the investigation of acute and chronic viral infection of the CNS and has uncovered basic concepts of virus-induced CNS disease that have been found to be applicable to other virus infections.

LCMV is characterized by its noncytolytic strategy of replication, which enables the virus to persist in vivo and in cultured cells without destruction of virally infected cells. Depending on the age, immunological competence, and genetic background of the host, the LCMV isolate, dose, and route of inoculation, experimental infection with LCMV

in the mouse can result in markedly different outcomes, including (1) a self-limiting acute infection that is efficiently cleared by a vigorous antiviral T cell response, (2) the classical lymphocytic choriomeningitis (LCM), a lethal acute infection following intracerebral inoculation that involves immune-mediated pathology, and (3) persistent infection either due to vertical transmission of virus *in utero* or infection of neonate animals. In contrast to the acute infection, chronic infection does not involve adaptive antiviral immune responses and occurs in absence of overt signs of inflammation, tissue destruction, and disease. A key determinant in these different scenarios is the host's antiviral immune response, which may be either protective, deleterious, or absent.

In the course of persistent infection of mice with LCMV strain Armstrong (ARM), distinct viral variants can be isolated from the brain and lymphoid tissue [108]. Whereas the parental clonal population ARM53b predominated in brain tissue (neurotropic), the variant LCMV ARM53b clone 13 that was isolated from spleen of persistently infected animals (lymphotropic) exhibits a remarkably changed pathogenic potential. Infection of immunocompetent mice with ARM53b elicits a vigorous immune response and the virus is efficiently cleared by the host immune system. In contrast, infection with clone 13 results in a generalized immunosuppression and persistence of the virus [109]. Clone 13 causes immunosuppression by infecting dendritic cells (DC), a crucial population of antigen-presenting cells in the spleen and in lymph nodes [110,111]. Viral infection renders DCs unable to present antigen to naïve T cells and B cells, resulting in a generalized immunosuppression and persistent viral infection. The exact mechanisms by which LCMV clone 13 and other immunosuppressive isolates of LCMV target DCs and block their function are currently unclear. Sequence analysis revealed only two amino acid changes in clone 13 as compared to the parental Armstrong strain, F260L in GP1 [112] and K1079Q in the viral polymerase encoded by the L gene [113]. The immunosuppressive potential of clone 13 was found to be associated with the single point mutation F260L in the viral glycoprotein, resulting in a 2–3 logs enhanced binding affinity to α-dystroglycan [114,115]. Studies on a large number of LCMV isolates demonstrated a consistent correlation between high receptor binding affinity and the immunosuppressive potential that is structurally reflected by a F260L or F260I mutation in LCMVGP1 [114]. LCMV isolates causing immunosuppression consistently exhibit a characteristic tropism in spleen tissue. Like the prototypic immunosuppressive variant clone 13, they efficiently target DCs in the T-cell area (white pulp) of the spleen. In contrast, the viruses that do not cause immunosuppression do not infect DCs and are restricted to the red pulp of the spleen [114,115].

Acute LCMV infection in the CNS: lymphocytic choriomeningitis

LCMV infection of the mouse illustrates the balance between immunological protection and immunopathology. Intracerebral (i.c.) inoculation of LCMV in adult immunocompetent mice results in fatal LCM, described originally by Armstrong and Lillie in 1945. Upon i.c. inoculation with LCMV, mice die within 6 to 9 days postinfection. Signs of disease appear after day 5 postinfection and manifest with a characteristic ruffling of the fur and a hunched posture. After day 6, animals deteriorate rapidly and go into fatal convulsions. Postmortem examination reveals massive inflammation of the meninges and the choroid plexus with marked mononuclear cell infiltration in these membranes. Within a few days of postinoculation, the virus replicates to high titers in non-neuronal cells lining the brain, including the leptomeninges and ependymal cells of the choroid plexus (Figure 5.2). At the same time, peripheral viral infection results in antigen-specific induction of cytotoxic $CD8^+$ T cells (CTLs) that specifically recognize viral antigens in the context of class I major histocompatibility complex (MHC). Peripherally expanded antiviral CTLs traffic to the CNS and attack infected cells of the meninges and the choroid plexus that present the cognate viral peptides in the context of MHC class I [116]. This results

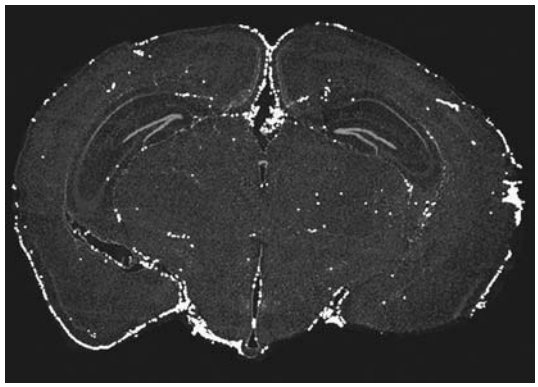

Figure 5.2. Virus distribution in the brain of mice suffering from acute LCM. Adult mice were inoculated i.c. with LCMV and sacrificed on day 5 of diseases. Viral antigen was detected in whole brain sections by immunofluorescence staining using hyperimmune guinea pig serum to LCMV and a FITC-labeled secondary antibody (white). Cell nuclei in brain tissue were counterstained with DAPI (grey). Image courtesy of Dorian B. McGavern. (For figure in color, please see color plate section.)

in the accumulation of large numbers of T cells in tissue lining the brain, while the brain parenchyma remains free of noticeable immune cell infiltrates. Accordingly, in fatal LCM, virus is restricted to cells lining the brain and no significant infection is found in the brain parenchyma. While lethal disease critically depends on $CD8^+$ T cells [117], the effector mechanism responsible for the observed pathology is currently unclear. Initial studies claimed dependence on the effector molecule perforin [118]. However, more recent studies revealed additional defects in effector T cells of perforin-deficient mice, leading to a re-evaluation of the role of perforin in lethal LCM. Despite the fact that virus-specific $CD8^+$ effector T cells from perforin-deficient mice are broadly impaired in their cytolytic function, these mice invariably succumb to lethal LCM, although a few days later than wild-type mice [119]. The delay in disease progression correlated with altered migration of virus-specific $CD8^+$ effector T cells to the CNS. However, once accumulated in sufficient numbers, virus-specific $CD8^+$ effector T cells induced fatal pathology in absence of perforin-mediated lysis.

Using high-resolution microscopy to analyze the molecular anatomy of antigen-specific T cell engagement in this experimental model, single $CD8^+$ T cells were found to engage up to three infected target cells forming immunological synapses at the site of engagement [120]. Immunological synapses contained crucial components involved in cell adhesion and signaling. Immunosuppression or depletion of specific T cell subsets efficiently prevents the fatal disease but also delays viral clearance and allows the establishment of a chronic persistent infection. Immune-compromised mice that are unable, or fail, to develop an anti-LCMV $CD8^+$ T cell response survive and do not show CNS pathology, indicating that immune-mediated pathology and not virus replication plays a central role in fatal disease.

Considering the immune-privileged nature of the CNS, the mechanisms of T cell recruitment in the LCM model are of great interest. Early studies revealed induction of the pro-inflammatory cytokines interferon (IFN)-γ and interleukin-6 (IL-6) [121,122]. Subsequent studies using extended probe sets revealed induction of a large number of cytokines after intracerebral inoculation with LCMV, including TNF-α, IL-1α, IL-1β, along with IFN-γ and IL-6 [123]. Importantly, these chemokines were also found increased after viral challenge in athymic mice that lack T cells, suggesting the CNS as the main source of these immunoregulatory factors. In addition to a role in recruitment of T cells, the prevention of lymphocytic choriomeningitis after depletion of type I IFNs indicates also a role of these cytokines in disease [124]. Examination of the chemokine gene expression in the CNS of lymphocytic choriomeningitis revealed a complex and highly dynamic expression pattern of different and at times overlapping classes of chemokine genes [125,126,127]. Significant expression of IP-10 and regulated on activation normal T cell expressed and secreted (RANTES) in the brain occurred at early time points after infection and preceded detectable increases in other chemokines and trafficking of leukocytes to the CNS [125]. Since IP-10 is highly expressed in CNS resident cells and involved in many different viral CNS infections, this suggests that induction of chemokine

expression may be an early host response to local virus infection involved in subsequent trafficking of T cells into the brain.

Despite major advances in our understanding of the role of the immune system in pathogenesis, the mechanism by which antiviral T cells kill the host are not fully understood. Despite the absence of an overt histological correlate, it is conceivable that the attack of CTLs on infected meningeal cells perturbs the blood-brain barrier, affecting normal CNS homeostasis and thus electrical brain activity [128]. The result would be a convulsive disorder with concomitant loss of brain activity resulting in cardiovascular death.

Persistent LCMV infection in the CNS

Inoculation of mice with LCMV at birth or congenital infection by vertical transmission from mothers to the fetus *in utero* leads to the establishment of a life-long persistent infection, LCMV-Pi [129,130]. In contrast to LCMV infection in adult immunocompetent mice, infection *in utero* or as neonates prevents the development of an efficient adaptive antiviral $CD8^+$ T cell response. The lack of antiviral $CD8^+$ T cells is a consequence of the continued presence of viral antigen during thymic selection of T cells that results in a failure to recognize virus as nonself. LCMV-Pi mice carry high loads of virus in most organs and body fluids and shed virus in urine and feces. Life-long persistent infection does not result in overt clinical symptoms in most mouse strains. However, mice of certain strains develop significant titers of antiviral antibodies over time. Although insufficient to clear virus, such antibodies, complexed with viral antigens, can deposit in the renal glomeruli, resulting in chronic glomerunephritis and arteritis and immune complex disease [131,132]. Likewise in some specific mouse strains certain LCMV isolates can induce growth hormone diseases syndrome related to the virus's ability to replicate in growth hormone-producing cells of the anterior pituitary [133].

In LCMV-Pi mice, viral antigen is found throughout the brain, with a high abundance in neocortex, hippocampus, and cerebellum, and to lesser levels the brain stem, thalamus, and basal ganglia [130, 134]. Within the brain parenchyma, the virus is primarily confined to neurons, and infection of astrocytes is negligible (Figure 5.3). In contrast to LCMV-Pi mice generated by intracranial inoculation at birth [130,134], congenital infection *in utero* results also in significant amounts of LCMV antigen in cells of the meninges, choroid plexus, and ventricle walls [135].

Despite the absence of inflammation or cytolysis within the brain parenchyma, LCMV-Pi mice exhibit impaired learning abilities and reduced tendency to explore a novel environment during their adult life (Figure 5.4) [136,137]. These behavioral abnormalities are associated with specific neurochemical alterations. Studies using behavioral pharmacological probes indicated that LCMV disturbs the cholinergic system [136], a finding consistent with previous reports documenting biochemical alterations in this neurotransmitter system in cells and mice persistently infected with LCMV [138]. Further, LCMV infection in neurons producing the neurotransmitter somatostatin resulted in a specific reduction of somatostatin mRNA, but not another neurotransmitter cholecystokinin [139]. More recent studies investigated the impact of persistent LCMV infection on synaptic density and plasticity, both of which have been implicated in cognitive function. Examination of the expression levels of the growth-associated protein-43 (GAP-43) and synaptophysin (SYN), which are well-established reliable markers of neuroplasticity and synaptic density, respectively, revealed that GAP-43 expression was greatly decreased in the hippocampus of LCMV-Pi mice while SYN immunoreactivity was unaffected [140]. Dissection of the molecular mechanisms by which persistent virus interferes with GAP-43 expression in a cell culture model revealed that LCMV-persistent infection affected both the rate of GAP-43 transcription and the posttranscriptional stabilization of GAP-43 mRNA induced in response to the prototypic neurotrophin nerve growth factor (NGF) [141]. This finding suggests that *in vivo* LCMV may affect the activity of neuronal signal transduction pathways that register extracellular cues, such as NGF, involved in modulation of neuronal activity. Together, these

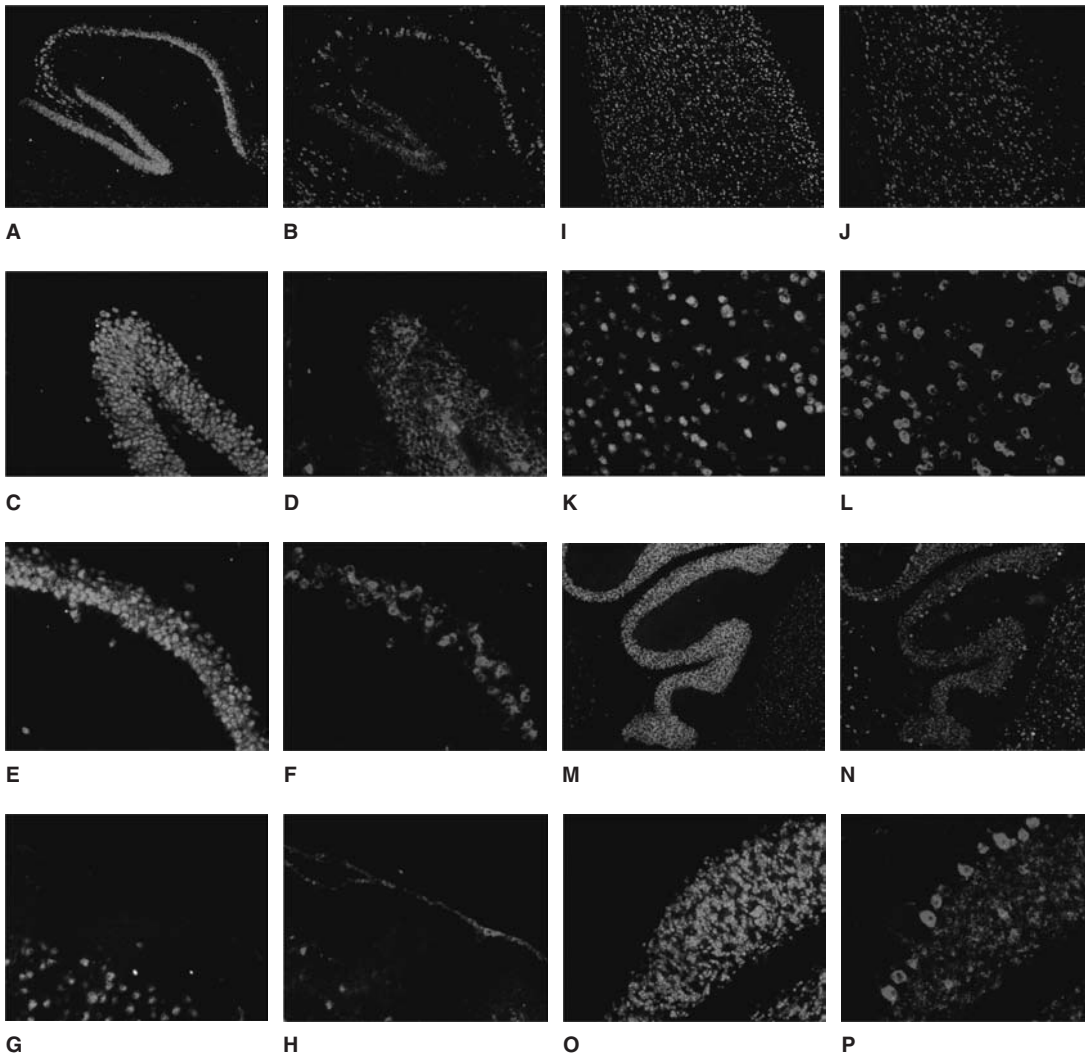

Figure 5.3. Virus distribution in the brain of LCMV-Pi mice. Viral antigen was detected in whole brain sections of 6-month-old LCMV-Pi mice by immunofluorescence staining using hyperimmune guinea pig serum to LCMV and a rhodamine red-X-labeled secondary antibody (B, D, F, H, and J, L, N, P). Neurons were labeled with an antibody to NeuN and an FITC-labeled secondary antibody (A, C, E, G, and I, K, M, O). Hippocampus (A, B), dentate gyrus (C, D), and C1 region (E, F). Meninges (G, H). Cerebral cortex (I – L), and cerebellum (M – P). Reprinted with permission from [135]. (For figure in color, please see color plate section.)

studies strongly suggested that chronic virus infection contributes to neuronal dysfunction by altering the host's gene expression profile. With the advent of DNA array technology allowing genome expression profiling, a comprehensive analysis of the impact of LCMV persistence on host gene expression in the CNS became feasible. Recent gene expression profiling studies using murine genome DNA arrays representing 39 000 host genes showed only 56 and 19 genes exhibiting significant induction or reduction,

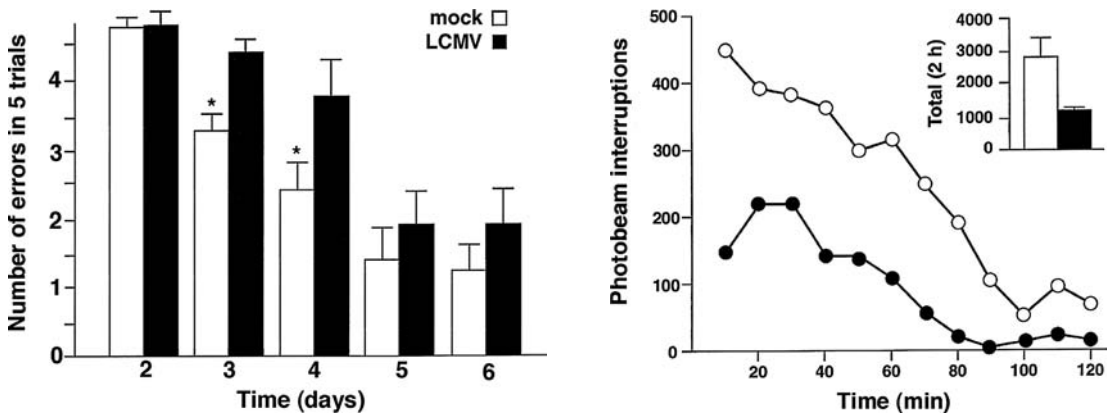

Figure 5.4. LCMV persistence in the CNS is associated with behavioral abnormalities. (A) Discriminated avoidance learning in LCMV-Pi and mock infected control mice. The behavioral abnormalities associated with LCMV-Pi mice were assessed in a nonconditional, spatial discrimination task. This test represents a short-term memory task involving trial-independent memory processes and is based on measuring the ability of the animals to learn a Y-maze spatial discrimination to avoid the onset of a mild foot shock. The number of errors, defined as entries into the wrong arm of the Y-maze or re-entering the start compartment before or after the onset of shock made by the mice (mean + SEM) during five trials/day/5 days is shown. (B) Locomotor activity during a 120-minute habituation session. LCMV-Pi and mock infected control mice.The time course of activation is presented using a 10-minute means. Values in the upper right corner represent mean + SEM for the total activity over 120-minute session.

respectively, in LCMV-Pi versus control mice, corresponding to less than 0.2% of the host genome [135]. Notably, the majority of the known genes with increased expression in LCMV-Pi mice belonged to the group of interferon IFN stimulated genes (ISGs) and included members known to play important roles in the host's innate and adaptive immune responses that contribute to the control of virus multiplication and spread. In addition, chronic upregulation of ISGs may also contribute to altered CNS function. Importantly, DNA array analysis on hippocampus tissue, a region implicated in learning and memory, of LCMV-Pi mice and uninfected controls produced similar results. The absence of changes in the expression of neuron-specific genes, although partially explained by the limited sensitivity of the screening assay, indicates that the virus causes only very subtle perturbations in the host's gene expression pattern. This striking ability of persistent LCMV to virtually merge into the genetic background of the host is likely the result of a long-term evolutionary relationship between pathogen and host species.

The most consistent changes in CNS gene expression in LCMV-Pi mice involved genes implicated in type I IFN (IFNα/β) response, which are known to play important roles in anti-viral defense and are also found up-regulated in other viral CNS infections. These genes included STAT1, IRF9, ISG15, UBP43, GARG49, and GARG16. The IFN-regulated transcription factors STAT1 and IRF9 are crucial components of type I IFN receptor signaling, which are expressed at low levels in the normal CNS, but can be induced under pathological CNS conditions including viral infection [142,143,144,145,146]. The protease UBP43 is involved in removal of ISG15, a small, ubiquitin-like protein strongly induced upon IFN stimulation from target proteins. Recent studies implicated UBP43 in the regulation of ISGylation [147] and innate immunity to viral infection [148]. Mice deficient in UBP43 were found to be resistant to lethal LCM or myeloencephalitis after

intracerebral inoculation with LCMV or vesicular stromatitis virus (VSV), respectively. The protection against LCMV-induced lethal LCM in UBP43$^{(-/-)}$ mice correlated with enhanced protein ISGylation and a concomitant reduction in viral replication. However, increased ISGylation may not be directly responsible for these findings [149]. The glucocorticoid attenuated response genes GARG39 and GARG49 have been found in the CNS after infection with Sindbis virus, rabies virus, and Japanese encephalitis virus [142,145,150], suggesting a role of these proteins in anti-viral defense in the CNS. Chronic regulation of specific genes of the type I IFN system likely contributes to curtailing the effects of virus replication and gene expression in LCMV-Pi mice and therefore provides the host with a certain degree of protection. However, since elevated levels of type I IFNs found in therapeutic setting, chronic inflammation, and persistent viral infections in the CNS are frequently accompanied by behavioral side effects [151,152,153], the up-regulation IFN-induced genes may act as a double-edged sword and contribute to CNS disturbances in the host.

Perinatal experimental LCMV infection of the rat as a model for human congenital LCMV infection

In contrast to the mouse, LCMV infection of the newborn and early postnatal rat leads to overt CNS disease that in some aspects resembles the congenital brain defects observed with intrauterine LCMV infection of humans. Three decades ago Monjan and colleagues observed that in the neonatal rat, LCMV specifically infects neurons of the cerebellum, olfactory bulb, and dentate gyrus of the hippocampus, while neuronal infection outside these regions was rare [154,155,156,157]. The selective infection of distinct neuronal populations likely explains the specific pathological changes that involve microcephaly, retinitis, and selective acute and permanent destruction of brain regions [155,156,158,159]. These neuropathologic changes result in abnormalities of movement, coordination, visual perception, and behavior.

Studies over the past years shed light on the basic mechanisms of neuroinvasion and pathology in this model that may be of relevance for LCMV congenital CNS disease in humans. The first cell types infected in neonatal rats after intracerebral inoculation with LCMV are astrocytes and Bergmann glia in proximity to the heavily infected ependymal and leptomeninges [160]. From these initial points of entry, the virus spreads continuously across the glial cell network through the parenchyma to reach specific neuronal populations susceptible to infection. The finding that glial cells are heavily infected with virus prior to neuronal infection indicates that neuronal infection is a result of viral replication and spread through glia and may involve glia-neuron interaction [160]. Glial cells are not only the preferred early target of LCMV in the CNS but also the main site of viral replication, as indicated by high virus titers in the CNS in early diseases where the virus shows predominantly glial tropism. After initial infection, spread, and replication of the virus in glia, these cells subsequently clear the infection while neurons remain persistently infected for a prolonged period of time, resulting in specific pathological changes in the affected brain regions. The selective preference of the virus for neuronal populations in cerebellum, hippocampus, olfactory bulb, and the paraventricular zone may be due to the fact that these particular regions in the neonatal rat brain contain mitotically active neuronal populations. It is conceivable that mitotically active neurons allow virus propagation and spread into adjacent postmitotic neurons like, e.g., the Purkinje cells in the cerebellum and the olfactory mitral cells. In each of the brain regions affected, the virus induces characteristic pathological changes via strikingly different mechanisms. The most apparent pathological changes occur in the cerebellum, where acute and permanent destruction of neurons is observed, resulting in a characteristic cerebellar hypoplasia [156,159,161]. More recent studies revealed important local differences in pathology within the cerebellar structure. The developmentally more immature dorsal lobules suffer complete disintegration and severe cell loss. Ventral structures in contrast show milder

pathology including disruption of granule cell migration along the infected Bergmann glia, resulting in ectopically located granule cells within the molecular layer of the cerebellar cortex. Overt tissue destruction in the cerebellum is observed during the second week of infection, is mediated by antiviral $CD8^+$ effector T cells, and can be prevented by T cell depletion [156,161]. In contrast to the cerebellum that suffers permanent damage, infected olfactory bulb tissue undergoes acute hyperplasia without losing its architecture and recovers fully with age [160]. The exact mechanism of this is unknown but likely involves specific regenerative capacities of olfactory bulb neurons. A particularly interesting pathology is observed in the hippocampus, where LCMV selectively infects granule cells of the dentate gyrus. While infection of granule cells in cerebellum and olfactory bulb results in marked acute hypoplasia, the hippocampus formation fully develops and shows initially no signs of neuronal loss. However, after a significant delay, loss of neurons from the dentate gyrus occurs that progresses over months and eventually results in a severe depletion of dentate granule cells. This delayed neuronal loss is not immune-mediated and may involve exitotoxicity [158,159]. The fourth brain region affected by LCMV infection in the neonate rat is the periventricular zone, which also contains mitotically active neurons after birth. In contrast to cerebellum, hippocampus, and olfactory bulb, no neuronal loss, perturbation of migration, or delayed onset pathology are observed in the periventricular zone [160].

Studies on different time points of LCMV infection of postnatal rats revealed a striking correlation between the time point of inoculation and the extent of neuronal infection [160]. While infection of neonates resulted in heavy virus loads in a variety of neuronal populations throughout the brain, later time points of infection resulted in a more restricted neuronal infection pattern, suggesting an inverse correlation between LCMV infection and neuronal differentiation.

A similar inverse correlation between LCMV infection and neuronal differentiation has been previously described in the rat pheochromocytoma line PC12, which represents a classical model for in vitro neuronal differentiation. PC12 cells exhibit several features of neuronal differentiation following exposure to NGF. These include the extension of neurites, and withdraw from cell proliferation becoming postmitotic cells with phenotypic properties of sympathetic neurons. While undifferentiated PC12 cells allow high levels of LCMV replication and virus production, viral multiplication is markedly impaired in PC12 cells that have undergone neuronal differentiation upon NGF stimulation [141].

Current therapy and vaccines against arenaviruses

The only licensed drug against arenaviruses, which is currently available is the nucleoside analogue ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) [162]. *In vitro* and *in vivo* studies have documented the prophylactic and therapeutic value of ribavirin against several arenaviruses, including LASV [163] and Junin virus [164]. The precise mechanisms by which ribavirin interferes with arenavirus multiplication remain to be determined, but it is highly likely that they involve disruption of different processes required for the completion of the virus life cycle. One of the problems associated with the use of ribavirin is that more than 40% of treated individuals develop haemolytic anemia, and the drug has been associated with congenital disorders [76] and hence should not be used with pregnant women (see also Chapter 20). In addition, oral ribavirin appears to be significantly less effective than the one administered intravenously, which poses additional logistic complications for its use in regions with limited clinical infrastructure.

Several ribavirin-related inhibitors of inosine monophosphate (IMP) dehydrogenase, including ribamidine (1-beta-D-ribofuranosyl-1,2,4-tiazole-3-carboxamide) [165], as well as acyclic and carbocyclic adenosine analogue inhibitors of the S-adenosylhomocysteine (SAH) hydrolase [166], have been shown to have also antiarenaviruses. Likewise, phenotiazines compounds [167], myristic

acid [168], several disulfide-based compounds, and brassinosteroids have been reported to have activity against several arenaviruses. However, none of these compounds has been tested in human trials, and their efficacy *in vivo* is currently unknown.

There is currently no licensed vaccine for LASV available. However, protection against LASV infection in nonhuman primates was conferred by vaccination with vaccinia constructs expressing LASVGP [169], demonstrating the feasibility of protective immunization against LASV infection. A more recent study using a DNA vaccine approach in a murine model evaluated the nature of the immunological correlate of protection [170]. Induction of protective $CD8^+$ T cell responses by a DNA minigene encoding a nine amino acid sequence from LASV nucleoprotein demonstrated $CD8^+$ T cell-mediated immunity to be crucial for protection. A promising novel LASV vaccine approach in nonhuman primates made use of an attenuated recombinant vesicular stomatitis virus vector expressing LASV glycoprotein (VSVΔG/LASVGP) [171]. Given the strongly attenuated nature of VSVΔG/LASVGP and the low prevalence of serum antibodies against VSV in human populations, the recombinant VSV vector used in this approach represents a promising vaccine platform for human trials.

Among the South American HF viruses, Junin virus is the only one for which a first candidate vaccine has been developed. After initial unsuccessful approaches based on killed virus, a live-attenuated Junin vaccine (Candid 1) has been developed, which is used in high-risk groups in endemic areas [90]. In the past years, more than 150 000 individuals at risk of exposure have received the vaccine, resulting in a significant reduction of AHF cases in the endemic region.

While no specific treatments have been established for Machupo and Guanarito, current therapy of Junin virus infection involves the administration of immune plasma from convalescent patients [172,173,174]. This treatment is only effective when administered during the first week of illness and critically depends on the titers of neutralizing antibodies. After treatment, approximately 10% of patients develop a self-limiting late neurological syndrome [174,175].

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6

Neurotropic alphaviruses

Diane E. Griffin

Introduction

Alphaviruses are members of the *Togaviridae* family of icosahedral, enveloped, single-strand, message-sense RNA viruses. The mosquito-borne alphaviruses are important causes of encephalomyelitis in the Americas and are on the category B list of agents of biodefense concern. Eastern equine encephalitis (EEE), western equine encephalitis (WEE), and Venezuelan equine encephalitis (VEE) viruses are the neurotropic alphaviruses of greatest importance as causes of human encephalomyelitis and were initially recognized for their ability to cause disease in horses. Semliki Forest virus (SFV) and Sindbis virus (SINV) do not usually cause encephalitis in humans, but are studied frequently in mice as model systems for alphavirus encephalomyelitis.

EEE virus (EEEV) was first isolated in 1933 from the brains of horses during an epizootic of equine encephalitis in Virginia and New Jersey [1] and was demonstrated to cause human encephalitis in 1938 [2]. In the summer of 1930 a similar equine epizootic occurred in the San Joaquin Valley of California and WEEV was isolated from the brains of affected horses [3], followed in 1938 by recovery of the same virus from the brain of a child with fatal encephalitis [4]. A related WEEV complex virus, Highlands J virus (HJV), was isolated in the eastern part of the United States in 1952 [5,6]. In 1936, an outbreak of equine encephalitis spread from Colombia into Venezuela, the virus isolated from the brains of affected horses was antigenically distinct from EEEV and WEEV and became the third encephalitic alphavirus identified in the Americas [7,8]. The first human cases of VEE to be recognized were in laboratory workers [9,10], and human disease was subsequently documented in the general population during equine outbreaks [10,11] (see also Chapter 17).

SFV was first isolated from mosquitoes collected in the Semliki Forest of Uganda [12] and is widely distributed in Africa [13,14,15]. Serosurveys indicate that infection is relatively common, but SFV-induced human disease has been recognized only twice – in a group of febrile patients in the Central African Republic and in a German laboratory worker who developed fatal encephalitis [14,16]. SFV can cause encephalitis in horses, mice, rats, hamsters, rabbits, and guinea pigs, and mice have provided an important model system for studies of alphavirus infection of the central nervous system (CNS) [12,17,18,19,20].

SINV was first isolated in 1952 from mosquitoes collected near Sindbis, Egypt, and is the most widespread alphavirus. Humans living in the Nile Delta at that time had a SINV seroprevalence of 27%, but no disease was associated with infection [21]. SINV was subsequently isolated in Europe, the Middle East, Africa, India, Asia, Australia, and the Philippines from a variety of mosquito and vertebrate species [22,23,24,25]. The first human isolates were from the blood of febrile patients in Uganda in 1961, and SINV was recognized in South Africa as a cause of rash and arthritis in 1963 [23]. Mice infected with neurovirulent strains of SINV have pathology in the brain and spinal cord similar to that observed in the CNS of humans dying of WEE, EEE, or VEE.

Natural cycles of infection, transmission, and evolution

Alphaviruses are maintained in a natural cycle between vertebrate and invertebrate hosts. The primary mode of transmission to vertebrates is through the bite of an infected mosquito (see also Chapter 20). Mosquitoes become infected by feeding on a viremic host, are able to transmit the virus 4–10 days later (external incubation), and remain persistently infected. Maintenance of this cycle requires an amplifying host that develops a viremia of sufficient magnitude to infect feeding mosquitoes. For many alphaviruses, humans are dead-end hosts with low-titered viremias. Mosquitoes salivate during feeding and deposit virus extravascularly in saliva [26]. Saliva virus titers are highest early after the mosquito is infected and decline, along with transmission rates, after 1 to 2 weeks, but mosquitoes remain infected for life [27]. Other modes of transmission are occasionally important. Horses infected with VEEV may shed virus in secretions, urine, and milk [9,28]. Aerosol transmission of VEEV has occurred in laboratory settings [9,10] and aerosolized VEEV has been developed as an agent of biological warfare [29,30]. Person-to-person transmission has not been documented [10,31].

Eastern equine encephalitis

EEEV is enzootic in North America along the Atlantic and Gulf coasts from New Hampshire to Texas, in the Caribbean, and in Central America. Birds are the primary reservoir hosts and many avian species are susceptible to infection [32]. In North America, the primary enzootic cycle is maintained in shaded swamps with the ornithophilic mosquito *Culiseta melanura* as the vector [33,34] (see also Chapter 20). The amplifying species are wading birds, migratory passerine songbirds, and starlings [35,36,37].

Outbreaks of equine, pheasant, and human encephalitis occur when the virus spreads from the enzootic cycle into mosquito populations that feed on a variety of hosts [33,34,38,39] (see also Chapter 20). In the absence of equine immunization, epizootics appear approximately every 5 to 10 years and are associated with increases in the populations of enzootic and epizootic mosquito vectors [40,41,42,43]. Most human cases are associated with exposure to wooded areas adjacent to wetlands where enzootic transmission is maintained [44]. Small numbers of human cases of EEE are diagnosed in the United States each year (Figure 6.1) [44]. There is no evidence that either horses or humans are important in the transmission cycle during epizootics. EEEV is also enzootic along the north and east coasts of South America and in the Amazon Basin, but human infections in these regions result in only mild or subclinical disease [45].

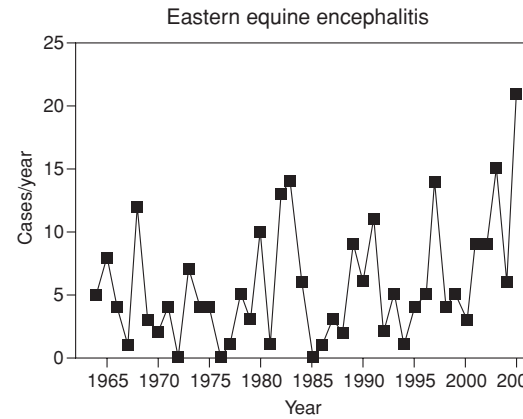

Figure 6.1. Numbers of human cases of eastern equine encephalitis reported annually in the United States since 1964. Data are from the Centers for Disease Control and Prevention.

Sequence comparisons indicate that EEEV has evolved independently in North and South America over the last 1000 years. Currently, there is one variety of EEEV in North America and the Caribbean and three varieties in South America [46,47]. North American isolates are highly conserved, differing by less than 2% in nucleotide sequence over 63 years, resulting in a calculated yearly nucleotide substitution rate of 1.6×10^{-4} [46,47]. The South American groups diverged about 450 years ago and comprise several genotypes that differ by up to 25% in nucleotide

sequences and are probably evolving locally within different vector-host relationships at a more rapid rate (4.3×10^{-4} substitutions/nt/yr) [47,48].

Western equine encephalitis

WEEV is endemic in the western portions of the United States, Canada, and South America. In North America WEEV is maintained in an endemic cycle involving domestic and passerine birds and *Culex tarsalis*, a mosquito adapted to irrigated agricultural areas [44] (see also Chapter 20). HJV is enzootic on the East Coast of the United States and is maintained in a cycle similar to that of EEEV with *Cs. melanura* the primary vector and migrating birds the primary reservoir. HJV can occasionally cause encephalitis in horses [49] and is a recognized pathogen for turkeys, pheasants, partridges, ducks, emus, and whooping cranes [47,50,51,52,53].

WEEV in North America has caused seasonal epidemics of encephalitis in humans, horses, and emus. Major epizootics occurred every 2–3 years in the western plains from 1931 to 1952 with attack rates up to 167 cases per 100 000 population [44]. An average of 34 human cases of WEE occurred per year in the United States from 1955 to 1984, but numbers of cases have steadily declined since that time (Figure 6.2). Seroprevalence in humans was 34% in rural areas of California endemic for WEEV in 1960 [54] and 1.3–2.6% in similar areas in 1993 to 1995 [55].

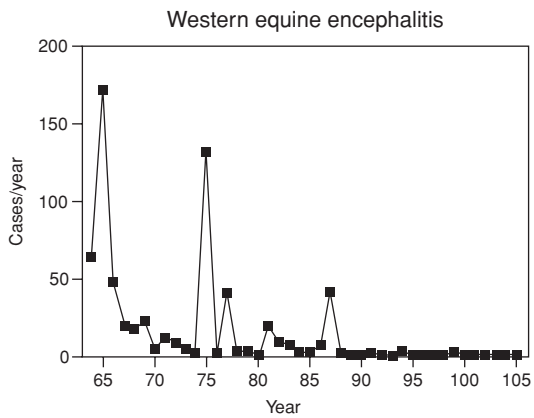

Figure 6.2. Numbers of human cases of western equine encephalitis reported annually in the United States since 1964. Data are from the Centers for Disease Control and Prevention.

WEEV is the result of a recombination between EEEV and a Sindbis-like virus [56] and has four major lineages; two in South America and two widely distributed in the Americas and the Caribbean [47,57]. In addition to these lineages of WEEV the WEEV complex includes HJV, Fort Morgan virus (FMV), and Aura virus [58]. HJV and FMV belong to lineages that diverged since recombination, while Aura is a "prerecombinant" virus. Rates of divergence of WEEV and HJV of 0.1–0.2% per year have been estimated [59]. Sequence analysis of the viruses found at the initial focus of a 1982 WEE epizootic in Argentina indicated that the enzootic virus was the source of a virulent variant that emerged by mutation or selection to cause the epizootic. The lack of significant human disease during equine outbreaks of WEE in South America may be related to the feeding habits of the vector or to a difference in virulence of South American strains of WEEV for humans and horses [60].

Venezuelan equine encephalitis

Enzootic VEE complex viruses are involved in perennially active transmission cycles in subtropical and tropical areas of the Americas. In enzootic areas isolates are primarily from *Culex (Melanoconion) spp.* mosquitoes that breed near aquatic plants [61,62,63,64] (see also Chapter 20). These mosquitoes feed on a wide variety of rodents, birds, and other vertebrates. Wild birds are susceptible to infection, but mammals are the most likely reservoir hosts [11,63,65,66,67,68].

Epizootic viruses cause significant disease in horses [11]. Virus isolations during epizootics are primarily from *Oc. Taeniorhynchus, P. confinnis*, and *Ae. sollicitans* mosquitoes, suggesting that the epizootic and enzootic transmission cycles differ [11,69,70]. VEE epizootics have occurred at approximately 10- to 20-year intervals in cattle ranching areas of Venezuela, Colombia, Peru, and Ecuador

when heavy rainfall leads to increased populations of epizootic mosquito vectors [31].

In addition, formalin-inactivated vaccines containing residual live virus initiated outbreaks in South America that spread to Central America and Texas between 1969–1972 and in Peru in 1973 [71,72]. During epizootics horses are an important amplifying species and availability of susceptible equines provides a means for virus spread [69]. Epizootic potential of the virus may be related to mutations that increase infectivity for the vector *Oc. taeniorhynchus* [73] and/or increase the level of viremia in equines [74].

The VEE complex includes six subtypes: VEE (I), Everglades (II), Mucambo (III), Pixuna (IV), CAB (V), and Rio Negro (VI). The greatest sequence divergence is in the E2 glycoprotein and the C-terminal region of nsP3 [71,75]. Studies of differential virulence of epizootic and enzootic strains of VEEV first suggested that, like WEEV, epizootic strains arise from nonpathogenic enzootic strains [76]. All epizootic IAB and IC strains are related to enzootic ID strains [11,71].

The virus, its life cycle, and cellular effects

Alphavirus virions are 60 to 70 nm in diameter and sensitive to ether and detergent. The RNA genome is approximately 11 700 nucleotides long, capped and polyadenylated and surrounded by multimers of a single capsid (C) protein arranged as an icosahedron with T = 4 symmetry (Figure 6.3). This nucleocapsid is enclosed in a lipid envelope that is derived from the host cell plasma membrane and contains the viral-encoded glycoproteins E1 and E2, which form heterodimers that are grouped as trimers to form 80 spikes on the virion surface [77]. Glycoproteins are arranged such that 240 copies of each interact with 240 copies of C. E2 is on the spike surface and involved in attachment to cellular receptors, whereas E1 forms a relatively flat skirt-like structure close to the virion surface and is important for fusion of the virus and cell membranes to initiate infection [78].

Binding of virus to the cell surface and entry into the cell is a multistep process that is dependent on E1 and E2 viral glycoproteins, cell surface molecules, low pH in the endosome, and fusion of membrane lipids. Variations in any of these components will affect the efficiency of infection and the likelihood that any particular cell will become infected *in vivo*. Because each alphavirus infects a wide range of hosts, often including birds, mammals, and mosquitoes, they must either use an evolutionarily well-conserved cell surface molecule or multiple molecules as receptors for initiation of infection. None of the receptors identified to date appears to be used exclusively, suggesting the possibility of several receptors. Alternatively, alphaviruses may use receptor-coreceptor combinations to achieve the wide host range and the specific tropisms observed *in vivo*. The receptor important for alphavirus binding and entry into neurons is unknown.

Initial binding is often through heparan sulfate (HS), a highly sulfated, negatively charged molecule [79,80,81]. The E2 glycoprotein of SINV contains a heparin-binding domain that overlaps a neutralizing epitope, and positively charged residues in this region increase the efficiency of attachment to cells in tissue culture [82,83]. Basic amino acids in other regions of SINV and VEEV E2 also contribute to HS binding, suggesting that the interacting site is conformational [82,84]. However, improved HS binding generally decreases virulence for mice, suggesting that amino acid changes that improve HS binding represent adaptations of the virus to replication *in vitro* [82,83,84].

Entry into the cell after initial binding requires endocytosis followed by a conformational change in the trimer of E1-E2 heterodimers induced by exposure to low pH in the early endosome [85–88]. This conformational change results in dissociation of E2 from E1 [89], the formation of E1 trimers [90], fusion of the viral envelope with the endosomal membrane, and delivery of the nucleocapsid into the cytoplasm. Fusion is a property of the E1 glycoprotein, a class II fusion protein with an internal fusion peptide [91,92,93,94]. Cholesterol and sphingomyelin are required for E1 binding and membrane fusion

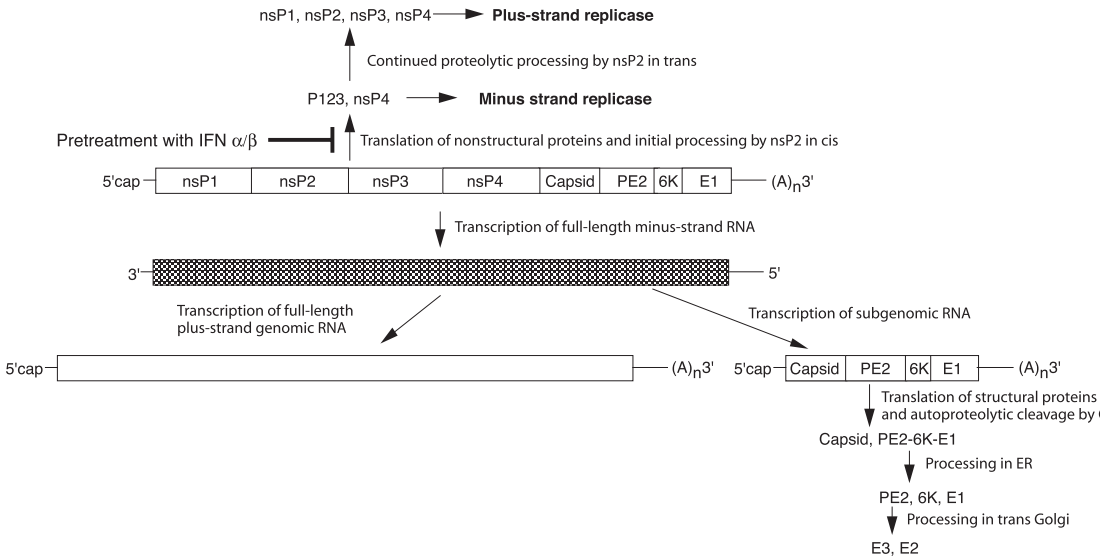

Figure 6.3. Schematic diagram of the replication of alphaviruses showing the transcription of a subgenomic RNA, processing of the nonstructural and structural proteins, and the stage of replication inhibited by pretreatment with type 1 interferon.

[95], which occurs through a process similar to that used by class I fusion proteins [96,97,98]. Amino acid changes in E1 can affect the fusion capacity, the lipid requirements for the target cell membrane, and the optimal pH for fusion [96,99,100,101,102,103].

The genome is released from the nucleocapsid by ribosomal removal of C [104]. The 5′ two-thirds of the message-sense genome encodes four nonstructural proteins (nsPs) that function in replication of the viral RNA and production of the subgenomic RNA [77]. nsPs are translated from genomic RNA as two large polyproteins (P123 and P1234) that form replication complexes that are tethered to cytoplasmic vacuoles formed from modified endosomal membranes [105]. The polyproteins are processed into individual proteins by a papain-like protease in the C-terminal portion of nsP2 [106]. NsP1 has methyl transferase and guanylyltransferase activities, is palmitoylated, and binds the replication complex to membranes [107,108,109]. The N-terminal domain of nsP2 has helicase, ATPase, GTPase, and 5′-triphosphatase activity [110,111]. nsP3 is a phosphoprotein that induces membrane remodeling necessary for the formation of cytoplasmic vacuoles [112]. nsP4 is the RNA-dependent RNA polymerase [77].

RNA transcription is initiated by synthesis of a full-length minus-strand that then serves as the template for the synthesis of both subgenomic mRNA and genomic RNA (Figure 6.3). Regulation of RNA synthesis is dependent on the processing of the nsPs [113,114,115]. Early in infection, nsP2 cleaves P1234 into the minus-strand replicase, P123 plus nsP4 [114,116]. Later in infection, P123 is cleaved into nsP1, nsP2, and nsP3. This processing changes the template specificity of the replicase to increase synthesis of plus strands and to shut off synthesis of minus strands [115,116,117]. The C-terminal half of nsP2 plays a critical role in the switch from minus- to plus-strand synthesis [118,119] and in regulation of the synthesis of subgenomic 26S RNA [120]. Only fully cleaved nsP1 + nsP2 + nsP3 + nsP4 complexes are functional in 26S RNA synthesis [116,121]. It is postulated that nsP2 binds directly or indirectly to the subgenomic promoter and may function both as part of the polymerase core and as a soluble

regulatory molecule for the transcription of subgenomic RNA [120,122].

The 26S subgenomic RNA is the mRNA for translation of the structural proteins as a large polyprotein NH2-C-E3-E2–6K-E1-COOH [123]. C is autoproteolytically cleaved from the nascent chain and is rapidly assembled with genomic RNA into nucleocapsids. Precursor of E2 (PE2, E3 + E2), 6K, and E1 are synthesized in association with the endoplasmic reticulum. The E3 protein serves as the signal sequence for E2, a transmembrane protein that has two or three N-linked glycosylation sites and contains the most important epitopes for neutralizing antibody. The cytoplasmic portion of E2 has a second stretch of hydrophobic amino acids that tethers it to the inner surface of the membrane. The 6K protein is the signal peptide for E1, is important for virion budding, and small amounts are incorporated into the virion [124,125,126]. E1 has one or two N-linked glycosylation sites, a short (one or two residue) intracytoplasmic tail, and a positionally conserved internal hydrophobic stretch of amino acids in the N-terminal portion that serves as the fusion peptide for virion entry into the cell.

PE2 and E1 are transported with 6K as a noncovalently-associated hetero-oligomeric complex through the cell secretory pathway to the plasma membrane. Late in the pathway PE2 is processed by a furin-like protease to E2 and E3, a small glycopeptide, which is shed from the cell surface. The N-terminal portion of C is conserved, basic, and is presumed to bind the viral genomic RNA while the C-terminal portion interacts with the cytoplasmic tail of E2 and with other copies of the C protein to form the nucleocapsid [77]. At the plasma membrane, the specific association of E2 tails with nucleocapsids [127,128] initiates a budding process that leads to the release of mature virions.

Alphaviruses replicate rapidly in most vertebrate cell lines with the release of progeny virus within 4 to 6 hours after infection. At the time of virus entry there is an increase in permeability, perhaps due to pore formation by the E1 and/or 6K proteins [129,130,131]. Infection causes extensive cytopathic effect characterized by cell rounding, shrinkage, and cytoplasmic blebbing with the death of infected cells within 24 to 48 hours [132,133]. Many alphaviruses kill cells by inducing apoptosis, a process associated with blebbing of the plasma membrane, condensation of nuclear chromatin, and formation of apoptotic bodies. Viral proteins are concentrated in the surface blebs from which budding continues to occur [134]. This process does not hamper, and may enhance, virus replication because inhibition of apoptosis usually decreases virus yield [135,136,137].

The mechanisms by which alphaviruses induce apoptosis are not completely understood. Apoptosis of cultured cells can be initiated during SINV fusion [138]. Membrane-bound sphingomyelinases are activated and sphingomyelin is degraded, releasing ceramide, an efficient inducer of cellular apoptosis [139,140]. Subsequent early events include activation of poly(ADP ribose) polymerase [141] followed by activation of cellular caspases, cleavage of caspase-3 substrates, and fragmentation of chromosomal DNA [142]. Alphavirus-induced apoptosis can be slowed or prevented, often in virus strain- and cell type-dependent ways, by expression of ceramidase [139], expression of a dominant inhibitory form of Ras [143], inhibition of constitutive expression of NFκB [144], overexpression of Bcl-2 family member and interacting proteins [132,136,137,145, 146,147,148], phosphorylation of PKCδ [149], and inhibition of caspase activity [150,151].

Alphavirus-induced vertebrate cell death can also occur by nonapoptotic mechanisms. Alphaviruses efficiently shut down host protein and mRNA synthesis [152,153,154], deplete nicotinamide adenine dinucleotide (NAD) and energy stores [142,155], and induce dysfunction of Na^+K^+ATPase, causing loss of membrane potential and a change in intracellular cation concentrations [156,157,158,159,160]. Although immature neurons die by apoptosis, mature neurons are more resistant to apoptotic cell death and, when infected by virulent strains of virus, can die by nonapoptotic pathways [161,162]. In particular, mature motor neurons become pale and swollen and are not protected from death by Bcl-2 family member proteins [161,163].

Clinical disease in humans

Eastern equine encephalitis

North American strains of EEEV are the most virulent of the encephalitic alphaviruses and cause high mortality in all age groups [44,164]. A prodromal illness consisting of 1–2 weeks of fever, chills, malaise, and myalgias begins days after the bite of an infected mosquito (see also Chapter 19). In cases of encephalitis these prodromal symptoms are followed by the onset of headache, confusion, vomiting, restlessness, and irritability leading to seizures, obtundation, and coma [164,165,166,167]. Children under 10 years of age are most susceptible [164] with 1 in 8 infections in children resulting in encephalitis compared with 1 in 23 infections in adults [168]. Meningismus is frequent and focal signs including cranial nerve palsies and paralysis are common [165,169]. Hyponatremia due to inappropriate secretion of antidiuretic hormone is a common complication, and edema of the face and extremities has been noted [165,166]. The case-fatality rate is 30–40%, with the highest rates in children and the elderly [44,164,165,166,169]. Death typically occurs within 2–10 days after onset of encephalitis.

Cerebral spinal fluid (CSF) is almost always abnormal. Pressure and protein are increased, glucose is low to normal, red blood cells and xanthochromia are commonly present, and white cell counts range from 10 to 2000/μL [166,169]. Polymorphonuclear leukocytes may be abundant early with a shift to mononuclear cells over the first few days [165]. Electroencephalograph (EEG) abnormalities are relatively nonspecific, usually showing slowing [165]. Computed tomographic (CT) scans may be normal or show only edema [165,169]. Magnetic resonance imaging (MRI) scans are more often abnormal with focal lesions most commonly observed in the thalamus, basal ganglia, and brain stem [165].

Poor outcome is predicted by high CSF white cell count or severe hyponatremia, not by the size of the radiographic lesions [165]. Recovery is more likely in individuals who have a long (5–7 day) prodrome and do not develop coma [169]. Sequelae, including paralysis, seizures, and mental retardation, are common and 35–80% of survivors, particularly children, have significant long-term neurological impairment [164,165,166].

Western equine encephalitis

WEEV causes encephalitis with signs and symptoms similar to those of EEEV, but the case fatality rate of 3% is lower [170]. There is a 3 to 5 day prodrome of fever and headache that may progress to restlessness, tremor, irritability, nuchal rigidity, photophobia, altered mental status, and paralysis [171,172,173] (see also Chapter 19). CSF pleocytosis is typical with 100 to 1500 cells/μL. Neutrophils are present early in disease and mononuclear cells later [172]. Infants often present with rigidity, seizures, and a bulging fontanel [171,172]. Transplacental transmission results in perinatal infection manifesting within the first week of life as fever, failure to feed, and seizures [172,174].

Clinically apparent disease is most common in the very young and those over 50 [170]. The estimated case to infection ratio is 1:58 in children under 5 years and 1:1150 in adults [44]. In older children and adults, males are 2 to 3 times more likely to develop disease than females [170]. Infants and young children are more likely to develop seizures, fatal encephalitis, and significant sequelae [171,173,175]. In infants of less than 1 year, approximately 60% of survivors have brain damage and, in some, the disease is progressive [172,176]. Common problems are mental retardation associated with quadriplegia, spasticity, recurring seizures, cortical atrophy, ventricular dilation, and intracranial calcification [171,175,176,177]. In older individuals recovery is typically rapid with remission of signs and symptoms within 5–10 days, and sequelae are less common [171].

Venezuelan equine encephalitis

Clinically evident human infection can occur with enzootic, as well as epizootic, VEE complex viruses [11,178,179]. Humans living in areas of enzootic

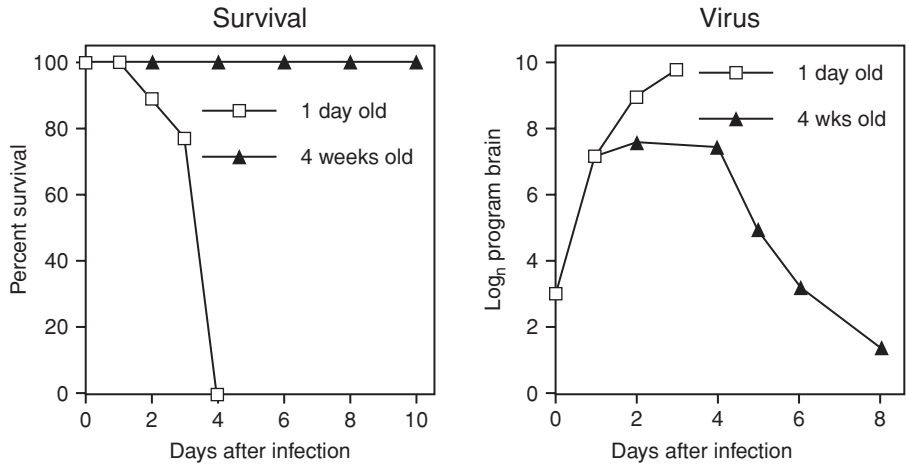

Figure 6.4. Age-dependence of alphavirus-induced encephalitis in mice. Young mice have high levels of virus replication in the central nervous system and die within a few days after infection while older mice are able to restrict virus replication, clear virus, and survive.

transmission have a high prevalence of antibody associated mostly with undiagnosed mild febrile illnesses [63,66,180]. Accidental laboratory aerosol infections of young adults with epizootic strains of VEEV caused a febrile illness with the abrupt onset of chills, headache, myalgia, somnolence, vomiting, diarrhea, and pharyngitis without evidence of encephalitis 2–5 days after exposure [9,10,179]. During epizootics human attack rates vary widely [31], and neurological symptoms tend to appear 4 to 10 days after onset of illness with headache and vomiting the most common initial symptoms followed by focal or generalized seizures, paresis, behavioral changes, and stupor or coma [31,181].

All ages and both sexes are equally susceptible to infection; however, disease manifestations vary with age (Figure 6.4) [31]. Individuals under the age of 15 are more likely to develop fulminant disease with reticuloendothelial infection, lymphoid depletion, and encephalitis. Children recovering from encephalitis may be left with neurological deficits, particularly seizure disorders [182]. Individuals over 50 are also prone to develop encephalitis, but most recover [179]. The incidence of encephalitis is generally less than 5% and the mortality less than 1% [31]. Essentially all deaths occur in children.

Laboratory studies often show lymphopenia. Fetal abnormalities, spontaneous abortions, and stillbirths may occur with infection during pregnancy [31,181]. Congenitally infected infants show severe neurological damage occasionally resulting in hydranencephaly [183].

Pathogenesis and determinants of outcome

The initial sites of virus replication probably vary with the virus and host. Mice have received most extensive study. After subcutaneous inoculation viruses may infect skeletal muscle at the local site (e.g., EEEV, WEEV, SFV, SINV) or be taken up by and infect Langerhans cells in the skin (e.g., VEEV) [184,185,186,187,188,189]. Infection of these antigen-presenting cells near the skin may be the most common mode of initiating natural infection. Langerhans and dendritic cells transport virus to lymph nodes, draining the site of inoculation where further replication may occur [190].

Initial replication is followed by a substantial plasma viremia in amplifying hosts and in hosts susceptible to disease. The ability to sustain a viremia is dependent on the continued efficient production of virus at a primary site of replication, delivery of virus

into the vascular system, and slow clearance from the blood. In mice, muscle, secondary lymphoid tissue, cardiac myocytes, osteoblasts, brain and spinal cord neurons, and brown fat cells are frequent secondary sites of replication [187,189,191,192,193]. EEEV, WEEV, SFV, and SINV cause encephalitis [187,194,195,196], and VEEV causes lymphoid depletion as well as encephalitis [197,198,199,200]. The ability to invade target organs is dependent in part on the duration and height of the viremia but also on other invasive characteristics of the virus [201].

The mechanism by which encephalitic alphaviruses enter the CNS is not entirely clear. Murine studies have shown infection or transport by cerebrovascular endothelial cells [184,202,203,204], infection of choroid plexus epithelial cells [187], infection of olfactory neurons [205,206,207,208], and transport by peripheral nerves [207,209]. Once within the CNS, virus can spread cell to cell or through the CSF [205,208,210]. For most encephalitic alphaviruses, the targeted cells within the CNS are neurons [187,195,208,211] and damage to this cell can be severe and irreversible. SFV and VEEV can also cause persistent CNS infection associated with infection of microglial and oligodendroglial cells leading to demyelination [17,184,202,212,213,214,215].

Outcome is influenced by characteristics of both the host and the virus. Most alphaviruses show an age-dependent susceptibility to disease [18,191, 208,216,217,218] (Figure 6.4). Resistance to fatal disease develops between 1 and 3 weeks of age in mice [18,184,219] and is associated with decreased virus replication at the site of virus inoculation and in target tissues (e.g., brain) and not with changes in the ability of infected mice to mount a virus-specific immune response [185,193,218,220,221, 222]. Genetic background of the host is an additional determinant of severity of encephalitis [205,223,224,225,226], but the specific genes determining susceptibility are just beginning to be identified [205,224,225]. For instance, defects in acid sphingomyelinase increase susceptibility to fatal disease [227]. For SINV, C57BL/6 mice are most susceptible to fatal encephalomyelitis and this is determined in part by a gene on chromosome 2 [228].

Virus strains differ in virulence, a measure of the ability of the virus to cause fatal disease reflecting the severity of neurological disease. Alphavirus strains with decreased virulence may replicate poorly even in newborn animals while virulent strains can replicate well and cause disease in adult, as well as newborn, animals. Peripheral replication, viremia, neuroinvasiveness (ability to enter the CNS efficiently), and neurotropism (ability to replicate in CNS cells) all contribute to virulence and are likely to be influenced by different molecular characteristics of the virus [229]. Many alphavirus strains lack neuroinvasiveness in that they can cause fatal disease after intracerebral or intranasal inoculation, but not after subcutaneous or intraperitoneal inoculation. Susceptibility to interferon (IFN)-mediated inhibition of replication also leads to decreased virulence.

Natural isolates vary in virulence. For instance, human encephalitis due to EEEV has not been recognized in South America [45], but occurs regularly in North America, and South American strains are also less virulent in experimental animals [34,230]. Likewise, epizootic strains of WEEV appear to be optimized for viremia and neuroinvasiveness and are generally more virulent for mice and guinea pigs than are enzootic strains, and North American strains are more virulent than South American strains [76,231,232]. The time course of virus clearance from the blood often correlates with virulence, virulent strains being cleared more slowly than avirulent strains [233,234,235,236].

Viruses with altered virulence have also been selected after chemical mutagenesis [237,238], by passage in tissue culture [239,240,241,242], by passage in mice [242,243], by isolation of plaque variants [244], and by manipulation of cDNA virus clones [240,245,246,247,248]. Nucleotide and amino acid changes affecting virulence have been mapped to the 5′ NTR and to nsP2, nsP3, E1, and E2 [71,74,184,229, 240,245,247,248,249,250,251,252,253,254,255,256, 257,258,259,260].

Eastern equine encephalitis

EEEV is neurovirulent for monkeys, mice, guinea pigs, and hamsters and can initiate infection in

the CNS by spread to choroid plexus epithelial cells [1,187,196,261,262]. Young mice have extensive neuronal damage and rapid death while older mice become relatively resistant to infection by the peripheral, but not the intracerebral, route of inoculation [187,196]. Hamsters develop fatal encephalitis, hepatitis, and lymphadenitis characterized by extensive vasculitis and hemorrhage [261].

Western equine encephalitis

Newborn mice experimentally infected with WEEV die within 48 hours with involvement of skeletal muscle, cartilage, and bone marrow. In weanling mice, brain, heart, lung, and brown fat appear to be the primary target tissues [191]. After intracerebral inoculation there is infection of the choroid plexus and ependyma with subsequent spread to neurons and glial cells in the brain, cerebellum, and brain stem and to motor neurons in the spinal cord [187]. After peripheral inoculation, WEEV replicates in skeletal and cardiac muscle and occasionally spreads to the CNS [187,192]. Infection of hamsters with relatively avirulent WEEV strains leads to progressive neuropathological changes consisting of perivascular inflammation, microcavitation, and astrocytic hypertrophy [263]. Macaques develop fever and encephalitis with infection of neurons and mononuclear inflammation [264].

Venezuelan equine encephalitis

Infection of macaques by aerosol or subcutaneous inoculation with enzootic and epizootic strains of VEEV elicits a biphasic febrile response – the first phase is coincident with the viremia and the second phase with termination of viremia, that is, the appearance of the immune response [197,265,266]. Leukopenia is common. Symptoms are usually mild, consisting of anorexia, loose stools, irritability, and occasionally loss of balance, tremor, or myoclonus [265].

Experimental infection of small laboratory animals with VEEV produces a variety of disease patterns. After subcutaneous inoculation of guinea pigs, rabbits, or hamsters with virulent strains of VEEV there is a viremia and virus spreads to bone marrow, lymphoid tissues, and brain. There is rapid destruction of myeloid and lymphoid cells, damage to the intestinal wall and pancreas, cerebral hemorrhage, and neuronal cell death [197,198,199,200]. Death occurs 2–4 days after infection and may be associated with ileal necrosis, bacteremia, and endotoxemia [267].

In mice, in addition to myeloid and lymphoid necrosis, there is encephalomyelitis leading to death in 6 to 7 days [197]. Virus replicates first in Langerhans cells that migrate to the draining lymph node after subcutaneous inoculation [190]. Virus enters the CNS by the olfactory route after respiratory or peripheral inoculation. There is initial infection of olfactory epithelium with spread to olfactory neurons and subsequent spread caudally to all regions of the brain, causing encephalitis and neuronal apoptosis [206,207,224,268]. Fatal disease has an immunopathologic component dependent on the strain of mouse infected [269,270]. There can also be transplacental transmission of infection [271].

Comparative studies of the virulent TRD and avirulent TC-83 strains of VEEV and construction of recombinant viruses identified the 5′ NTR and the E2 glycoprotein as important determinants of VEEV virulence for mice [240,249]. Attenuated viruses infect dendritic cells less efficiently and replicate less well in lymphoid tissue and in the CNS than virulent viruses [190,224,272]. Virulence for guinea pigs is determined by both envelope and non-envelope genes [273,274]. Analysis of the E2 sequences and construction of recombinant endemic and epidemic VEE viruses indicate that determinants of equine virulence are different from determinants of murine virulence, but that they also lie largely within the envelope genes [71,74,254]. Changes most frequently associated with acquisition of equine virulence are a Thr to Met change at position 360 of nsP3 and replacement of uncharged residues with Arg at positions 193 and 213 of the E2 glycoprotein [253].

Sindbis virus

In young mice, virus replicates to high titer and spreads rapidly, causing death in 3–5 days. In older

mice, virus replication is more restricted and animals often recover [275] (Figure 6.4). After peripheral inoculation virus replicates in muscle, produces a viremia, and then spreads to the brain and spinal cord where the primary target cells are neurons [210].

Strains differing in virulence have been derived from independent isolates from Egypt (AR339), South Africa (SR86), and Israel (SV-Peleg). Variants of AR339 and SV-Peleg have been derived by passage in mice and in tissue culture [243,276]. Virulence is determined primarily by the 5′ NTR and the E2 glycoprotein but can be influenced by changes in E1 and the nsPs [245,255,256,257,277]. A change in nucleotide 5 or 8 from A to G increases neurovirulence by unknown mechanisms [229,256]. A number of amino acid changes in the E2 glycoprotein affect virulence by altering efficiency of virus entry into the CNS or by enhancing neuronal infection [247,255,258,259]. Neuroinvasion is affected by changes at residues 55 and 190 of E2 [229].

The neuroadapted strain, NSV, was derived from AR339 [21] by passage through mouse brain and causes fatal encephalomyelitis in 4- to 12-week-old mice [243]. NSV has the same cellular tropism (*i.e.*, neurons) as AR339 but replicates to higher titers in the CNS [195,205,243]. Motor neurons in the brain and spinal cord are particularly susceptible to infection, and paralysis is a frequent manifestation of disease [195,210]. Changes in the AR339 genome that lead to increased neurovirulence have been identified in the E1 and E2 glycoproteins, nsP2 and the 5′ NTR [247,248,255]. A Gln to His change at E2–55 increases efficiency of infection of neurons and is a major determinant of increased virulence in older mice [247,258,278,279].

In vitro studies of cultured cortical neurons showed that SINV infection induces both apoptotic and lytic neuronal cell death and that bystander death of uninfected neurons contributes substantially to death in these cultures. Treatment with *N*-methyl-D-aspartate (NMDA) receptor antagonists of glutamate excitotoxicity protect from early lytic death [280]. These results provide evidence that alphavirus infection damages neurons by activating neurotoxic pathways that result in excessive glutamate receptor stimulation, as well as by the direct effects of virus replication.

Semliki Forest virus

Isolates from mosquitoes collected in 1942 in Uganda (V12, V13, and L10) [12], in 1948 in Nigeria [13], and in 1959 in Mozambique (A7 and A7–74) [15,18], have given rise to a variety of strains of SFV with differing levels of virulence. Virulent and avirulent strains differ in their ability to invade and replicate in the CNS of weanling mice and rats after peripheral inoculation [17,281], but all strains cause fatal disease in newborn or suckling mice [17,184,185,188]. In 3- to 4-week-old mice avirulent SFV is restricted in replication and spread in the CNS compared to virulent strains of virus and compared to avirulent strains in younger mice [219]. This difference in replication is associated with decreased budding of infectious virus and is independent of the host immune response [184,222]. In general, reduced virulence correlates with reduced replication in neurons [194]. Mature neurons can be made more susceptible to virus replication by treatment with aurothiolate compounds that induce intracellular membrane proliferation [184,282].

In vitro studies of the differences between virulent and avirulent strains of SFV have shown differential replication in mouse neuroblastoma cells and primary cultures of rat neurons [283]. Efforts to identify specific nucleotide and amino acid changes important for virulence have utilized comparative sequence analysis and an infectious SFV cDNA clone pSP6-SFV4 derived from the prototype virulent L10 strain. Construction of SFV4/A7 chimeric viruses has shown that determinants of virulence reside in both the structural and nonstructural regions of the genome [246]. Subsequent studies showed that E2, nsP2, and nsP3 are important determinants of virulence [184,250,251,252,260].

Focal areas of demyelination are found 14–21 days after infection and are characterized initially by swelling and vacuolation of oligodendrocytes and loss of myelin sheaths followed by remyelination [212]. Demyelination is macrophage-mediated and appears to be the result of oligodendrocyte

infection, the immune response to infection, and induction of an autoimmune response to myelin [284,285,286]. SJL mice have more prolonged inflammatory responses and demyelination after infection than other strains of mice [223,287]. SFV infection of the CNS can also increase the susceptibility of mice to induction of experimental autoimmune encephalomyelitis [288] apparently by damaging the blood-brain barrier, increasing adhesion molecule expression on endothelial cells and facilitating entry of autoimmune T lymphocytes into the CNS [204,289,290].

Immune responses and virus clearance

Alphaviruses induce robust immune responses that include early innate cytokine responses that control initial virus replication followed by antibody and cellular immune responses that lead to viral clearance.

Innate responses

Type I (α/β) IFN is abundantly induced after alphavirus infection of experimental animals [281,291,292, 293,294,295,296] and presumably humans. Alphaviruses and strains of alphaviruses vary in their ability to induce IFN [293,297,298], and the amount of IFN produced is usually linked to the level of virus replication [216,292]. The primary source of early IFN *in vivo* may be plasmacytoid dendritic cells [299, 300].

In vitro, induction of IFN requires viral entry and RNA synthesis and results in activation of IFN regulatory factor-3 (IRF-3) [301,302]. Study of temperature-sensitive *(ts)* mutants suggests that formation of dsRNA is necessary for IFN induction because viruses with mutations in the protease domain of nsP2, which cannot process the nonstructural polyproteins, and thus cannot initiate plus-strand RNA synthesis, do not induce IFN [90,303,304]. SINV stimulates formation of the IRF-3/CBP/p300 transcriptional activation complex for immediate early IFNs [301]. Production of IFN follows the initial release of virus from infected cells by 2 to 3 hours [305].

Treatment of cells with IFN inhibits alphavirus replication [292,306,307,308,309,310], but the mechanism by which this occurs, and therefore the IFN-induced host responses important for control of replication, are not known. Attachment and entry are not affected and input mRNA is translated [308,311]. However, later replication steps, including formation of replication complexes, structural protein synthesis, and morphogenesis, are inhibited [310,311].

IFN-induced proteins shown to have an effect on alphavirus replication are human MxA, a large cytoplasmic GTPase [312], IFN-stimulated gene (ISG)-15, and zinc finger antiviral protein (ZAP) [313]. Transgenic expression of the human MxA protein in IFN-α/β receptor-deficient mice results in decreased SFV replication by preventing accumulation of genomic and subgenomic RNA and provides some protection against fatal disease [314]. The RNA-binding protein ZAP blocks translation of incoming viral genomic RNA [313,315]. Well-studied antiviral proteins PKR and RNase L have limited roles in the IFN-induced antiviral response *in vitro* or *in vivo* [307]. PKR does not have a major role in alphavirus-induced inhibition of host protein synthesis [316]. Interestingly, SINV-infected RNase L-deficient fibroblasts fail to shut off minus-strand RNA synthesis or to form stable replication complexes. The cells become persistently infected, suggesting a direct or indirect role for RNase L in virus replication [317]. Virus strains vary in their sensitivity to the antiviral activities of IFN and this may or may not correlate with virulence [318,319,320]. Mutations associated with altered sensitivity to IFN have been mapped to the 5′ NTR, nsP1, and nsP2 [318,321, 322,323].

IFN is an important part of the host response to alphavirus infection. Animals can be protected from lethal infection if treated with IFN or IFN inducers before or soon after infection [292,324,325,326,327]. IFN limits virus replication early, during the time the specific immune response is being induced. Animals unable to respond to IFN due to deletions of the IFN receptor or crucial IFN-signaling molecules develop fatal infections even when infected with normally avirulent strains of virus [318,328,329]. Absence of

an IFN response allows virus replication in cells previously resistant to infection [330].

In the CNS, neuronal infection leads to production of IFN-β by both neurons and glial cells, and IFN-β has a role, independent of IFN-α, in early control of virus replication in the CNS [331]. Injured neurons also produce IFN-γ interleukin (IL)-6, and chemokines and macrophages and glial cells become activated in response to neuronal infection and rapidly produce an additional array of cytokines and chemokines [332]. Production of these factors results in the upregulation of major histocompatibility complex (MHC) molecules on microglial cells and increased adhesion molecule expression on capillary endothelial cells, which facilitate subsequent entry of inflammatory cells into the CNS.

IFN may also contribute to alphavirus-induced disease. Fever during the viremic phase of infection is probably a response to the IFN induced early after infection. It has also been postulated that the rapidly fatal disease in newborn mice may be due to the production of large amounts of IFN and other cytokines [294]. Acute-phase responses induced by alphaviruses prior to the virus-specific immune response include increases in tumor necrosis factor (TNF)-α, IL-1, and IL-6, and levels generally correlate with the extent of virus replication [294,332,333,334]. Adult mice deficient in IL-1β have reduced mortality after CNS infection with a neurovirulent strain of SINV, again suggesting the possibility that cytokine effects may contribute to mortality [335].

Virus-specific adaptive responses

Both humoral and cellular immune responses are induced by infection. In experimentally infected adult mice, antiviral antibody is usually detected in serum within 3 to 4 days after infection [217,220,261]. The cellular immune response, manifested by the presence of virus-reactive lymphocytes in draining lymph nodes and blood and the infiltration of mononuclear cells into infected tissues, also appears within 3 to 4 days after infection [336,337,338]. These responses appear later (7–10 days after infection) in neonatal mice that survive infection [293]. Both appear to play a role in recovery from infection and protection against reinfection.

Humoral immunity

Virus-specific IgM antibody is produced very early in human disease and often provides a means for rapid diagnosis of infection [339,340,341,342]. IgG antibody appears in serum after 7–14 days and is maintained at relatively high levels for years [202,343,344]. Appearance of antibody correlates with cessation of viremia and many lines of evidence support the hypothesis that recovery from alphavirus infection is dependent in large part on the antibody response [243,345,346,347]. Rapidity of host antibody synthesis is predictive of outcome from encephalitis; patients without evidence of antibody at the time of onset of illness are most likely to die [340].

The most extensive experimental studies to define the antibody specificity and the mechanisms of antibody-mediated recovery and protection have been done using VEEV, SFV, and SINV infections of mice. Passive transfer of antibody before or after infection can provide protection. Both neutralizing monoclonal antibodies (MAbs), mostly anti-E2, and nonneutralizing MAbs, both anti-E1 and anti-E2, can protect against alphavirus challenge and promote recovery [348,349,350,351,352,353,354,355,356,357,358], suggesting that virus neutralization per se is not the only mechanism of protection. Protection often correlates with the ability of the MAb to bind to the surface of infected cells, but this is not absolute [354]. Complement may play an auxiliary role by promoting virus clearance by the reticuloendothelial system [359,360].

Antiviral antibody can also inhibit alphavirus replication intracellularly and therefore promote clearance and recovery. Treatment of immunodeficient mice persistently infected with SINV or SFV with antiviral antibody clears infectious virus from the CNS without causing death of infected neurons or neurological damage [222,361]. MAbs specific for either of the neutralizing epitopes on SINV E2 can downregulate intracellular virus replication *in vivo*

and *in vitro* by a nonlytic mechanism [361]. Antibody against an N-terminal peptide of VEEV E2 that is not neutralizing can limit virus replication *in vivo* [362] and a nonneutralizing MAb to SFV E2 can limit virus replication *in vitro* [348]. Anti-E1 MAbs may also be able to alter intracellular virus replication, but this has been less extensively studied [363].

Antibody-mediated inhibition of intracellular virus replication requires bivalent antibody but does not require the Fc portion of the MAb, complement, or other cells [361,364,365,366]. Soon after antibody binding, virion budding from the plasma membrane is inhibited, perhaps by restoring cellular Na^+K^+ATPase function and K^+ flux or host protein synthesis [367]. The effects of antibody on control of virus production *in vitro* can be amplified by treatment of infected cells with IFN [308]. *In vivo* studies also show that IFN and antibody act synergistically to promote recovery from alphavirus infection, but the mechanisms by which these systems interact have not been identified [328,368,369]. Mechanisms of virus clearance that depend on antibody may be particularly important in the CNS where limited expression of major histocompatibility complex (MHC) antigens may restrict the role of T lymphocytes in clearance [361,370].

Antibody also is important for protection from infection [371]. Inactivated vaccines protect against EEE, VEE, and WEE [372,373]. Delivered before or shortly after infection, passive transfer of antibody can protect from acute fatal disease but may predispose to late disease [243] associated with persistent infection, inflammation, and neuronal degeneration [223,243,374,375].

Cellular immunity

Alphavirus infection induces virus-specific lymphoproliferative, cytokine, and cytotoxic T lymphocyte responses [337,376,377,378,379]. After epidermal virus inoculation, Langerhans cells increase expression of MHC class II antigens, as well as accessory and costimulatory molecules that enhance activation of naïve T cells [380]. These cells travel to local lymph nodes where immune responses are induced. After activation, T cells enter the circulation to sites of virus replication. Activated T cells routinely cross the blood-brain barrier as part of normal immunologic surveillance of the CNS [381]. During CNS infection, the entry of activated cells is enhanced [382,383]. Infiltration of mononuclear cells into the CNS can be detected within 3 to 4 days after infection [220,338] and includes natural killer cells, $CD4^+$ and $CD8^+$ T lymphocytes, B cells, and macrophages [336,384,385,386,387]. Retention of T cells is immunologically specific [338,381]. Proportions of different mononuclear cell populations in the CNS vary with time after infection [384,385].

T cells have many roles during alphavirus infection. VEEV-specific T lymphocytes can inhibit virus replication *in vitro* [202,378] and play a role in virus clearance and in protection from aerosol challenge [388]. Viral RNA levels in the CNS of SINV-infected mice decrease more rapidly when $CD8^+$ T cells are present [370]. IFN-γ is an important effector cytokine produced by T cells. Mice lacking the ability to produce antibody can clear infectious virus from some populations of neurons through production of IFN-γ [389]. IFN-γ treatment of infected mature neurons improves cellular protein synthesis and decreases viral RNA and protein synthesis [306]. The intracellular effectors of IFN-γ action are not known, but synthesis of nitric oxide may play a role [225].

Mice deficient in production of both IFN-γ and antibody develop persistent SINV infection, but titers are lower than those found in mice with severe combined immunodeficiency, suggesting additional factors important for virus clearance [331].

Immunopathology

Data from several model systems of alphavirus encephalomyelitis suggests that the virus-specific cellular immune response in the CNS can also contribute to neuronal damage [269,270,375,390,391]. In fatal encephalomyelitis, paralysis and death of neurons are initiated by infection with a neurovirulent strain but carried out through contributions of the host [390,391]. Mice infected with a neuro-adapted strain of SINV (NSV) become paralyzed and die 7–8

days after infection, during the process of viral clearance. Studies with knockout mice have suggested that T cells contribute to fatal disease after infection with NSV.

Mortality is decreased in mice deficient in β2microglobulin, αβ T cells, or $CD4^+$ T cells but is not affected by deficiencies in antibody, perforin, Fas, TNF-α receptor-1, IL-6, or IL-12 [370,390]. A detrimental role for T cells in outcome of NSV infection was also suggested by study of B6 mice protected from fatal disease by passive transfer of immune serum 24 hours after infection. These mice clear infectious virus, but viral RNA and antigen persist. Brains at 2–4 weeks show progressive loss of parenchyma associated with mononuclear cell infiltration, and the number of $CD4^+$ T cells and macrophage/microglial cells in the hippocampal gyrus was correlated with terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL)-positive pyramidal neurons, suggesting that the cellular immune response can promote progressive neuronal death and tissue injury despite control of replication of infectious virus [375].

Interestingly, protection of NSV-infected mice from paralysis and death by AMPA glutamate receptor antagonists is associated with delayed virus clearance and a decrease in the inflammatory response [391]. This further suggests a role for inflammation in neuronal damage during alphavirus encephalomyelitis.

Pathology

Much of the pathology observed during alphavirus encephalomyelitis is associated with the inflammatory response to infection and neuronal damage. In addition, demyelination has been described as a consequence of EEEV and WEEV infection in humans [176,392,393] and of WEEV and SFV infection of mice, probably as a result of infection of oligodendrocytes [212,286].

Eastern equine encephalitis

Histopathology on fatal cases of EEE demonstrates a diffuse meningoencephalitis with widespread neuronal destruction, perivascular cuffing with polymorphonuclear as well as mononuclear leukocytes, and vasculitis with vessel occlusion in the cortex, basal ganglia, and brain stem. The spinal cord is frequently spared [166,394]. Pathologic changes in the CNS of humans with fatal neurologic disease and mice with experimentally induced encephalomyelitis begin with infiltration of mononuclear and occasional polymorphonuclear inflammatory cells into perivascular regions [338,385,393]. This phase often includes perivascular extravasation of red blood cells and endothelial cell swelling and hyperplasia [393]. Lymphocytes and monocytes then move from the perivascular regions to infiltrate areas of the parenchyma that contain virus-infected neurons. This inflammatory process is accompanied by gliosis and evidence of inflammatory and glial cell apoptosis [395].

Virus antigen is localized to neurons and neuronal death is marked by cytoplasmic swelling and nuclear pyknosis [395]. Apoptotic glial and inflammatory cells are frequently found in the regions of affected neurons [43,166,395]. Neonatal mice and human infants may die with widespread virus-induced neuronal cell death before the inflammatory process, a manifestation of the cellular immune response, can be initiated [396].

Western equine encephalitis

Pathology of acute cases of WEE shows leptomeningitis and perivascular cuffing with polymorphonuclear leukocyte infiltration in the earliest cases and lymphocytes, plasma cells, and macrophages at later times. Inflammation is accompanied by endothelial hyperplasia, petechial hemorrhages, and glial nodules in areas of neuronal degeneration. Lesions are found primarily in the basal ganglia, brain stem, cerebellum, cerebral cortex, and spinal cord [171,393]. In addition, there are areas of focal necrosis and demyelination, particularly in the subcortical white matter and basal ganglia [176,393]. Occasionally in infants and children, there is pathologic evidence of progressive disease consistent with persistent infection [172,176,393,397]. Individuals

surviving months to years after onset of encephalitis (often with progressive disease) may have cystic lesions, gliosis, and demyelination with areas of active mononuclear inflammation [176,393].

Neuronal cell death may be apoptotic, particularly in young animals [398], or may be characterized by cytoplasmic swelling, vacuolation, membrane breakdown, and cellular degeneration suggesting necrosis [161,395,396].

Venezuelan equine encephalitis

Examination of tissues shows lymphocyte depletion early, mild hepatitis, myocarditis, and encephalitis. Death is uncommon, but in fatal cases, pathology has shown myocarditis, focal centrilobular hepatic necrosis and inflammation, and generalized lymphoid depletion [399]. Lesions in the brain are found primarily in the olfactory cortex and basal ganglia and consist of perivascular cuffing and glial nodules [197,400]. Congenitally, infected infants show severe neurologic damage with widespread necrosis, hemorrhage, and hypoplasia that can result in hydranencephaly [183].

Persistence

Persistent infection can occasionally be established in mammalian cell cultures *in vitro*. Mutations in the nsP2 protein can lead to reduced SINV RNA synthesis and persistent infection [322,401]. Mouse fibroblasts producing IFN can be persistently infected with SINV [402,403]. Persistent infection can also be established if the cell infected, such as a mature neuron, is resistant to virus-induced apoptosis [132,306,366,404].

In vivo, there is substantial evidence that alphaviruses can persist in the CNS after appearance of an immune response, clearance of virus from the circulation, and apparent clearance of infectious virus from tissue [405]. Several progressive cases of WEEV in humans have been reported and pathologic examination of CNS tissue months to years after resolution of acute encephalitis has shown an active inflammatory process [393,406].

Mice infected with viruses that do not cause fatal disease clear infectious virus from the CNS within 7–8 days and recover uneventfully [275]. The disappearance of infectious virus is rapid while the decline in viral RNA occurs more slowly [361]. Viral RNA and proteins can be detected in the nervous system long after recovery of mice from SINV or SFV-induced encephalitis and reactivation is common for several weeks after initial clearance [202,223,331,405,407,408,409]. It is postulated that this persistence is due to failure of the virus or the immune system to eliminate the infected cells. Therefore, one consequence of a nonlytic mechanism for clearance of virus from tissue is that the virus genome is not completely eliminated if the originally infected cells survive [405]. This leads to a need for long-term control of virus replication, and reactivation of infection appears to be prevented by continued presence of T lymphocytes and antibody-secreting B cells within the CNS [405,408,410,411].

Prevention, diagnosis, and treatment

A formalin-inactivated vaccine derived from a North American strain of EEEV (PE-6) is available for horses and emus and for investigational use to protect laboratory workers. This vaccine does not induce significant neutralizing or anti-E2 antibody to South American strains of EEEV [412]. An inactivated WEEV vaccine is available for horses and as an experimental preparation for laboratory workers [373]. Yearly booster doses are required for both [413]. The earliest VEEV vaccines to be developed for horses and laboratory workers were also formalin-inactivated preparations [372,373]. These vaccines had repeated problems with residual live virus-producing disease and with poor immunogenicity and are no longer in use [70,71,72]. A live attenuated vaccine (TC-83), developed by serial passage of the virulent TRD strain in guinea pig heart tissue culture cells [239], is protective for horses and laboratory workers, but 15–30% of recipients develop fever and pharyngeal viral shedding [413,414]. Therefore, a formalin-inactivated TC-83 vaccine (C-84) was produced [413]. Both the

live and inactivated vaccines are immunogenic, but live TC-83 provides better protection against aerosol challenge in hamsters than C-84 and is therefore preferred despite its reactogenicity [415]. Several experimental VEEV vaccines are currently under development [416,417]. In both horses and humans, prior vaccination against one alphavirus can interfere with development of neutralizing antibody to subsequent alphavirus vaccines [379,418,419].

Protection of human populations relies primarily on personal protection from mosquito bites. Infection in mosquito populations can be monitored by virus isolation, by nucleic acid amplification, or by seroconversion of sentinel pheasants or chickens. This information can be used to guide insecticide spraying to reduce adult and larval mosquito populations [40]. VEE epizootics can be controlled by immunizing equines with TC-83, limiting equine movements from regions of infection, applying larvacides to mosquito breeding sites, and spraying insecticides to control adult mosquitoes [11,31,69].

Diagnosis is based on virus isolation or detection of antibody (see also Chapters 17 and 18). Virus can be isolated from CSF, blood, or CNS tissue by inoculation into newborn mice or onto a variety of tissue culture cells. For VEEV, diagnosis can also be made by virus isolation from blood or pharynx [9,10,181]. Direct virus detection and identification in field and clinical samples can be accomplished through various nucleic acid amplification assays [420]. Antibody is usually measured by enzyme immunoassay with detection of IgM in serum and CSF particularly useful [340,421].

No successful specific antiviral therapy has been identified for CNS infection and the mainstay of treatment remains vigorous supportive therapy including respiratory assistance, maintenance of electrolyte balance, and control of seizures and increased intracranial pressure [422].

Summary and future directions

Alphaviruses are mosquito-borne causes of acute encephalomyelitis in the Americas with potential to spread to new regions of the world. Alphavirus infections of experimental animals have provided important model systems for understanding the pathogenesis of viral infections of the CNS and mechanisms of noncytolytic clearance of viruses from neurons. An important future goal is determination of the atomic structure of the surface glycoproteins and nonstructural proteins. This information will help determine the mechanisms by which single amino acid changes affect virulence and alter virus replication. In addition, there is a need to determine the mechanisms by which different components of the innate and adaptive immune responses control replication and clear infectious virus from neurons without damage to the infected cells. Lastly, treatments that interfere with virus replication and protect neurons from immune-mediated damage are needed.

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7

Flaviviruses

Barbara W. Johnson

Flaviviruses are small, positive-strand RNA viruses that are transmitted from infected to susceptible vertebrate hosts primarily by arthropods [1]. Flavivirus infections cause seasonal disease syndromes corresponding to mosquito and tick activity throughout the temperate and tropical areas of the world. These seasonal disease outbreaks have been recognized since the 1800s, although flaviviruses were not identified as the etiological agents and arthropods as the transmission vectors until early in the twentieth century, when virus isolation and characterization techniques were developed [1,2,3,4]. The *Flavivirus* genus consists of nearly 80 viruses, approximately half of which are associated with human disease [1,5]. Flaviviruses were originally classified in the *Togaviridae* family as group B arboviruses, to the group A arboviruses, now classified as alphaviruses (see Chapter 6), because they are both arthropod-borne viruses, or arboviruses, and they can cause similar disease syndromes. Both are also positive-strand RNA viruses; however, they have different genome organizations (see Chapter 6, and Figure 7.3) [5,6]. The genus *Flavivirus* was later reclassified within the *Flaviviridae* family, which also includes the *Pestivirus* and *Hepacivirus* genera.

The majority of flaviviruses are arboviruses, with over half transmitted by mosquitoes and approximately one-third transmitted by ticks (Figure 7.1). Five flaviviruses have no known vector [1]. Flaviviruses are hypothesized to have derived from a monophyletic lineage, possibly a plant virus, which entered the transmission cycle of a common ancestor to both ticks and mosquitoes, or to ticks and then later mosquitoes [1,7,8].

Flaviviruses were originally characterized serologically and divided into eight antigenic complexes and twelve subcomplexes based on cross-neutralization assays with hyperimmune antisera [1,6,9,10]. More recently, flaviviruses have been classified based on molecular phylogenetics [1,11]. Although there are regions of the flavivirus genome that are highly conserved, there is also considerable genetic diversity within the genus, with the most distantly related flaviviruses having only about 40% sequence homology (Figure 7.1) [1,9].

In regions where multiple flaviviruses co-circulate, a person may be at risk of serial flavivirus infections. Prior flavivirus infection does not prevent infection by a subsequent flavivirus, but antibodies raised in the primary flavivirus infection may modulate the second infection, resulting in milder or more severe illness [12,13,14]. Experimental evidence suggests that within a sero-complex, antibodies from the first infection may partially neutralize the virus during the secondary infection, leading to reduced clinical symptoms [15]. There may be some degree of cross-protection between flaviviruses from different sero-complexes, but this has not been documented. The lack of West Nile encephalitis cases in Central and South America, where many flaviviruses are known to occur, compared to the United States and Canada, where West Nile virus infections are primary flavivirus infections, has been hypothesized to be due to the differences in prior flavivirus infections between these two populations. In contrast, although the four

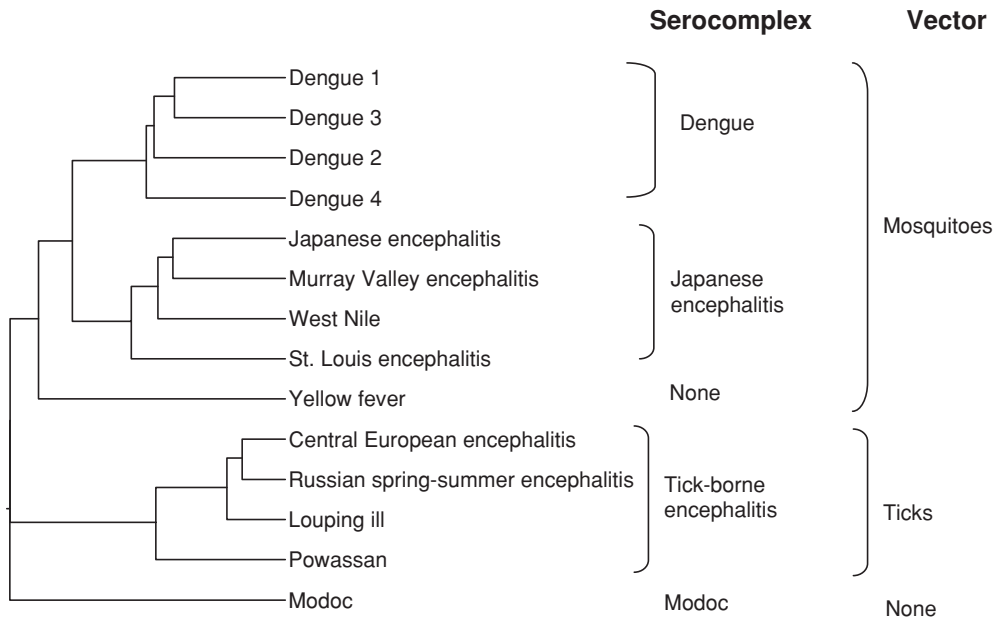

Figure 7.1. General organization of a selection of viruses in the *Flavivirus* genus based on phylogenetic analysis of complete genome sequences from Genbank. The serological relationships and arthropod vectors are shown on the right [1,11,16].

dengue virus serotypes are closely related, antibodies raised against the primary dengue virus infection may function to enhance the entry of the second dengue serotype virus into cells, which is hypothesized to contribute to the more serious dengue hemorrhagic fever/dengue shock syndrome seen in secondary dengue infections [12,13,14].

Medically important flaviviruses are associated with three clinical syndromes: encephalitis syndrome; hemorrhagic fever; or fever, arthralgia, and rash [1]. All flaviviruses are neurotropic to some degree, which is probably due to evolutionary conservation of the regions on the envelope protein involved in host cell receptor interactions [1,10,16,17]. However, only the neurotropic flaviviruses, which cause neuroinvasive disease, will be described in this chapter. These belong primarily to two groups: mosquito-borne viruses in the Japanese encephalitis serocomplex and the tick-borne encephalitis viruses [1]. The most important human pathogens in these two groups, in terms of number of cases, include Japanese encephalitis virus, West Nile virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, and Russian spring-summer and Central European tick-borne encephalitis virus subtypes [1,10]. In this chapter, the general features of the neurotropic flaviviruses will be reviewed. Clinical disease syndromes, epidemiological and ecological aspects, as well as prevention strategies of specific medically important flaviviruses will be described in individual sections at the end of the chapter. Although in certain cases dengue virus infections can present as encephalitis, these and other hemorrhagic or fever flaviviruses will not be discussed here.

Ecology and epidemiology

Japanese encephalitis complex viruses are maintained in enzootic cycles between birds and mosquitoes, primarily ornithophilic *Culex* spp.

Humans become infected when they are bitten by an infected mosquito, but they are incidental hosts, as viremia is brief and low, and of insufficient titer to infect a mosquito through a blood meal. Similarly, ticks of the genus *Ixodes* are the vectors of the tick-borne encephalitis viruses, with small mammals such as rodents serving as vertebrate hosts [9,18,19]. During transmission season, when either the temperature or rainfall provides favorable mosquito or tick breeding conditions, humans become infected when they are bitten by an infected arthropod vector. (A more detailed, complete description of arbovirus transmission and ecology is given in Chapter 20). Although in general these viruses are not transmitted in nature directly from host to host, cases of human-to-human transmission of West Nile virus have been reported to occur through blood transfusions and organ donations [20,21,22,23,24].

Flaviviruses from the two neurotropic flavivirus groups are distributed widely throughout temperate and tropical regions of the world (Figures 7.6 and 7.7). In Asia alone over 3 billion people are at risk of being infected with Japanese encephalitis virus. Although Japanese encephalitis virus has circulated in Asia for over 100 years, the virus is emerging in new areas where changing agricultural practices have brought arthropod vectors and vertebrate hosts into closer contact with one another and into contact with naïve susceptible human hosts. As a result, despite the availability of effective, safe vaccines, Japanese encephalitis virus infection has become the leading cause of pediatric encephalitis throughout Asia. In addition, flaviviruses, such as West Nile virus, have emerged for the first time in areas where competent arthropod vectors and susceptible vertebrate hosts have provided the conditions necessary for establishment of virus transmission in new ecological niches. An example of this is the introduction of West Nile virus into New York in 1999 and the spread of the virus throughout North America since then, which has resulted in the largest outbreaks of meningitis and encephalitis in the Western Hemisphere [25].

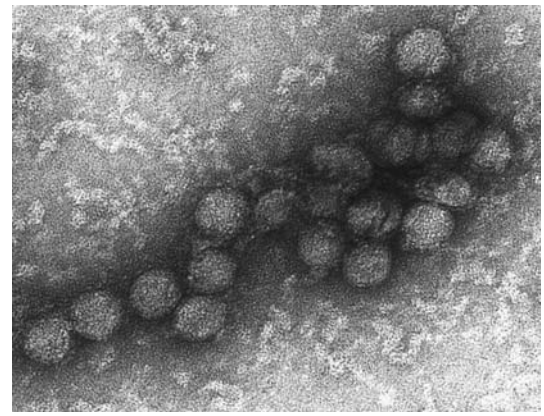

Figure 7.2. Negative staining electron micrograph of West Nile spherical virions approximately 45 nm in diameter (×216,000). The virus was isolated in Vero E6 cells from an organ transplant recipient who died in 2002 [90].

Flavivirus structure and replication

Flaviviruses are small, spherical viruses with icosohedral symmetry, approximately 50 nm in diameter [26,27] (Figure 7.2). The virion is smooth, with no spikes or surface projections, and it is comprised of viral envelope and membrane proteins arranged in head-to-tail heterodimers, embedded in a host cell-derived lipid bilayer, which surrounds a nucleocapsid core. The nucleocapsid consists of multiple copies of the capsid protein, arranged in an icosohedral, surrounding and anchoring the RNA genome [28,29]. The single-stranded, positive-sense RNA genome, approximately 11 kb in length, functions as an mRNA, and is comprised of a single, long open reading frame. The open reading frame is flanked at both the 5′ and 3′ ends by untranslated regions and capped at the 5′ end (Figure 7.3). Viral proteins are translated in a polyprotein that is co- and post-translationally cleaved by cellular and viral proteases and glycosylated by cellular glycosyltransferases [27]. This process produces the three structural proteins, capsid protein, premembrane protein, and envelope glycoprotein, and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A,

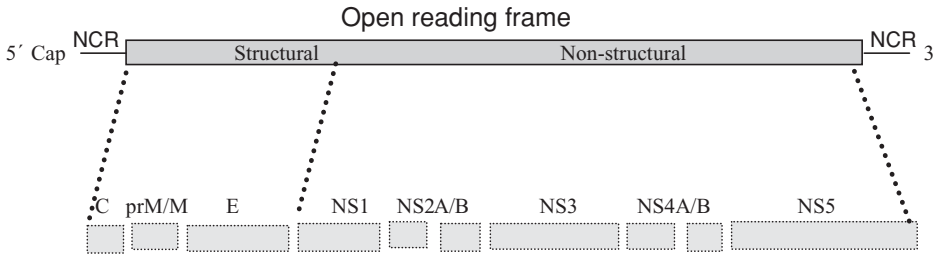

Figure 7.3. Flavivirus genome organization and translation and processing of the polyprotein. NCR, noncoding region; C, capsid; prM, premembrane; E, envelope; NS, nonstructural.

NS4B, and NS5 [30]. The capsid, premembrane, and envelope structural proteins make up the virus particle; the nonstructural proteins function primarily in viral replication and virion assembly, together with host cell factors [26,27]. The envelope protein is the major surface protein of the viral particle. It interacts with specific host cell receptors in the initial binding of the virus to the cell surface, is involved with membrane fusion and entry, and thus is an important determinant of tissue tropism and virulence [6,31]. The envelope protein is also the major viral antigen against which host protective antibodies are produced [6,31,32]. Comparison of flavivirus envelope protein sequences has shown both highly conserved and very divergent subregions [1,11]. However, despite the lack of proofreading in RNA viruses during replication, the envelope gene is one of the most slowly evolving sites, which is probably due to the selective pressure of infecting and replicating in both vertebrate and arthropod cells.

Flavivirus replication takes place in the host cell cytoplasm. The virus binds to the surface of host cells through an interaction between the envelope protein and specific cellular receptors [26,27,33,34]. Following attachment, fusion and entry of the virus is carried out via receptor-mediated endocytosis, where the envelope protein undergoes an acid-catalyzed conformational change, resulting in membrane fusion, uncoating, and release of the nucleocapsid into the cytoplasm [27,31]. Following translation and processing of the viral proteins, a viral replicase is assembled from viral nonstructural proteins and host factors. Viral replication is initiated with the synthesis of a genome-length minus-strand RNA intermediate, which serves as a template for the synthesis of genomic RNA [27]. Virion morphogenesis is hypothesized to occur in association with intracellular membranes, probably the endoplasmic reticulum [26,27]. Immature virions assemble in membrane-bound vesicles in a premembrane and envelope protein heterodimer conformation, which prevents viral envelope protein fusion activity. Virions accumulate in the rough endoplasmic reticulum and are transported to the cell surface in acidic vesicles through the host secretory pathway, possibly used for synthesis of host plasma membrane glycoproteins [26]. The virions fuse with the plasma membrane and are released by exocytosis after the membrane protein is cleaved from the precursor premembrane protein in the Golgi by a Golgi protease, signaling virus release and envelope protein activation [32].

Pathogenesis and immune response

Flavivirus infection of arthropod cells in culture may show cytopathic effects such as syncytia formation, but infection of mosquito cells is generally persistent, not cytopathic (see Chapter 20). Virus infection of vertebrate cells in culture shows cytopathic effects such as cellular rounding, and as virions accumulate in the rough endoplasmic reticulum, proliferation, hypertrophy, and fragmentation of the membranes. Mitochondrial damage, rarefaction of cytoplasm, formation of vacuoles and inclusion bodies, and an increase in lysosomal enzymes have also been

shown to occur in cultured cells following flavivirus infection [1,27].

In natural infections, an infected arthropod inoculates the flavivirus into vertebrate skin along with the saliva during a bite. Initial virus replication occurs in the skin cells at the site of inoculation [35]. The virus is then transported in migrating dendritic Langerhans cells to lymph nodes and from the lymphatic system to the thoracic duct and into the bloodstream [1,17,35]. This is the primary viremia, which is the source of infection of connective tissue, skeletal muscle, myocardial tissue, smooth muscle, lymphoreticular tissues, and endocrine and exocrine glands. Viremia continues for several days due to release of the virus from these tissues back into the bloodstream [1].

Most infections by neurotropic flaviviruses are self-limiting. The vast majority of infections are either asymptomatic or the person may have a mild subclinical fever syndrome. In the primary immune response viremia is of low titer and brief, modulated by macrophages, and then cleared following the rise of the humoral immune response, usually within a week of infection (Figure 7.4) [1]. In addition, the T-cell helper and cytotoxic immune response are elicited against infected lymphoblastoid cells [6,34]. Virus is usually not detectable in serum specimens collected at the time of onset of symptoms (Figure 7.4).

Neuroinvasive disease occurs in approximately 1 in 100 to 1 in 1000 neurotropic flavivirus infections. The viral factors that are hypothesized to contribute to neuroinvasive flavivirus infections include the level of viremia and the genetic differences in virus strain neurovirulence [1,10,16,17]. Single mutations in the envelope gene have been shown to alter neurovirulence phenotype [10]. Arthropod vector competence is another factor that contributes to neurovirulence and is described in detail in Chapter 20. In the human host, age, gender, genetic susceptibility, pre-existing herpesvirus or heterologous flavivirus infection or immunization, and concomitant parasite infection are factors that contribute to susceptibility to infection and may affect disease severity [1,36]. Generally, the highest proportion of neuroinvasive disease is seen in the very young and the elderly. In areas where Japanese encephalitis and Murray Valley viruses are endemic, children make up the largest proportion of cases, and it has been demonstrated experimentally that younger neurons are more susceptible to virus infection. However, in areas with low flavivirus seroprevalence, such as North America, it has been shown that the risk of St. Louis encephalitis and North American West Nile virus infections resulting in neuroinvasive disease is higher in those over 55 years of age [1,37]. The reasons for this are unclear but may include factors such as the impaired integrity of the blood-brain barrier caused by cardiovascular or other age-related diseases [4].

The exact mechanisms by which flaviviruses enter the central nervous system are unknown. Most of the data regarding the regions of the central nervous system infected by flaviviruses come from postmortem studies of pediatric Japanese encephalitis cases in Asia and West Nile cases from North America and experimental infections in animal models. Possible mechanisms of introduction include (1) infection of cerebral endothelial cells and migration across the cell to the brain parenchyma, (2) migration of infected leukocytes through the tight junction formed by endothelial cells, (3) direct choroidal virus shedding, (4) axonal transport up the olfactory nerve, (5) increased permeability due to tumor necrosis factor α induction by attachment of double-stranded RNA to Toll-like receptors, or (6) retrograde transport along peripheral nerve axons [1,10,37,38,39,40]. Whether the virus enters at a single site or at multiple locations has yet to be determined.

Once in the central nervous system, the virus replicates and spreads rapidly. The resulting pathogenesis is due to direct, virally mediated damage to neurons and glial cells, cytotoxic immune response to infected cells, the inflammatory immune response in perivascular tissue, and microglial nodule formation [1,11,38,41].

In histopathological studies, West Nile virus has been shown to directly infect and destroy neurons in the brain stem, deep nuclei of the brain, and anterior horn cells in the spinal cord (Figure 7.5) [22]. The inflammatory immune response of natural killer cells, macrophages, and T-lymphocytes

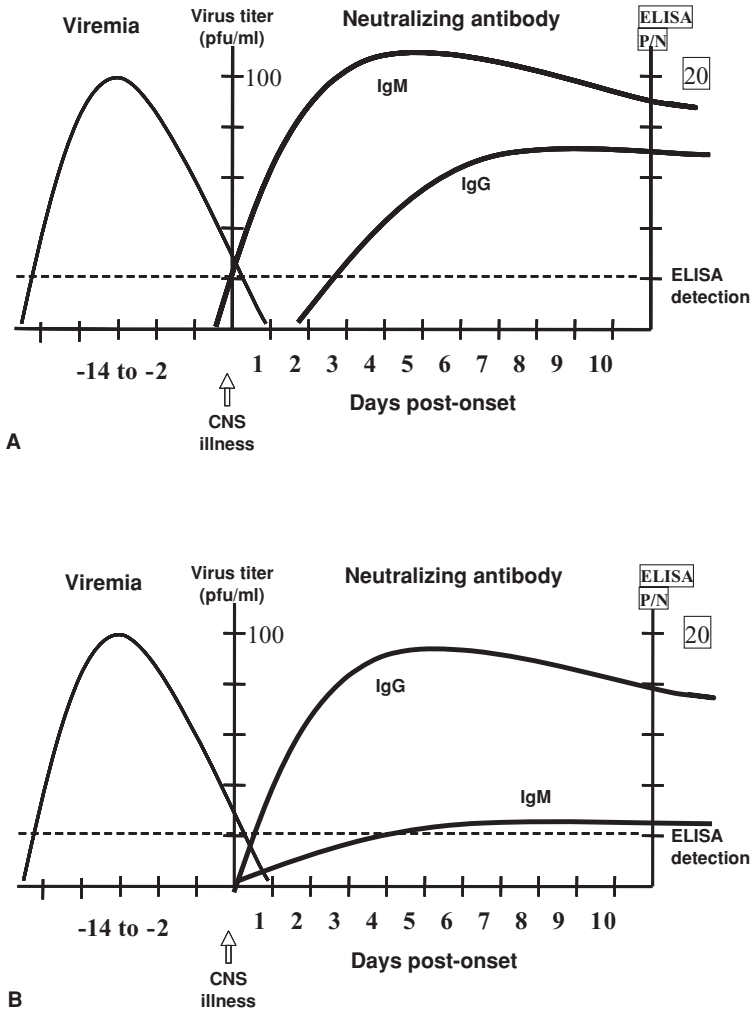

Figure 7.4. Graphical representation of the course of viremia and IgM and IgG antibody immune response in a (A) primary flavivirus infection and (B) secondary flavivirus infection.

results in lysis of neuronal tissue and diffuse perivascular inflammation of the brain stem and anterior horn cells of the spinal cord, and immune-mediated damage to bystander nerve cells, glial cells, as well as other surrounding tissue [39]. Damage to these neuronal cells is characterized by central chromatolysis, cytoplasmic eosinophilia, cell shrinkage, neuronophagia, and cellular nodule formation composed of activated microglia and mononuclear cells [1,37,38,39,40].

Persistent and long-term pathological changes are often seen following flavivirus neuroinvasive infection, such as residual neurological deficits, electroencephalographic changes, and psychiatric disturbances [1]. Long-term follow-up studies in Japanese encephalitis cases in children have shown neuronal loss and dense microglial scarring resulting in recurrent neurological disease [2,16,41]. Chronic progressive encephalitis has been observed years after infection with tick-borne encephalitis virus

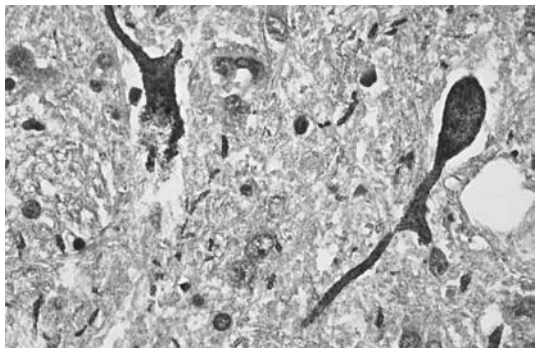

Figure 7.5. Photomicrograph of immunohistochemical staining of brain tissue from a fatal West Nile encephalitis case, showing West Nile antigen-positive neurons and neuronal processes in the brain stem and anterior horn cells (dark areas). (From W.-J. Shieh and S. Zaki, CDC, 1999.) (For figure in color, please see color plate section.)

[1,17]. In West Nile virus encephalitis patients in North America, the majority experience long-term neurological deficits, with only 13% reporting full recovery in physical cognitive and functional abilities 1 year after illness.

Clinical presentation

The majority of flavivirus infections are asymptomatic or subclinical [2,40]. Clinical disease ranges from a mild febrile illness to a severe neurological syndrome following an incubation period of 2 to 14 days [7]. Febrile illness is characterized by fever, chills, headache, back pain, myalgia, and anorexia, as well as eye pain, pharyngitis, nausea, vomiting, and diarrhea [7,38]. A transient maculopapular rash over the trunk and limbs is also common [38]. Acute illness usually lasts from 3 days to several weeks. Most patients with uncomplicated fever completely recover within days to months, but prolonged fatigue is often seen [4,37,38].

Fever symptoms may be followed in 1 to 4 days by acute or subacute appearance of meningeal and neurological signs (Table 7.1). The neurological syndrome depends on which part of the nervous system is infected: the parenchyma of the brain, which causes encephalitis, the meninges, which causes meningitis, or the anterior horn cells of the spinal

Table 7.1. Clinical syndromes associated with neuroinvasive flavivirus infections

Encephalitis, meningoencephalitis, encephalomyelitis[a]
Acute febrile illness[b]
Mental status changes (confusion, disorientation, delirium, seizures, stupor, coma)
Motor weakness (flaccid weakness in comatose patients, acute flaccid paralysis in conscious patients)
Movement disorders (parkinonsian syndrome, including mask-like facies, tremors, cogwheel rigidities; cerebral ataxia)
Other neurologic dysfunction (convulsion, cranial nerve palsy, dysarthria, rigidity, abnormal reflexes, tremor)
Meningitis[a]
Acute febrile illness[b]
Signs of meningeal irritation (nuchal rigidity, photophobia, phonophobia)
Absence of other signs of neurologic dysfunction
Myelitis
Limb paralysis (asymmetric muscle weakness resulting in monoplegia or less commonly, quadriplegia)
Central bilateral facial weakness
Diaphragmatic and intercostal muscle paralysis leading to respiratory failure
Sensory loss, numbness

[a] CSF pleocytosis (≤500 cells/mm3; mostly lymphocytes), elevated protein concentration (80–105 mg/dL), normal glucose concentration.

[b] Fever (37.8°C), headache, fatigue, myalgia, nausea/vomiting.

Table modified from Burke, D. and Monath, T.P., Flaviviruses. In D. Knipe and P. Howley (Eds.), Fields Virology, 4th ed. Vol. 1, Lippincott Williams and Wilkins, Philadelphia, 2001, pp. 1043–125 [1,2,16,37,38].

cord, which causes myelitis [1,4,37,39,42,43,44,45]. The primary clinical presentations may overlap and include a reduced level of consciousness, often associated with seizures, flaccid paralysis, and parkinsonian movement disorders [4,46]. Encephalitis is more common than meningitis, with 50–85% of patients presenting with encephalitis, compared to 5–50% with meningitis [4]. Seizures are more common in children, with approximately 85% Japanese encephalitis or Murray Valley encephalitis pediatric patients and 10% of adult West Nile encephalitis patients experiencing seizures [4,7,47]. Motor weakness occurs in 10–50% of flavivirus neuroinvasive cases, with acute asymmetric flaccid paralysis similar to that seen in poliomyelitis [4,39]. Coma occurs in approximately 15% of patients.

The case fatality rate among hospitalized patients with neuroinvasive disease ranges from approximately 9% of those infected with North American West Nile virus to 30% of pediatric Japanese encephalitis cases. The cause of death is primarily due to neuronal dysfunction, respiratory failure, and cerebral edema [38]. Multiple or prolonged seizures in Japanese encephalitis patients are associated with a poor outcome, as are changes in respiratory pattern, flexor and extensor posturing, and pupillary and occulocephalic reflex abnormalities [4,47]. About one-half of survivors have long-term neurological sequelae, including motor deficits, cognitive and language impairment, and convulsions, with children making up the largest proportion of this group [39,47,48]. In addition, even those who were considered to have good recovery may have subtle long-term effects such as learning disorders, behavior problems, and other neurological deficits. Follow-up studies of pediatric Japanese encephalitis cases show that a high proportion of patients experience persistent movement disorders 3–5 years later [49]. Many patients with poliomyelitis do not recover, although limb strength may improve over time [39].

Laboratory diagnosis

Flavivirus infections may present with clinical symptoms similar to those of other bacterial or virological infections, such as a flu-like illness, encephalitis, or polio-like myelitis [1]. In addition, viruses within the Japanese encephalitis or tick-borne encephalitis sero-complexes cause similar disease syndromes and may be indistinguishable from one another clinically. Therefore, laboratory diagnosis is necessary to identify etiology and differentiate between other bacterial or viral pathogens. However, because of the brief transient viremia and the cross-reactivity of the elicited antibodies to other flavivirus antigens in serological assays, identification of the specific flavivirus can be difficult. This is especially true in secondary flavivirus infections, where the immune response may be greater to the primary infecting flavivirus than to the most recent infection [14,50].

Diagnosis is usually made serologically by detection of virus-specific antibody, ideally from paired acute and convalescent specimens, with the rise in antibody titer indicative of a recent infection [51,52]. In practice, however, only a single acute specimen is usually obtained. In these cases, specific immunoglobulin M (IgM) antibody-capture enzyme-linked immunosorbent assay (ELISA) can be used for rapid detection of acute flaviviral infections, as IgM antibody is produced early in infection, rises rapidly to detectable levels, and is less cross-reactive than IgG antibodies (Figure 7.4) [51,52,53,54,55,56].

Cerebral spinal fluid (CSF) is the preferable diagnostic specimen in neuroinvasive disease, as antiflavivirus IgM antibody may be present in serum, such as in inapparent or mild infections or following a flavivirus vaccination, but may not be the cause of encephalitis [57,58]. This is especially true in areas where there is high background immunity in the population, such as in Asia where a large proportion of the population has been exposed to or vaccinated against Japanese encephalitis virus. Anti-Japanese encephalitis IgM antibody has been shown to be detectable in serum as much as 6 months following vaccination with the live attenuated vaccine virus [59]. However, IgM antibodies elicited in non-neuroinvasive flavivirus infections or following flavivirus vaccination do not enter the CSF, so by testing the CSF, the effect of background IgM

antibodies in the serum is eliminated [7,51,53,57, 60,61]. IgM antibody is usually detectable in the CSF by onset of illness or within a few days thereafter, except in very acute, sudden-onset encephalitis, when the IgM antibodies may not have reached detectable levels at hospital admission, in which case the IgM ELISA may result in a false negative (Figure 7.4) [53,57]. Therefore, it is critical for diagnosis that a second specimen be collected and tested if possible 7 days after onset of illness or at hospital discharge [51,52]. Viral nucleic acid detection in CSF by nucleic acid amplification testing has also proved useful in approximately 50% of these acute infections, which when used together with IgM ELISA enhances sensitivity [62,63,64]. Virus isolation in CSF or tissue is specific and the gold standard for diagnosis and identification of a viral infection, but it is not sensitive in neurotropic flavivirus infections, as low levels ($\leq$100 infectious particles per ml) of viremia are usually cleared by the onset of illness (Figure 7.4). However, detection of flavivirus antigen in brain tissue by immunohistochemistry is useful for diagnosis in fatal cases, as these patients may not have detectable IgM or IgG antibodies in serum or CSF [22].

Cross-reactivity in serological assays, including the IgM ELISA, is a problem in flavivirus infections. This is due to the heterologous population of antibodies produced in the infection against different epitopes of the flaviviral envelope protein, some of which are virus species-specific, and others of which are conserved across the flavivirus serocomplex or genus. Antibodies elicited against these conserved regions cross-react in serological assays with other flaviviral antigens and cause false-positive results in the IgM ELISA. Antibodies within a flavivirus serocomplex are highly cross-reactive; those between serocomplexes are less cross-reactive but still may confound accurate diagnosis [61]. For example, dengue and West Nile viruses, which are not in the same serocomplex, cocirculate in Africa and Asia. Dengue virus may have a clinical presentation of encephalopathy, and a specimen from a dengue patient submitted for West Nile testing may test positive in a West Nile virus IgM ELISA [65,66]. The plaque-reduction neutralization test is a more specific assay and is used to confirm or differentiate conflicting IgM ELISA results in primary flavivirus infections.

Serological diagnosis of secondary flavivirus infections remains problematic, however, and neutralization assays may not discriminate between the flaviviruses, as neutralizing antibody titer from the primary flavivirus infection rises quickly and may be equal to or higher than that of the antibody titer from the acute flavivirus infection (Figure 7.4B) [50]. In addition, IgM antibody to the second infecting virus may not rise to levels detectable by the IgM ELISA, producing a false-negative result.

Diagnostic assays such as the microsphere immunoassays have been shown to have improved specificity and sensitivity [54,55,56]. These assays, based on Luminex^{TM} technology, can be run in a one-well multiplex format, which reduces the sometimes very limited specimen volume needed, and have a statistical-based results interpretation.

Use of the hemagglutination inhibition and complement fixation assays has decreased in recent years as they require paired specimens and lack specificity. The computed tomography (CT) scan has not been shown to be an effective diagnostic method for identifying flaviviral encephalitis cases, and magnetic resonance imaging (MRI) yields characteristic abnormal results in only 25% to 35% of cases, and these may be nonspecific [37,38,39,47,67].

Treatment

Treatment of encephalitis is supportive and includes pain control for headaches, rehydration, antiemetic treatment for nausea and vomiting, reduction of intracranial pressure, and control of seizures [68]. In patients with paralysis, the airway is managed to reduce aspiration and obstruction. Ventilation support may be required in patients with neuromuscular respiratory failure. Antivirals and other treatments such as ribavirin interferon-α and immunoglobulin have not been found to be effective, and high-dose corticosteroid treatment may be contraindicated

because of the risk of depressing the immune system before the virus is cleared [39].

Prevention

Vector control programs up to the early 1970s successfully eradicated *Aedes aegypti*, the primary mosquito vector of dengue and yellow fever viruses, from most of Central and South America (see Chapter 20). However, these programs were not sustainable, and as a result *Aedes aegypti* has reinfested these areas and dengue and yellow fever epidemics have reemerged. Most vector control programs are organized on a local rather than national level and it becomes difficult to maintain the funding for these programs in the absence of cases once the outbreaks or epidemics have passed. Insecticide treatment is expensive, must be periodically applied, and may have deleterious effects on other species, including humans, and resistance to classes of insecticides develops quickly in the arthropod vectors, which complicates this effort. In addition, barrier systems such as bed nets, which have been used successfully to disrupt transmission of malaria, are not effective methods with *Culex* spp. mosquitoes, as these mosquitoes typically feed at dusk when human activity is high.

Vaccination remains the most effective method to prevent flavivirus infection. Vaccination has been used successfully to control Japanese encephalitis virus and tick-borne encephalitis virus and will be described in more detail in their respective sections [68,69,70,71].

Brief descriptions of specific flaviviruses

Japanese encephalitis virus

Molecular phylogenetic analysis suggests that Japanese encephalitis virus possibly originated in the Indonesia-Malaysia region from an ancestral virus common to both Japanese encephalitis and Murray Valley encephalitis viruses [72]. The first recorded epidemics of summer-fall encephalitis, which became known as Japanese encephalitis, were recorded from 1871 in Japan [1,46,73]. Japanese encephalitis virus was isolated from a fatal case and shown to cause encephalitis in infected monkeys in 1934. The first epidemics of Japanese encephalitis were documented in Korea in 1949, with over 5500 cases, and in China in 1940; in 1966 over 40 000 cases were reported throughout China [73]. The first cases of Japanese encephalitis in India were reported in 1954, and by 1978 there were severe epidemics in the northeastern states. In a recent outbreak in 2005 in India and Nepal there were almost 9000 cases and over 1700 deaths [73,74]. In the southern tropical regions of Asia, such as Malaysia and Indonesia, Japanese encephalitis virus is endemic and causes disease year around or in association with monsoons. In the northern temperate regions of Nepal and China, epidemics are seasonal and correspond to the summer mosquito activity [68,73]. In the last 30 years, epidemic activity reported from Southeast Asia, India, and Sri Lanka has shown that the geographical range of Japanese encephalitis virus has been expanding, probably due to changing agricultural practices that bring humans into contact with the enzootic transmission cycle (Figure 7.6A). As a result, Japanese encephalitis has become the leading cause of pediatric encephalitis in Asia, with 30 000 to 50 000 cases and 10 000 to 15 000 deaths reported to the World Health Organization annually [47,68].

Japanese encephalitis is transmitted naturally in an enzootic cycle between *Culex* mosquitoes, primarily *Culex tritaeniorhynchus*, which breeds in rice paddy fields and other pools of stagnant water (see Chapter 20). *Culex tritaeniorhynchus* feeds preferentially on wild wading birds, which carry the virus to new areas, domestic birds, and large domestic animals, but will feed on humans. Pigs are important vertebrate hosts in the epidemiology of Japanese encephalitis virus, as they are reared in close proximity to human dwellings and rice paddies, maintain high prolonged viremia capable of infecting mosquitoes, do not become ill, and produce many immunologically naïve offspring that provide a continual supply of susceptible hosts [46,68,73].

Japanese encephalitis is considered to be mainly a disease of children, and serosurveys have shown

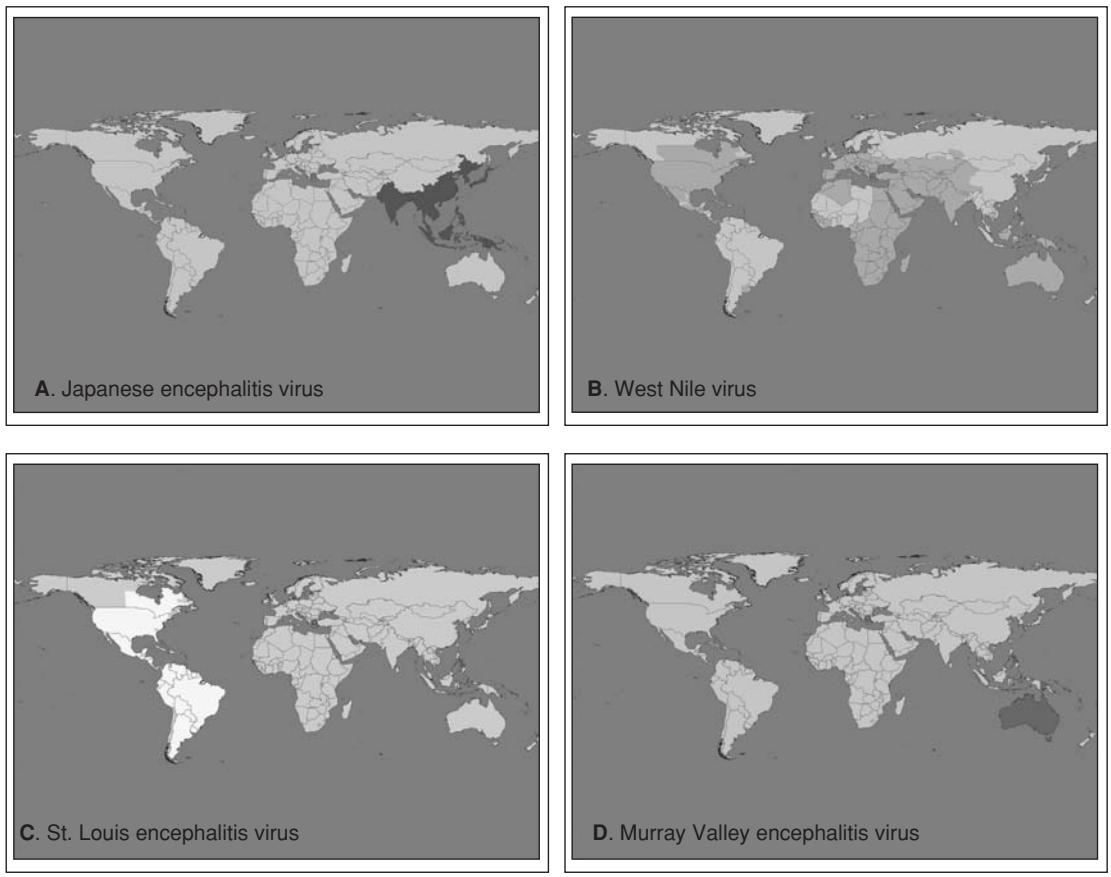

Figure 7.6. Geographical distribution by state or province of the Japanese encephalitis serocomplex viruses: (A) Japanese encephalitis virus, (B) West Nile virus, (C) St. Louis encephalitis virus, and (D) Murray Valley encephalitis virus. The distribution of St. Louis virus in Canada in Ontario, Manitoba, and Quebec was based on a single North American outbreak in 1975 to 1976. No activity has been documented since that time (M. Drebot, Public Health Canada, personal communication).

that by age 15 years, the majority of adults living in Japanese encephalitis endemic areas have been exposed to the virus [4]. However, adults may become infected when Japanese encephalitis virus moves to new areas or when susceptible adults, particularly travelers, enter an area of transmission activity.

The overall risk of travelers being infected with Japanese encephalitis virus is very low but depends on the destination and season of travel, as well as the level of outside activities of the traveler. Travelers who spend extended periods of time in rural Japanese encephalitis endemic areas are at greatest risk, although there have been reports of travelers infected at resorts and in urban areas [46,75].

As with other encephalitic flaviviruses, the majority of Japanese encephalitis virus infections are asymptomatic, with approximately 1 encephalitis case to 300 inapparent infections [47]. A mild illness with clinical symptoms consistent with other flavivirus infections, such as fever and headache, may result; however, because of the emphasis on encephalitis cases, these may be underreported [76].

In neuroinvasive disease, severe encephalitis is characterized by rapid onset of headache, high fever, neck stiffness, stupor, disorientation, coma, tremors, and in children, gastrointestinal pain and dysfunction, seizures, convulsions, and parkinsonian movement disorders. Pediatric clinical symptoms also include dull flat mask-like facies with wide unblinking eyes, tremor, generalized hypertonia, and cogwheel rigidity [47]. Acute flaccid paralysis may occur in comatose patients or in those with a normal level of consciousness [68]. The mortality rate of hospitalized patients is approximately 20–30%. The cause of death is due to aspiration, seizures, or increased intracranial tension [46]. Up to one-half of survivors may have severe long-term neurological sequelae, including movement disorders, paralysis, behavioral changes, memory loss, and cognitive impairment [25,47].

Vaccine campaigns with an inactivated mouse brain-derived Japanese encephalitis vaccine initiated in the 1960s have nearly eliminated Japanese encephalitis in Japan, Korea, and Taiwan [69]. In China, with the addition of a live attenuated Japanese encephalitis vaccine (SA-14-14-2) in 1988, the incidence of Japanese encephalitis has been reduced from 10 to 20 per 100 000 in the 1960s to 1970s to 0.6 to 0.8 today [77]. Japanese encephalitis vaccines are part of the immunization programs in many areas of Asia; in other areas vaccination campaigns are implemented in years following outbreaks.

Three types of Japanese encephalitis vaccines are currently available [69,78,79]. Japanese encephalitis-VAX® is an inactivated mouse brain-derived vaccine manufactured by Biken in Osaka, Japan, that is licensed and distributed in the United States by Sanofi Pasteur [69,75,78]. There are several mouse brain-derived inactivated vaccine manufacturers in Asia; however, distribution of this vaccine is limited, as it is difficult and expensive to produce sufficient quantities of doses in suckling mouse brains [78]. Another disadvantage to using inactivated vaccines is that they have limited duration of protection and require multiple booster doses [69]. A cell culture-derived inactivated Japanese encephalitis vaccine based on the Beijing P-3 strain has been developed but has not been used outside of China. Other inactivated cell-derived Japanese encephalitis vaccines are being developed, including ICSI, of which SA-14-14-2 is a live attenuated vaccine virus that was developed and licensed in China in 1988 [80]. Fifty million doses are produced annually in China for domestic use, and SA-14-14-2 was recently licensed for distribution in Nepal, South Korea, Sri Lanka, Thailand, and India [68,80]. Vaccination remains the only effective long-term method of protection against Japanese encephalitis virus infection, and as a consequence there are several candidate vaccines in development, including live attenuated chimeric vaccines based on the attenuated yellow fever 17D virus vaccine and DNA vaccines [70,78,81,82,83].

West Nile virus

West Nile virus was first isolated from the blood of a febrile patient in Uganda in 1937 and is considered a common childhood infection in Africa (Figure 7.6B). The virus is separated into two distinct lineages based on phylogenetic analysis of the complete West Nile virus genomes [84,85]. Lineage 1 consists of strains from Western Africa, Eastern Europe, the Middle East, and, recently, North America, and includes Kunjin virus from Australia [84]. West Nile lineage 1 virus strains are associated with neuroinvasive disease and have caused encephalitis epidemics throughout Western Africa, Eastern Europe, the Middle East, and North America and epizootics with high equine mortality in Europe and North America [4,37].

Lineage 2 strains are less pathogenic than lineage 1 strains, and their geographical range is restricted to sub-Saharan Africa and Madagascar. Infections with lineage 2 West Nile virus strains may cause a febrile illness but rarely are associated with neuroinvasive disease. West Nile virus in endemic areas of Africa follows a similar pattern to that of Japanese encephalitis virus in Asia. Children are most likely to become infected, with a small percentage of infections developing symptoms of West Nile fever and very few cases of West Nile encephalitis [1].

The virus is transmitted in an enzootic cycle between mosquitoes, primarily *Culex* spp., and birds,

particularly water birds and birds in the corvid family (see Chapter 20). Humans and horses become infected through the bite of an infected mosquito, but neither serves as an amplifying host, as viremia is low and brief. Most cases occur in the mid- to late summer in temperate regions, which corresponds to the transmission activity of the mosquito vector (see Chapter 20).

West Nile virus was first detected and identified in the United States in August of 1999 during a meningoencephalitis epidemic of 59 cases and 7 deaths in New York [38]. The North American West Nile virus is most closely related to and probably originated from a lineage 1 strain from Israel [85]. Since its introduction, the range of West Nile virus has extended down the eastern coast of the United States, west across the country, and north into Canada. Human West Nile virus infections have now been reported from throughout the continental United States, with the exception of Maine, where virus has been detected in mosquitoes but no human cases have occurred. Through 2006 there have been more than 25 000 West Nile infections, with approximately 10 000 of those neuroinvasive disease cases, over 200 acute flaccid paralysis cases, and nearly 1000 deaths (Centers for Disease Control [CDC], unpublished data) [39]. In Canada there have been 2300 West Nile infections reported since the first cases were detected in Ontario in 2002. West Nile virus was recently isolated from horse brain tissue in Argentina, and probable human West Nile cases based on serology have been identified in Central and South America, but large encephalitis epidemics have not been reported from these areas [86,87,88,89].

Since its introduction in 1999, the ecology, epidemiology, and pathology of North American West Nile virus has been intensely studied. The many competent mosquito vectors and susceptible vertebrate hosts, primarily birds in the corvid family, as well as the virulent North American West Nile virus strain, may have contributed to the rapid spread and establishment of the virus in North America (see Chapter 20). This strain has been shown to be highly virulent in birds, and the higher viremia in this vertebrate host may also contribute to more active transmission [90]. The highest annual and cumulative incidence of neuroinvasive disease has occurred primarily in the northern Great Plains states and in the Louisiana-Mississippi Gulf region (CDC, unpublished data). This may be due to the predominance of the highly competent vector, *Culex tarsalis*, combined with the flood irrigation practices and the outdoor lifestyle in the Great Plains. The multiple determinants of vector-borne disease make it difficult to predict whether the epidemic cycle of West Nile virus will be similar to that of St. Louis virus, which is characterized by discrete epidemics followed by long periods of senescence, or to Japanese encephalitis virus, with annual epidemics. To date there has been a seasonal epidemic pattern of continuous cases similar to that of Japanese encephalitis virus, but seroprevalence in North America and Europe remains low, between 2–3% a year, which is similar to that of St. Louis virus in North America [38].

Similar to other flaviviruses in the Japanese encephalitis serogroup, the majority of West Nile virus infections are asymptomatic, with one in five experiencing a mild illness characterized by acute onset of fever, headache, stiff neck, fatigue, malaise, muscle pain and weakness, gastrointestinal symptoms, and a transient macular rash on the trunk and extremities [38,39,43]. The higher reported rates of West Nile fever, compared to those of Japanese encephalitis and St. Louis encephalitis virus infections, may be due to the increased awareness of the disease in the United States. Generally, symptoms resolve within 60 days; however, long-term effects have been reported and it is an active area of research.

Neuroinvasive disease, including encephalitis, meningitis, paralysis, and seizures, develops in approximately 1 in 140 infections, with encephalitis making up the largest proportion, which is similar to that of other Japanese encephalitis antigenic complex virus infections [38]. Acute asymmetric flaccid paralysis has been reported in approximately 13% of patients with West Nile neuroinvasive disease [39]. Although the range of illness is found across all age groups, younger persons tend to have milder West Nile fever, whereas the elderly are more likely to

proceed to the more severe West Nile encephalitis [37,45]. The case fatality rate is approximately 4–18% of hospitalized patients, with mortality higher in the elderly and in immunocompromised persons [37,91]. Long-term neurological problems in survivors and muscle weakness patients with paralysis have been reported [37,39].

Human-to-human transmission of West Nile virus through transfusion of blood products and transplantation of solid organs was identified in 2002 among asymptomatic donors in areas where there was intense West Nile virus transmission [24]. As a result, routine blood screening by highly sensitive and specific nucleic acid amplification tests has been implemented. A single case of transplacental infection has also been reported [37].

Vaccines to protect against West Nile virus infection have been developed for the veterinary market and are commercially available for horses and birds [92]. Commercial development of a human West Nile virus vaccine is ongoing, with the risk-benefit ratio being carefully considered. The pattern of future outbreaks of West Nile virus in North America will be an important component of West Nile virus vaccine development.

St. Louis encephalitis virus

St. Louis encephalitis virus was first identified as the causative agent in encephalitis epidemics in Illinois and in St. Louis and Kansas City in Missouri in 1932 and 1933 [1,93]. Since then periodic encephalitis outbreaks interspersed with long periods of sporadic cases have occurred throughout North and South America and in the United States primarily in the central and eastern states (Figure 7.6C). The largest epidemic, with nearly 2000 cases, occurred throughout the Midwest and southern United States in 1975 [1,94]. The most recent epidemic was in 1990, with most of the nearly 250 cases occurring in Florida and Texas [1]. Since the introduction of West Nile virus to the United States in 1999, surveillance of arboviruses has increased, and subsequently, St. Louis encephalitis cases not associated with epidemics have been identified. These may be a result of increased surveillance rather than a true increase in St. Louis encephalitis cases.

Maintained in enzootic cycle between birds and *Culex* spp. mosquitoes, St. Louis encephalitis virus also infects horses and humans; however, neither horses nor humans play a role in transmission cycle, and there is no morbidity or mortality associated with St. Louis encephalitis virus infection in horses [1,93]. St. Louis encephalitis virus circulates throughout North, Central, and South America, but the rates of transmission to humans are low, although intense mosquito-bird transmission can presage epidemics. Historically St. Louis encephalitis has been considered a disease of rural agricultural areas, although there have been small urban outbreaks. However, even in these cases, such as those that occurred in Los Angeles in 1984, vegetated parkland areas were shown to be the sites of transmission [1]. Transmission is seasonal, with most cases occurring during the late summer months, corresponding to mosquito activity (see Chapter 20).

St. Louis encephalitis is in the Japanese encephalitis serocomplex and very closely related to West Nile virus (Figure 7.1) [93]. In the Americas, where St. Louis encephalitis virus and West Nile virus cocirculate, it may not be possible to differentiate between the two viruses in serological assays, although this is the primary method of diagnosis, as virus isolation from either West Nile or St. Louis virus infected human patients is rare. Although the majority of human St. Louis encephalitis virus infections are subclinical, the ratio of encephalitis cases to asymptomatic infections is approximately 1:85 to 1:800 in adults and children, respectively [1]. There are three clinical syndromes associated with St. Louis encephalitis neuroinvasive infections: encephalitis, aseptic meningitis, and febrile headache. Case fatality rates increase with age, from 2% in young adults to 22% in elderly patients [1]. Adults are more likely to become infected in North America due to low seroprevalence rates. From 30–50% of survivors experience slow, complete recovery, whereas 20% experience long-term neurological symptoms, including gait and speech disturbances, sensorimotor impairment, psychoneurotic complaints, and

tremors [1,93]. Vaccines have been developed to protect against St. Louis encephalitis virus infection. However, given the long time between epidemics, the low seroprevalence rate, and the cost of bringing a vaccine to market, commercial vaccine development is unlikely.

Murray Valley encephalitis virus

Outbreaks of encephalitis reported in the Murray Valley of Australia in the early 1900s are thought to have been caused by Murray Valley encephalitis virus, although the virus was not identified or characterized as being distinct from Japanese encephalitis virus until 1951 [1,4,95]. Like Japanese encephalitis and West Nile viruses, Murray Valley encephalitis virus infection can also result in a polio-like acute flaccid paralysis, and early Murray Valley encephalitis virus outbreaks were thought to be poliomyelitis.

Murray Valley encephalitis virus is transmitted in an enzootic cycle between water birds and the principal mosquito vector *Culex annulirostris*, which breeds in transient pools [95] (see Chapter 20). Similar to Japanese encephalitis virus, large water birds such as herons, egrets, and pelicans are the primary vertebrate hosts [7,95]. Mammals such as kangaroos and rabbits also may be significant viremic hosts in the transmission cycle [1]. The range of Murray Valley encephalitis virus extends throughout the tropical northern parts of Australia and New Guinea, and in these areas, Murray Valley encephalitis virus infection is the most common cause of viral encephalitis (Figure 7.6D). Similar to the other neurotropic flaviviruses, humans are infected incidentally and do not contribute to the transmission cycle [48].

Febrile illness due to Murray Valley encephalitis infection is not reported, but the ratio of subclinical infections to encephalitis cases is estimated to be 1:1000 [48]. Similar to Japanese encephalitis infections the clinical patterns of the disease include rapid onset of fatal encephalitis, flaccid paralysis, tremor, or encephalitis with complete recovery [48]. Clinical illness is generally seen in children and non-immune adults [2,48].

There have been approximately 40 cases reported in the last 25 years, with a 31% fatality rate [4]. About a third of survivors experience long-term neurological deficits [48]. Because of the large proportion of inapparent infections and high seroprevalence in adults, large-scale vaccination programs against Murray Valley encephalitis virus have not been considered necessary or economically feasible.

Tick-borne encephalitis viruses

Tick-borne encephalitis was first described in the far-eastern region of the Soviet Union in 1934. The Russian spring-summer encephalitis virus was isolated from a human brain and ticks were shown to be the arthropod vector in 1937 [1,96]. During an epidemic in 1948 in Czechoslovakia, another virus, later named Central European encephalitis virus, was isolated from a patient and was found to be closely related to but distinct from Russian spring-summer encephalitis virus. Since then, 12 species of tick-borne flaviviruses have been identified. These are divided into two groups: seabird and mammalian [18]. The mammalian tick-borne encephalitis flaviviruses that are human and animal pathogens include louping ill virus, Russian spring-summer encephalitis virus, Central European encephalitis virus, Omsk hemorrhagic fever virus, Langat virus, Kyasanur Forest disease virus, and Powassan virus [1,9,18,27,93,96,97]. The geographic ranges of the tick-borne encephalitis viruses are shown in Figure 7.7. The virus is maintained in nature between ticks and small mammals, with rodents shown to be important asymptomatic amplifying, reservoir, and overwintering hosts (see Chapter 20). Although not amplifying hosts, cattle, sheep, and goats infected with tick-borne encephalitis viruses may excrete virus in their milk so that humans can become infected by ingesting raw milk or cheese [96].

Tick-borne encephalitis viruses have been isolated from ticks throughout Northern, Central, and Eastern Europe, and outbreaks have occurred in Central and Eastern Europe, Scandinavia, France, Germany, Italy, Greece, and Albania, which corresponds to the geographical distribution of the *Ixodes*

Figure 7.7. Geographical distribution of the tick-borne encephalitis viruses.

spp. tick vectors (Figure 7.7) [1,96]. Human infections from louping ill virus, Powassan virus, and Negishi virus have been reported in Ireland and the United Kingdom; Russia and North America; and China, respectively [1,98,99]. There are several thousand tick-borne encephalitis cases a year in Europe; since 1958, 31 cases of Powassan encephalitis virus infections have been detected in North America [98,99].

The clinical course of the disease is distinct from the mosquito-borne flaviviruses, as many of the tick-borne encephalitis infections take a biphasic course. The Central European encephalitis viruses generally are associated with milder disease, and after an incubation period of 3–7 days, in the first phase, patients may experience an influenza-like illness for approximately 1 week, with fever, headache, malaise, and myalgia [96]. Following an asymptomatic period of up to a week, 20–30% experience a second phase of the disease involving the central nervous system, the clinical symptoms of which are meningeal irritation, meningitis, meningoencephalitis, or meningoencephalomyelitis [1,96]. Generally, symptoms are more severe in children than in adults. The case fatality rate ranges from 1–5%. Approximately 10–20% of survivors, generally those with the more severe clinical forms, have long-term neurological problems similar to those resulting from other neuroinvasive flavivirus infections.

The more severe course of disease results from infection by the far-east Russian spring-summer virus subtypes [96]. In these infections, onset of illness may be more gradual, with a prodromal phase

consisting of symptoms similar to those of the Japanese encephalitis complex virus infections: fever, headache, anorexia, nausea, vomiting, and photophobia. Infection of the brain stem and upper cervical cord produce stiff neck, ataxia, sensorial changes, convulsions, and in about 20% of cases, flaccid paralysis. The case fatality rate is approximately 20%, with 30–60% of survivors experiencing residual neuronal damage [96,100].

A formalin-inactivated tick-borne encephalitis vaccine is commercially available in Europe and may be required for travelers to endemic areas in Eastern Russia, where the seroprevalence rate may be as high as 51% [96]. However, because of adverse events attributed to the vaccine, particularly in children, the vaccine is recommended only in areas where there is a high risk of infection. The vaccine is not available in the United States.

Other flaviviruses causing encephalitis

Other flaviviruses generally associated with enzootic transmission may cause sporadic encephalitis cases or may be emerging. The etiological agent may be difficult to identify in regions where there are multiple flaviviruses circulating, due to the cross-reactivity in serological assays and the infrequency of obtaining a virus isolate. These viruses may emerge as significant human pathogens as deforestation and changing agricultural practices bring humans into areas of enzootic transmission cycles of these viruses.

Rocio virus

Rocio virus was isolated from a fatal human case during an encephalitis epidemic in 1975 in Brazil. Between 1975 and 1976, 971 cases were reported; however, since then only one case has been detected. Clinical symptoms are similar to those of infections from St. Louis encephalitis virus, which circulates in the same geographical area, and serological cross-reactivity makes Rocio virus infection difficult to identify without a virus isolate [1].

Modoc virus

Modoc virus was first isolated from deer mice in Modoc, California, in 1958. It was reported as the etiological agent in one human case. Rodents are the primary host of Modoc virus; no arthropod vector has been identified [1].

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Note

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SECTION II

Introduction: retroviruses, DNA viruses, and prions

Carol Shoshkes Reiss

This section of the book includes a more diverse collection of infectious agents when compared to the first section. But, in contrast to the acute nature of most RNA virus infections, all of these persist, sometimes over many decades of a host's life.

Two retroviruses are included that primarily infect peripheral cells, but in a minority of patients the retroviruses are brought into the brain as passengers on circulating cells that cross the microvascular endothelium to reach distinct cell types in the central nervous system (CNS). These diseases, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and neuro-AIDS, caused by human T cell leukemia virus-1 (HTLV-1) (Chapter 8) and human immunodeficiency virus (HIV) (Chapter 9), respectively, are serious clinical problems involving millions of patients. In HTLV-1, it is the host's immune response that leads to the pathology, while with HIV, the progressive loss of immune function leads to susceptibility to opportunistic infections and tumors. Like RNA viruses, the RNA-dependent DNA polymerase (reverse transcriptase) lacks the proofreading function of host DNA replication, and mutations are frequently inserted, which leads to evasion of both host defenses and antiviral drug treatment.

JC virus is a ubiquitous small DNA virus, a papovavirus, but it causes disease in immunocompromised patients (Chapter 10). Most patients today are comorbidly infected with HIV given the sheer numbers of patients with that disease. A recent small cluster of patients with progressive multifocal leukoencephalopathy (PML) was found among multiple sclerosis patients receiving a monoclonal antibody, which restricted access of T cells to the CNS, thus enabling the latent JC virus to replicate and cause disease.

Herpes simplex virus can remain latent in the trigeminal ganglion (or ganglia innervating the genitourinary [GU] system) and repeatedly spontaneously reactivate from latency causing acute infection in epithelial tissue, which is innervated by the ganglion, in spite of a strong adaptive immune response in the host. Far more serious is the very rare encephalitis of newborns or even rarer, of adults (Chapter 11). A vaccine is currently in clinical trials for genital herpes, which is both an STD and the principal source for newborn disease.

Shingles or zoster is the reactivation from latency of another herpes virus, varicella-zoster virus (Chapter 12). This virus infection, generally acquired as chickenpox in childhood, remains latent for decades in a ganglion. Reactivation leads to infection of a dermatome patch of skin that is supplied by that ganglion. This can also lead to postherpetic neuralgia, a painful condition. A vaccine, Zostavax™, made by Merck, was licensed by the U.S. Food and Drug Administration (FDA) in 2006, to prevent the adult reactivation from latency.

In contrast to viruses, transmissible spongiform encephalopathies (Chapter 13) are caused by an

"infectious" endogenous prion protein that is malfolded. The native protein is ubiquitous, but either oral exposure (ingestion) or injection of the protease-resistant scrapie form of the protein leads to self-catalyzed transformation of the endogenous protein, leading to disease. There are also inherited forms of this family of diseases. Although they occur in many species, with virtually the same symptoms, they have been given different names (scrapie, mad cow, kuru) but are collectively termed transmissible spongiform encephalopathies. Vaccines are in development to slow disease progression.

8

Human T-lymphotropic virus type 1 and disease in the central nervous system

Angelina J. Mosley and Charles R.M. Bangham

Introduction

The exogenous human retrovirus human T-lymphotropic virus type 1 (HTLV-1) results in a highly dynamic persistent infection that has a significant clinical impact in endemic areas. HTLV-1 usually causes an asymptomatic infection, but in a small proportion of individuals disease may develop: adult T-cell leukemia or a range of inflammatory diseases. Of these inflammatory diseases HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is the most studied. HAM/TSP is a chronic inflammatory disease of the central nervous system. It is rarely fatal, but can be severely debilitating. HTLV-1 is not a classical neurotropic virus and does not directly infect the cells of the central nervous system (CNS). Instead, HTLV-1 is found primarily within infiltrating, infected $CD4^+$ T lymphocytes. $CD4^+$ T-cell infiltration into the CNS is currently believed to be the pivotal event for the pathogenesis of HAM/TSP. Here we describe the immune control of HTLV-1 infection in the periphery and discuss its relationship with inflammation in the CNS. We explore the current hypothesis of HAM/TSP pathogenesis, identify crucial factors, and suggest a new hypothesis focused on why the majority of HTLV-1-infected individuals do not develop neuroinflammatory disease.

HTLV-1

HTLV-1 was the first exogenous replication competent human retrovirus to be identified over 20 years ago by Poiesz *et al.* [1]. HTLV-1 virions were isolated from a cell line established from a cutaneous T-cell lymphoma. HTLV-1 has since been associated with two different types of disease: lymphoma/leukemia and chronic inflammatory diseases [2,3,4,5]. In this chapter, we discuss the impact of HTLV-1 infection at both the population and the cellular level. Specifically, we focus on immune control of HTLV-1 infection and a current hypothesis of inflammatory disease pathogenesis.

HTLV-1 infection

Epidemiology and associated diseases

It is estimated that 10 to 20 million people are infected worldwide with HTLV-1 [4,6]. These individuals can be found in many geographically restricted populations [4,7]. Endemic areas include southern Japan, the Caribbean, Central and West Africa, and Central and South America. Pockets of infection and sporadic cases have been described in other countries [4,8]. Within endemic areas seroprevalence in adults ranges from 1–30%.

Successful transmission of HTLV-1 to an uninfected individual requires the transfer of infected cells. Consequently, cell-free blood products carry an extremely low infectious risk and HTLV-1 is considered to have a low level of transmissibility, especially compared to that of HIV-1. HTLV-1 screening of blood donors has been routine in Japan, France, Brazil, and the United States for some years and was introduced in the United Kingdom in 2003.

Infection with HTLV-1 is widely believed to be lifelong and in most cases (~95%) remains asymptomatic. There is some evidence that infection with HTLV-1 results in a degree of immunosuppression, which imposes a significant disease burden in areas where other infections, like tuberculosis, also have a high prevalence [9,10,11,12,13]. In the remaining minority (~5%) of HTLV-1-infected individuals disease develops after a long, clinically asymptomatic phase. Disease manifests as either adult T cell leukemia/lymphoma (ATLL; a rapidly fatal malignancy of $CD4^+$ T cells) or one of a range of chronic inflammatory diseases including polymyositis, uveitis, alveolitis, arthropathy, and the highly studied HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [5]. HAM/TSP is a chronic inflammatory disease of the CNS and is the main focus of this chapter.

Exactly what determines disease development, and in which organs, is unknown. In fact, one of the main questions in HTLV-1 biology that remains unanswered is: Why do only a very small proportion of HTLV-1-infected individuals develop disease?

HTLV-1 virology

HTLV-1 is a large (100–110 nm diameter), enveloped virus with C-type virion morphology (Figure 8.1A) [1,3]. The infectious genome consists of two identical plus-strand RNA molecules, 9032 nucleotides in length. HTLV-1 is a complex retrovirus and thus, in addition to the usual gag, pro, pol, and env genes, HTLV-1 contains an additional X region, which encodes regulatory proteins, namely Tax, Rex, Rof, Tof, and at least two other putative proteins (Figure 8.1B) [14]. Further, HTLV-1 encodes a gene, HBZ, which is believed to act as a protein and as a regulatory RNA [15,16,17].

The viral proteins Tax, Rex, and Rof are essential for HTLV-1 infection and persistence *in vivo*, as they mainly control HTLV-1 viral gene expression [14]. Tax is a pleiotropic transcriptional transactivator with no DNA binding ability of its own [18], but it enhances the inherent DNA binding function of cellular transcription factors [19,20]. In this way, Tax up-regulates the expression of both viral and cellular genes [21,22]. However, almost nothing is known about the control of the activation of the HTLV-1 provirus *in vivo*.

In vitro, HTLV-1 has been found to infect a broad range of cell types and cell lines from different vertebrate species. However, as its name suggests, HTLV-1 is confined to T-lymphocytes *in vivo* [23,24]. HTLV-1 mainly infects $CD4^+CD45RO+$-activated T cells, but a significant proportion of the *in vivo* viral burden has also been found in $CD8^+$ T cells [25,26]. There have also been reports that HTLV-1 infects monocytes, B cells, DC, epithelial cells, and neural cells [24,27,28,29,30,31]. However, evidence for productive infection of these cell types is limited.

HTLV-1 cell biology

Like other replication-competent exogenous retroviruses, HTLV-1 has two potential routes of replication: the infectious replication cycle or replication of the integrated provirus upon host cell division. HTLV-1 is highly cell-associated (Figure 8.2): no cell-free virions have been found in the serum of HTLV-1 seropositive individuals, cell-cell contact is required for infectious transmission via a viral synapse [32], and the infectivity of virus particles produced *in vitro* is extremely low (in the order of one infectious particle per 10^5 to 10^6) [33]. The HTLV-1 sequence remains remarkably stable over time – both within and between individuals – but this stability cannot be explained only by the fidelity of the HTLV-1 reverse transcriptase (RT), which is of a similar order to that of other RTs [34].

If HTLV-1 only replicated passively through mitosis of the host cell it would be impossible to establish an infection in a new host. Additionally, env would not be conserved, antiretroviral therapy would have no effect on viral burden [35], and expansion of infected cells would be solely dependent on antigen exposure or chance contact with certain cytokines such as IL-2 or IL-15. This is clearly not the case. The observed size of HTLV-1-infected $CD4^+$ T cell clones is much greater than that seen in antigen-driven expansion [36,37], suggesting an active role for HTLV-1 in driving its own persistence within a host,

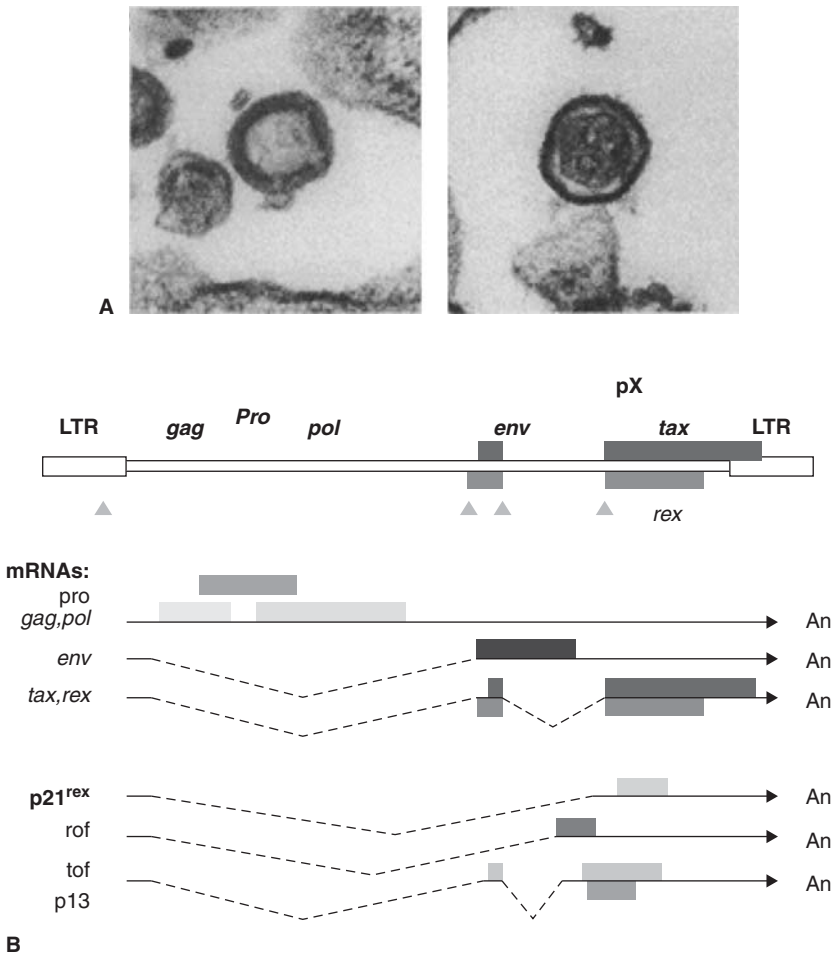

Figure 8.1. HTLV-1 virion morphology and genome structure. (A) HTLV-1 is a large enveloped C-type retrovirus. The left-hand panel shows an immature particle, which undergoes proteolytic maturation to form the mature particle (right-hand panel) with an electron dense core. (Adapted from [215]). (B) The HTLV-1 genome is an RNA molecule, 9032 nucleotides long. HTLV-1 is a complex retrovirus and thus encodes many regulatory proteins in addition to the gag, pro, pol, and env genes. (Adapted from [216].)

most likely through the activity of the Tax protein, which upregulates expression of a range of T-cell activation and proliferation genes [38], and Rof, which alters early cell activation and LFA-1-mediated cell-cell contact [39].

In the chronic phase of infection, where the strong anti-HTLV-1 immune response is active, it is likely that mitotic replication is the predominant mode of HTLV-1 persistence [36,40]. But this does not preclude the occurrence of continued *de novo* (infectious cycle) infection in this chronic phase. Indeed, the relative importance of infectious and mitotic replication of HTLV-1 is likely to vary between individuals and is an area of much research interest.

Finally, because cell-free virus is undetectable *in vivo*, it is most appropriate to measure HTLV-1 burden as the HTLV-1 proviral load. That is, we must measure the frequency of HTLV-1

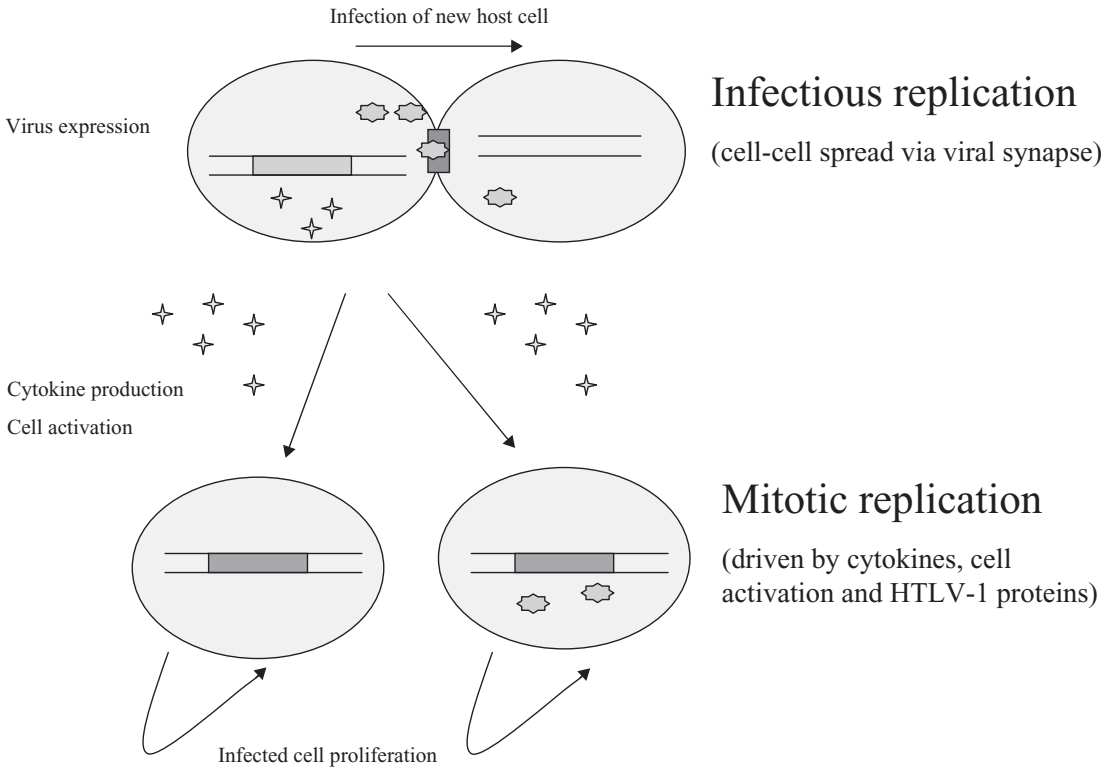

Figure 8.2. HTLV-1 viral life cycle. HTLV-1 (big stars) exhibits an unusual life cycle for a retrovirus such that its primary mode of replication is by mitotic replication of infected host cells (colored DNA), driven either by HTLV-1 proteins directly or by cytokine secretion (small, 4-point stars) and immune activation (mitotic replication). *De novo* infection requires cell-cell contact and transfer of viral particles through a specialized viral synapse (gray box; infectious replication). Cell-free virions are not found *in vivo* and are poorly infectious *in vitro*.

provirus-carrying cells within the circulating peripheral blood mononuclear cell (PBMC) population. While HTLV-1 proviral load remains stable within an individual, it can vary by more than 10^4-fold between infected individuals (ranging from 0.001% to 50%) [41]. But even the lower HTLV-1 proviral loads seen are typically higher than those found in HIV-1-infected individuals.

HTLV-1 establishes a persistent, chronic infection at a given equilibrium "set point." What determines an infected individual's HTLV-1 set point remains a vital, but as yet incompletely answered question. The dynamic interaction between HTLV-1 and the host immune response to infection is likely to play a major role. The ability of the immune system to control HTLV-1 replication, and thus proviral load (discussed in detail below), is in turn likely to be determined by the level of viral activity and virus gene expression [42]. Virus gene expression (at either the mRNA or protein level) is almost undetectable in circulating $CD4^+$ T cells directly *ex vivo* [43,44,45]. However, after a short (6 hour) *ex vivo* incubation, significant levels of Tax expression are easily detected [23]. This abundant *ex vivo* Tax expression is decreased if infected $CD4^+$ T cells are co-incubated with autologous $CD8^+$ cells, thus suggesting efficient immune-mediated control of productively infected cells. Using an *in vivo* DNA labeling technique, we

have recently estimated that approximately 0.03–3% of HTLV-1-infected cells express Tax *in vivo* per day [46]. However, the question remains as to whether this low level of expression is sufficient to maintain proviral load and the immune response.

HAM/TSP – the clinical entity

HAM/TSP pathology

History

Six years after its identification, HTLV-1 was found to be the etiological agent of HAM/TSP. Gessain and de Thé reported an association between HTLV-1 seropositivity and possession of an unusual neurological syndrome – tropical spastic paraparesis (TSP) – in the Caribbean [47]. They found that 10 of 17 TSP patients were HTLV-1-positive. Simultaneously, Osame reported that the prevalence of HTLV-1 seropositivity was high in patients with defined neurological symptoms in Japan [48]. Thus, HTLV-1-associated myelopathy (HAM) was identified. Subsequently, TSP and HAM were shown to be the same clinical entity, and the syndrome was therefore termed HAM/TSP.

HAM/TSP – diagnostic criteria and disease progression

HAM/TSP is a chronic inflammatory disease of the CNS that has much in common with the symptomatology of certain forms of multiple sclerosis (MS). However, HAM/TSP is a progressive degenerative syndrome and does not exhibit the relapsing-remitting phenotype that is usual in MS [49].

The onset of HAM/TSP is generally insidious, and clinical symptoms are slowly progressive. Symptoms are neurological in nature, mainly consisting of spasticity, hyperreflexia, and muscle weakness in the lower extremities (Table 8.1) [49]. Disturbed gait is often accompanied by persistent back pain and cerebellar symptoms as well as urinary and bowel disturbances [50]. In addition, somatosensory disturbances are observed in about half of HAM/TSP cases,

Table 8.1. World health organization criteria for HAM/TSP diagnosis

Incidence
Mostly sporadic and adult
Mostly female
Onset
Usually insidious, may be sudden
Main neurological findings
Chronic spastic paraparesis
Weakness of lower limbs
Bladder disturbance, constipation, impotence
Sensory symptoms
Low lumbar pain with radiation to the legs
Vibration senses frequently impaired
Hyperreflexia of the lower and upper limbs
Exaggerated jaw jerk in some patients
Less frequent neurological findings
Cerebellar signs, optic atrophy, deafness, nystagmus
Rarely: convulsions, cognitive impairment, dementia
Other possible neurological findings
Muscular atrophy, polymyositis, cranial neuropathy, meningitis
Possible systemic non-neurological findings
Pulmonary alveolitis, uveitis, Sjorgen's syndrome
Arthropathy, vasculitis, cryoglobulinemia
Monoclonal gammaopathy
Adult T-cell leukemia or lymphoma
Laboratory diagnosis
Detection of HTLV-1 antibodies or antigens in blood/CSF
Mild lymphocyte pleocytosis in CSF
Lobulated lymphocytes in blood or CSF
Mild-to-moderate increase in protein in CSF

(Adapted from Hollsberg and Hafler [49].)

and HAM/TSP may also be accompanied by mild peripheral neuropathy and other inflammatory diseases [51,52,53].

The progression of HAM/TSP (as measured by a worsening disability score) varies between individuals but is generally characterized by an initial phase of clinical deterioration followed by a steady course, gradually reaching a plateau at a fixed level of disability.

Little is known about the reasons for this variation in the rate of HAM/TSP progression, but the rate of progression is known to be associated with HTLV-1

proviral load [54,55] and with age at onset of disease. HAM/TSP onset generally occurs in the 4th to 5th decades but can range from 6 to over 70 years. The reasons for this broad range are unknown, but may be related to viral dosage and immune system maturity at the time of HTLV-1 transmission.

Histopathology and MRI of HAM/TSP lesions

Direct visualization of the CNS of HTLV-1-infected individuals is limited to histochemistry of a small number of autopsy samples and small-scale magnetic resonance imaging studies, both chiefly on HAM/TSP patients and frequently on single patients. Very few systematic studies of the CNS have been done across all manifestations of HTLV-1 infection.

Histological studies of HAM/TSP patients have shown that the spinal cord, particularly at the upper thoracic and lower cervical level, is mainly affected by inflammatory lesions [56,57]. These inflammatory lesions are associated with perivascular and parenchymal T lymphocyte infiltration, accompanied by foamy macrophages, demyelination and loss of axons, some neuronal degeneration, and gliosis (proliferation and scarring by glial cells) [58,59]. Thus, both the white and gray matter of the CNS appear to be affected. The reasons for the focus of lesions in the thoracic spinal cord is unknown, but might be related to a slower blood supply. Aye *et al.* have shown that the sites of inflammation in HAM/TSP correlate with sites of the slowest blood flow in the spinal cord and with the distal part of the medullary arteries in the brain, where inflammatory lesions are also often detected [60].

HAM/TSP lesions are usually categorized into early (active) and late (inactive) lesions depending on the duration of disease. In early spinal cord lesions, histochemical cell staining has shown that $CD4^+$ cells tend to predominate in the perivascular mononuclear cell infiltrate [61,62,63]. However, one study has shown that $CD4^+$ and $CD8^+$ T cells, as well as macrophages, are evenly distributed in active lesions [63]. Cells undergoing apoptosis in active lesions are mostly $CD4^+$ $CD45RO^+$ activated T cells [64].

In contrast, histochemical staining of inactive ("burnt-out") lesions shows a predominance of $CD8^+$ T cells over $CD4^+$ T cells [56,63]. Macrophages remain present. In lesions of much longer duration (>10 years of disease), $CD8^+$ T cells are mostly absent and the lesions appear degenerative rather than inflammatory [61]. Fibrotic change in blood vessel walls (both capillaries and larger vessels) is also seen in late, inactive lesions. Natural killer (NK) and B cells are rarely present in high frequencies in either active or inactive lesions, suggesting that HAM/TSP is primarily mediated by T cells.

Several studies of magnetic resonance imaging (MRI) in HAM/TSP have been carried out. However, the spatial resolution of the information is low and no information can be provided at the cellular level. There remains some debate over the usefulness of MRI in clinical diagnosis or monitoring of HAM/TSP patients' progression [65]. Generally, abnormal lesions within both the spinal cord and brain of HAM/TSP patients are identifiable by MRI. These lesions may be multifocal and disseminated throughout the CNS (similar to MS) [66].

The involvement of the brain at different stages of HAM/TSP is less clear [67,68]. Cerebral white matter lesions and spinal cord atrophy are considered the main MRI findings. Lesions in the brain tend to be subcortical and in the deep cerebral white matter [69,70]. There is some suggestion that, with a longer duration of disease (5–10 years), inflammatory lesions disseminate from the spinal cord to the brain, but there is also evidence that there are simultaneous acute changes in the spinal cord and brain of HAM/TSP patients, as identified by MRI [60], suggesting widespread foci of inflammatory damage.

Risk factors for HAM/TSP

Since only a small fraction (0.1–2%) of HTLV-1-infected individuals develops HAM/TSP, infection with HTLV-1 is clearly necessary but by no means sufficient to cause disease. The disease develops only after a long clinically asymptomatic phase. In Japan, the lifetime risk of developing HAM/TSP among

asymptomatic carriers (ACs) is estimated to be 0.23% [71]. In the absence of an effective treatment, cure, or vaccine against HAM/TSP it would be helpful to identify significant risk factors for HAM/TSP development, both for the clinic and for our basic understanding of HAM/TSP pathogenesis.

Few risk factors for HAM/TSP have been identified in most populations studied with endemic HTLV-1 infection except age, sex, and proviral load [59,72]. A high HTLV-1 proviral load has long been known to be associated with an increased risk of HAM/TSP. In southern Japan the prevalence of HAM/TSP rises exponentially with proviral load once it exceeds 1% PBMCs infected (Figure 8.3) [41,72]. However, the range of HTLV-1 proviral loads seen in both the AC and HAM/TSP groups is large with an extensive overlap [73,74,75].

It is of course likely that HTLV-1 gene expression, not the proviral load per se, causes the manifestations of HTLV-1-associated diseases. Consistent with this, we have recently shown that, at a given proviral load, productively HTLV-1-infected $CD4^+$ cells from HAM/TSP patients express the Tax protein at a significantly higher rate than cells from ACs [76]. We also found that the rate of Tax expression was a better predictor of HAM/TSP than proviral load alone, correctly identifying HAM/TSP in over 80% of cases.

No neuropathic strain of HTLV-1 has been identified associated with HAM/TSP [77,78,79,80], although in Japan there is a higher prevalence of the Cosmopolitan subgroup A serotype in HAM/TSP patients than ACs [81]. Thus, it is likely that host factors determine the clinical outcome of HTLV-1 infection.

Asymptomatic HTLV-1-infected genetic relatives of HAM/TSP patients were found by Nagai *et al.* to have a median proviral load nine times higher than that of unrelated ACs [41]. This observation suggested that genetic factors contribute significantly to the control of proviral load and hence, the risk of HAM/TSP. In southern Japan, a detailed study of host immunogenetics revealed significant independent risk effects of certain genetic polymorphisms within the HLA genes and the TNFA and SDF1 loci [74,82,83], which are discussed below.

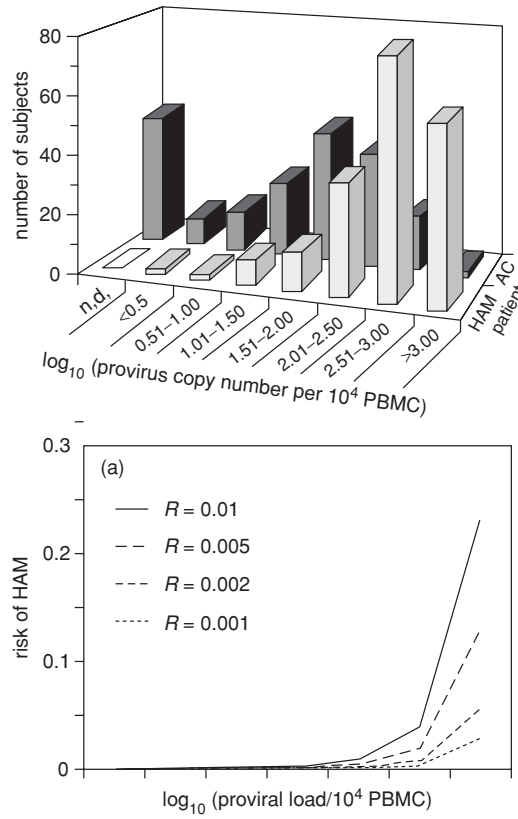

Figure 8.3. The risk of HAM/TSP is associated with HTLV-1 proviral load. A strong risk factor for the development of HAM/TSP is HTLV-1 proviral load in peripheral blood mononuclear cells. The incidence (top panel) and risk (lower panel) of HAM/TSP rises exponentially in patients with a proviral load greater than 1% of PBMCs infected [72].

HAM/TSP – an immune-mediated disease

Thus far, we have established that it is likely to be the host response to HTLV-1 that chiefly determines the clinical outcome of infection. What is the pathogenic mechanism of HAM/TSP? Infiltration of $CD4^+$ and $CD8^+$ T cells into the CNS is likely to be a major contributor, but is HAM/TSP an autoimmune disease?

The study of the pathogenesis of HAM/TSP is severely limited for three main reasons. First, HAM/TSP only manifests after a long clinically

asymptomatic phase so patients often already have well-established disease at the time of diagnosis. The frequency of conversion from AC status to HAM/TSP is so low as to be unhelpful when following a cohort longitudinally. Second, it is not practically or ethically possible to gain direct access to the cells and tissues in the CNS, except in autopsy cases. Third, no good animal model of HAM/TSP exists. The rat model of HAM/TSP is of limited use, as the CNS lesions in the rat do not contain large infiltrates of T lymphocytes [61,84,85,86,87]. A squirrel monkey model of HTLV-1 infection is hampered by the absence of T lymphocyte-specific antibody reagents and the animals do not develop signs of neurological disease [88,89]. Thus, pathogenesis of HAM/TSP can only be inferred from limited *in vivo* work, *ex vivo* and *in vitro* experiments, and by analogy with other chronic infections and neurological diseases.

In this section, we focus on the systemic immune response to HTLV-1 itself and its probable relationship with neuroinflammation in the CNS.

The role of CD8$^+$ T-cells in HAM/TSP

The CD8$^+$ T cell response to HTLV-1 is unusual in its magnitude and its restricted specificity: up to 10% of circulating CD8$^+$ T cells can be specific for a single Tax peptide [73,74]. CD8$^+$ cells specific for the other HTLV-1 proteins have also been detected, but at a much lower frequency [73,90,91]. Not only are HTLV-1-specific CD8$^+$ T cells abundant, they are also chronically activated, actively cytotoxic and are capable of producing IFN-γ and matrix metalloproteinases without the need for antigenic re-stimulation *ex vivo* [92,93]. HTLV-1-specific CD8$^+$ T cells have also been found to be preferentially infected by HTLV-1, and such infected CD8$^+$ T cells can themselves be killed by other HTLV-1-specific CD8$^+$ T cells, a process known as T cell fratricide.

The role of the CD8$^+$ T cell response in HTLV-1 infection has been somewhat controversial for many years [94,95]. HTLV-1-specific CD8$^+$ cells have the potential to be both beneficial and detrimental such that they may control HTLV-1 proviral load or they may promote neuroinflammation. Since these two mechanisms are not mutually exclusive, an important possibility is that both harmful and beneficial effects are exerted simultaneously. We shall now explore the evidence supporting each argument.

CD8$^+$ T cells protect against neuroinflammation

HTLV-1-specific CD8$^+$ T cells are postulated to protect against HAM/TSP disease primarily by reducing proviral load. CD8$^+$ cells from HTLV-1-infected individuals are cytotoxic directly *ex vivo* [23,93,96] and have been shown to lyse Tax-expressing autologous CD4$^+$ cells in a perforin-dependent manner without the need for prior re-stimulation [23]. However, as with HIV-1 infection, the relative importance of cytotoxic CD8$^+$ cells and their impact on the infected cell population has been open to much debate. What is the evidence that CD8$^+$ cells influence HTLV-1 proviral load?

As mentioned briefly above, possession of the *HLA-A*02* allele was shown to halve the odds of HAM/TSP while, within ACs, possession of *HLA-A*02* was associated with a proviral load one-third that of ACs without *HLA-A*02* [74]. However, reductions in proviral load were not seen in *HLA-A*02*+ HAM/TSP patients. This may be related to a recent observation that the protective effects of *HLA-A*02* are only observed in Japanese individuals infected with the Cosmopolitan subgroup B strain of HTLV-1, not the subgroup A strain, which is itself associated with a higher prevalence of HAM/TSP [81]. In addition to *HLA-A*02*, possession of *HLA-Cw*08* and heterozygosity at all three HLA class I loci was associated with a reduced proviral load [83]. In contrast, possession of the *HLA-B*5401* allele was associated with a higher susceptibility to HAM/TSP in the same Japanese population [83].

Smaller scale immunogenetic studies have been performed on the HTLV-1-infected Iranian population where a novel single nucleotide polymorphism (SNP) in the gene for the cytotoxic protein perforin was found to be associated with HAM/TSP [97]. Additionally, possession of the *HLA-A*02* and *HLA-Cw*08* alleles were not found to protect against HAM/TSP

in this Iranian population [98], perhaps because the Iranian subjects studied were infected with the Cosmopolitan subgroup A strain of HTLV-1.

It is clear from the evidence described above that genetically determined variation in a MHC class I-restricted $CD8^+$ T cell response can influence the disease outcome of HTLV-1 infection. If an HTLV-1-infected individual with a low proviral load possesses a strong $CD8^+$ cell immune response two predictions can be made. First, it would be expected that $CD8^+$ cell-mediated immune selection pressure upon key viral epitopes should result in detectable changes in amino acid sequence. Indeed, by calculating the ratio of nonsynonymous to synonymous changes (dN/dS) in Tax sequences Niewiesk *et al.* found a stronger net positive selection for amino acid change in Tax in ACs, who generally have lower proviral loads, than in HAM/TSP patients [99]. Second, if $CD8^+$ cells control proviral load, the gene expression profile of circulating $CD8^+$ cells would be expected to be associated with proviral load. Using a DNA expression microarray we have recently shown that $CD8^+$ cells from individuals with a low HTLV-1 proviral load over-express a core group of nine genes [100]. These nine genes show strong functional coherence since the majority encode proteins involved in target cell recognition and cytotoxicity.

Together, the immunogenetics, dN/dS ratio, and microarray studies suggest that a "strong" $CD8^+$ cell response suppresses HTLV-1 proviral load. In an acute viral infection, a "strong" $CD8^+$ cell response is a large response (i.e., a high frequency of virus-specific cells), but this is not necessarily the case for the $CD8^+$ cell response to chronic viral infections [101,102,103]. In some studies of HTLV-1 infection, HTLV-1-specific $CD8^+$ cells were detected only in HAM/TSP patients [93,104,105]. However, other studies, using a variety of experimental techniques, have identified substantial HTLV-1-specific $CD8^+$ cell responses in both ACs and HAM/TSP patients, with no significant difference in the median frequency of $CD8^+$ cells between the two groups at a given proviral load [75,106,107]. There appears to be a large range in the size of the HTLV-1-specific $CD8^+$ cell response with a significant overlap between ACs and HAM/TSP patients, although the frequency of the cells tends to be higher in HAM/TSP patients.

The frequency of HTLV-1-specific $CD8^+$ cells is positively correlated with proviral load [108,109]. However, correlations between virus-specific $CD8^+$ cell frequency and viral load can be at best difficult to interpret and at worst uninformative in persistent infections. Kubota *et al.* have found both positive and negative correlations between $CD8^+$ cells and proviral load in HTLV-1 infection, depending on the $CD8^+$ cell phenotype [110]. Similarly, in HIV-1 infection, both positive, negative, and zero correlations have been found [111,112,113]. In order to resolve this difficulty, Nowak and Bangham developed a mathematical model that described the relationship between viral burden and the $CD8^+$ cell response [114]. Using this model it was found that control of virus load was not necessarily reflected by the magnitude (frequency) of the $CD8^+$ cell response, but rather by the ability of individual $CD8^+$ cells to respond (i.e., the activation threshold). That is, two infected hosts can have the same frequency of virus-specific $CD8^+$ cells, but the individual whose $CD8^+$ cells have a higher activity (lower activation threshold) will have a lower virus burden (Figure 8.4A). Similarly, HTLV-1-infected ACs and HAM/TSP patients can have similar frequencies of HTLV-1-specific $CD8^+$ cells but still have different proviral loads depending on differences in $CD8^+$ cell responsiveness.

$CD8^+$ cell responsiveness can be defined as a composite function of the rate at which an individual mounts a $CD8^+$ cell response (proliferation rate) and the rate at which $CD8^+$ cells can kill infected cells (rate of lysis). We have recently developed a functional *ex vivo* assay in order to quantify experimentally an HTLV-1-infected individual's $CD8^+$ cell rate of lysis [96]. Using a simple flow cytometry-based experimental protocol and a novel mathematical model we estimated the $CD8^+$ cell-mediated rate of lysis, as defined by the proportion of Tax-expressing $CD4^+$ cells killed per average $CD8^+$ cell per day, for both ACs and HAM/TSP patients attending an HTLV-1 clinic in the United Kingdom. We found that, as predicted, individuals varied greatly in their $CD8^+$

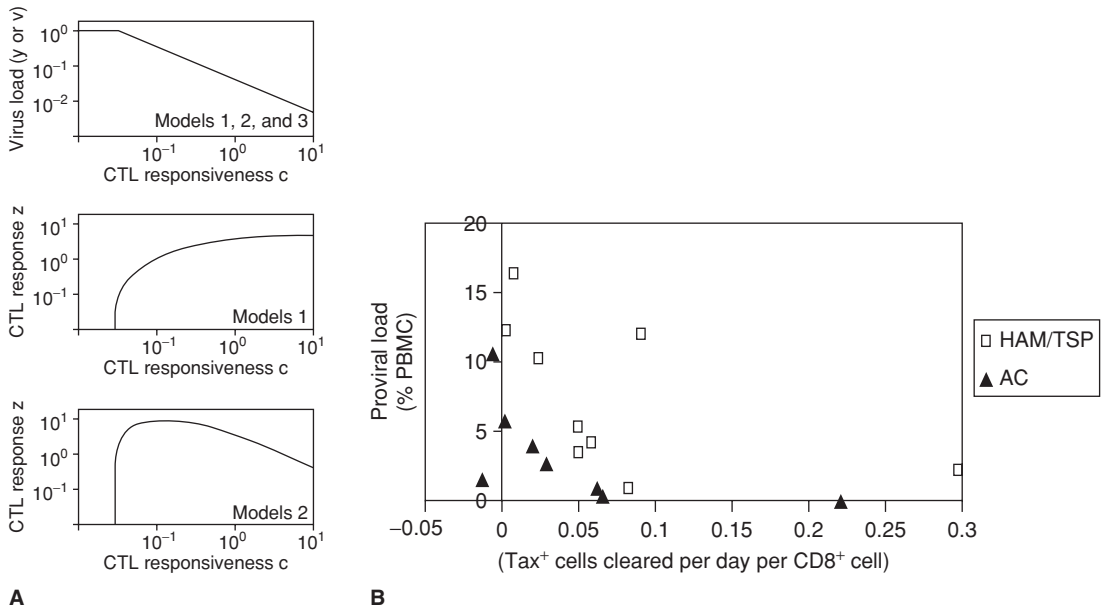

Figure 8.4. CD8$^+$ cell "responsiveness" determines proviral load. (A) A mathematical model predicted that CD8$^+$ cell (CTL) responsiveness (c) determines virus load (y or v), but responsiveness is not reflected in virus-specific CD8$^+$ cell frequency (z)[114]. (B) CD8$^+$ cell responsiveness, defined as the percentage of Tax+ CD4$^+$ cells killed per CD8$^+$ cell per day, was measured in a group of ACs and HAM/TSP patients using a flow cytometry-based functional assay and a novel mathematical model [96]. We found that CD8$^+$ cell rate of lysis was significantly negatively correlated with HTLV-1 proviral load in both clinical groups.

cell rate of lysis. Importantly, the CD8$^+$ cell rate of lysis correlated negatively with proviral load in *both* ACs and HAM/TSP patients (Figure 8.4B). This observation suggested that, regardless of disease status, a high rate of CD8$^+$ cell-mediated lysis decreases HTLV-1 proviral load whereas a low rate of CD8$^+$ cell-mediated lysis fails to control a high proviral load. CD8$^+$ cells from both ACs and HAM/TSP patients are, surprisingly, equally capable of killing infected CD4$^+$ cells. Further, we calculated that 40–50% of between-individual variation in HTLV-1 proviral load was determined by variation in CD8$^+$ cell rate of lysis [96].

From these genetic, molecular, theoretical, and functional studies we conclude that the HTLV-1-specific CD8$^+$ cell response is a major determinant of an individual's HTLV-1 proviral load and therefore protects against HAM/TSP by controlling virus replication in the host. However, what determines the remaining 50% of between-individual variation in proviral load remains to be elucidated.

CD8$^+$ T cells promote neuroinflammation

Although HTLV-1-specific CD8$^+$ cells are seen in PBMCs from both ACs and HAM/TSP patients their frequency tends to be positively correlated with HTLV-1 proviral load [108,109], suggesting that CD8$^+$ cells may play a detrimental role in HAM/TSP pathogenesis. This proposal is supported by the histopathological observation (discussed earlier) that CD8$^+$ T cells are present in the spinal cord lesions seen in HAM/TSP patients [56,63]. The function of these spinal CD8$^+$ cells remains unclear.

Similar to the presence of CD8$^+$ cells in HAM/TSP lesions, a high frequency of CD8$^+$ cells is also found in the cerebral spinal fluid (CSF) of HAM/TSP

patients [105,115,116]. Indeed, HAM/TSP is characterized by a pleiocytosis of the CSF (Table 8.1). The frequency of HTLV-1-specific $CD8^+$ cells has been found to be higher in the CSF than in the periphery and especially focused against epitopes in the immunodominant viral protein Tax [117,118]. The increased frequency of HTLV-1-specific $CD8^+$ cells in the CSF compared to the periphery has prompted the suggestion that there is specific expansion or recruitment of these cells in the CNS. The latter suggestion seems more likely at present, especially given the observations that $CD8^+$ cells in the CSF of HAM/TSP patients are predominantly $CD11a^+$, $CD45RO^+$, $CD28^-$ memory cells, and they express the migratory β2 integrin molecule CD18 more frequently in HAM/TSP than in other neurological diseases [116,119].

There is clearly an increase in the presence of $CD8^+$ cells in the CNS of HAM/TSP patients, but presence does not necessarily mean pathogenic participation. HTLV-1-specific $CD8^+$ cells from HAM/TSP patients have been shown to be more frequently pro-inflammatory than those from ACs. That is, the frequency of inflammatory (IFN-γ-producing) $CD8^+$ cells correlates positively with proviral load within HAM/TSP patients [108,120]. By antigenic stimulation and intracellular cytokine staining, the HTLV-1-specific $CD8^+$ cell production of IFN-γ, TNF-α, and IL-12 has been shown to be higher in HAM/TSP patients than in ACs [104]. Also, possession of the *TNFA -863A* allele in the promoter region of TNFα was found to be associated with an increased risk of HAM/TSP in the Japanese population [82].

$CD8^+$ cells therefore represent an important source of pro-inflammatory soluble mediators as well as provide cytolytic effector functions in clearing HTLV-1-infected $CD4^+$ cells. Indeed, CSF concentrations of IFN-γ are elevated in HAM/TSP patients [121], and the CSF levels of neopterin (an accepted molecular marker of inflammatory damage of the CNS) correlate with proviral load [122]. TNF-α and IFN-γ are generally considered neurotoxic [123,124], and thus it has been suggested that, in HAM/TSP, $CD8^+$ cells produce an excess of neurotoxic cytokines and cause the neuronal degeneration seen in HAM/TSP lesions. How do TNF-α and IFN-γ cause neurodegeneration?

TNF-α is a pleiotropic molecule with direct effects on cells that express either of its two receptors (TNFR1, TNFR2) including neurons, oligodendrocytes, astrocytes, and microglia. The action of TNF-α on the glia also has indirect effects on neurons: TNF-α-mediated toxicity toward oligodendrocytes results in damage to neuronal structure and the myelin sheath [125]. Interestingly, low concentrations of TNF-α have also been shown to promote neuronal cell survival and proliferation [126]. It is still controversial as to how and under what conditions TNF-α is neurotoxic rather than neuroprotective [126,127,128].

IFN-γ is a critical regulator of T cell-mediated inflammation in the periphery, but in the CNS it acts chiefly on microglia (reviewed in [125]). Upon activation by exposure to IFN-γ microglia become phagocytic, express antigen presentation molecules, and produce cytotoxic oxygen radicals. Interestingly, all four receptors for IL-2 – which is neurotoxic alone – and its related cytokine IL-15 are expressed on activated microglia such that, in combination with IFN-γ, IL-2 enhances microglial nitric oxide (NO) production, whereas IL-15 attenuates it [129,130]. Microglia-derived NO and reactive oxygen species (ROS) are likely to be directly toxic to both neuronal cells and other glia [131,132].

The combination of excess TNF-α and IFN-γ production by $CD8^+$ cells in HAM/TSP patients is likely to be particularly damaging to the CNS as IFN-γ and TNF-α activity upon and production by microglia can establish a self-perpetuating cycle of activation and neurotoxicity. IFN-γ induces microglial production of TNF-α, which can then interfere with the homeostatic function of astrocytes and microglia [125].

In summary, cytokines in the CNS are both produced and received by glial cells and are rarely directly neurotoxic. The establishment and perpetuation of neuroinflammation by cytokines in neurodegenerative disease is likely to center on the activity of the microglia and deserves further study, especially in the context of chronic stimulation where

it is unknown how long a microglial cell can tolerate stimulation without damage [125]. Until the normal homeostatic mechanisms by which glia control inflammation within the CNS are elucidated, it is difficult to predict the exact mechanism by which $CD8^+$ cells induce neuroinflammation in HAM/TSP patients.

$CD8^+$ T cells in balance

In order to reconcile the opposing views on the role of $CD8^+$ T cells in HAM/TSP pathogenesis, Asquith *et al.* developed a mathematical model describing the interaction between infected cells and virus-specific $CD8^+$ cells [94]. This model rests on the observation by Valitutti *et al.* that $CD8^+$ cells respond differently to different concentrations, of antigen [133]. At low concentrations, the $CD8^+$ cells are cytotoxic: as the antigen concentration rises, they successively acquire more functions, viz. cytokine secretion and proliferation. Using this model, a key prediction was made: differences in HTLV-1 antigenic loads could explain how two HTLV-1-infected individuals with a similar proviral load could have different clinical outcomes. That is, below a given HTLV-1 antigen load, $CD8^+$ cells are cytotoxic, but above that, threshold cytokine production is much increased, thus allowing $CD8^+$ cells both to lyse infected cells and to contribute to inflammation simultaneously. This has been found to be the case for influenza- and Murray Valley encephalitis-specific $CD8^+$ cells [133,134] and we have recently shown that the rate of Tax expression, at a given proviral load, is higher in HAM/TSP patients than in ACs [76].

Role of $CD4^+$ T cells in HAM/TSP pathogenesis

$CD4^+$ T helper (T_H) cells are of central importance in supporting both the cell-mediated and the humoral arms of the adaptive immune response by producing appropriate cytokines. The $CD4^+$ T cell response to HTLV-1 has been relatively little studied. Since HTLV-1 is primarily carried by activated $CD4^+$ T cells and the viral protein Tax is a powerful cell activator (including cytokine production and infected cell proliferation) it was difficult to take a traditional approach to studying the T_H response.

Very little is known about the humoral T_H2 and B cell responses to HTLV-1 infection. There is a chronic antibody production with high titers of HTLV-1-specific IgM, IgA, and IgG that positively correlate with proviral load [135,136]. This high titer might simply result from the strong antigenic drive given by a high proviral load. Given the absence of cell-free virus and the recent evidence that HTLV-1 spreads by cell-cell contact [32], the role of the antibody and T_H2 responses in controlling HTLV-1 infection are unknown and may be difficult to determine.

Here we will summarize the current evidence that HTLV-1-infected and HTLV-1-specific $CD4^+$ cells play a role in HAM/TSP pathogenesis.

The role of the $CD4^+$ T cell response in HAM/TSP

We have already discussed above that $CD4^+$ T cells are present in HAM/TSP lesions of the spinal cord at numbers as high as, or higher than, those of $CD8^+$ cells [56,63]. This is particularly the case in early, active lesions in HAM/TSP patients with short disease duration, suggesting that $CD4^+$ T cells play a role in the initiation of HAM/TSP disease.

Using a short-term (6 hour) enzyme-linked immunospot (ELISpot) assay, we recently showed that the frequency of HTLV-1-specific (IFN-γ-producing) $CD4^+$ cells in the peripheral blood was not correlated with proviral load, but was differentially associated with disease status; at a similar proviral load, HAM/TSP patients were found to have a 25-times higher frequency of HTLV-1-specific $CD4^+$ cells than ACs [137]. In addition, immunogenetics studies of HTLV-1-infected individuals have shown an association between possession of the MHC class II *HLA-DRB*0101* allele and an increased susceptibility to HAM/TSP [74,138,139], but only in the absence of the *HLA-A*02* allele. However, *HLA-DRB*0101*$^+$ HAM/TSP patients were also found to have a lower proviral load than *HLA-DRB*0101*- HAM/TSP patients [74]. This may be because $CD4^+$ cells can support the $CD8^+$ response, which may in

turn reduce HTLV-1 proviral load. Or alternatively, possession of *HLA-DRB*0101* may lower the threshold of development of HAM/TSP meaning that *DRB*0101*$^+$ HAM/TSP patients will, on average, have a lower proviral load than *DRB*0101*- HAM/TSP patients.

The mechanism of action of *HLA-DRB*0101* in HAM/TSP susceptibility is complex, but, together with histochemical and specific-cell frequency data, a significant role for the CD4$^+$ cell response in HAM/TSP is suggested. The question is then, how could CD4$^+$ cells cause neuroinflammation and CNS degeneration?

A plausible mode of CD8$^+$ cell-mediated neuroinflammation in HAM/TSP (see above) is through the elevated production of inflammatory cytokines, both in the peripheral blood, the CSF, and the local environment, and the same is also likely to be the case for CD4$^+$ cells. CD4$^+$ cells from HTLV-1-infected individuals preferentially exhibit a T_H1 (cell-mediated) cytokine profile, producing IFN-γ, TNF-α, and IL-2 [140,141], suggesting that CD4$^+$ cells are an additional source of proinflammatory cytokines. Furthermore, serum and CSF levels of both IFN-γ and IL-12 (a T_H1 cytokine that induces IFN-γ production by immune cells) have been found to be higher in HAM/TSP patients than in ACs [121].

In addition to IFN-γ, HTLV-1-infected cells produce IL-15 and IL-13 [142,143]. IL-15 promotes T cell proliferation and CD8$^+$ cell survival [144], but, as mentioned earlier, it also decreases NO production by IFN-γ-activated microglia [125]. Possession of the *IL-15 +191C* allele has been found associated with the odds of possessing HAM/TSP, but this effect appears to act through HTLV-1 proviral load [82]. IL-15 has been postulated to play a role in HAM/TSP pathogenesis [143,145,146]. IL-13, which is produced by Tax-expressing T cell lines and HTLV-1-infected PBMCs [142], has anti-inflammatory, immunosuppressive, and neuroprotective activities.

Moreover, single nucleotide polymorphisms in the anti-inflammatory cytokine IL-10 and the proinflammatory cytokines IL-1 and IL-6 have been associated with HAM/TSP [147,148,149]. However, in an independent study no significant difference was found in serum and CSF levels of IL-10 between HAM/TSP patients and controls [121].

Interestingly, CD4$^+$ cells found in the CNS are known to be neurotrophic (i.e., neuroprotective), such that they secrete nerve growth factors and other neuronal repair-supporting molecules [150,151,152,153]. It is possible that CD4$^+$ cells are recruited to early HAM/TSP lesions in order to promote the repair of the CNS, following damage by other infiltrating CD4$^+$ or CD8$^+$ cells. However, almost no work has been done investigating a possible neuroprotective role of CD4$^+$ cells in HAM/TSP. There is some data suggesting that there is no significant difference in the CSF levels of neurotrophic factors between HAM/TSP patients and controls, although no correction was made for duration of disease [154].

From the evidence discussed above, it is clear that it is somewhat difficult to predict what role CD4$^+$ cells play in HAM/TSP pathogenesis. There is likely to be a neuroinflammatory-neuroprotective dichotomy, as there is for the CD8$^+$ cells. The activity and function of T cells in the CNS of HAM/TSP patients is complicated by the fact that both CD4$^+$ and CD8$^+$ cells carry and respond to HTLV-1 infection.

HTLV-1 Tax expression in HAM/TSP

As described earlier, one of the well-established risk factors for HAM/TSP is a high proviral load (>1% PBMCs infected), but proviral load is a static measure of viral carriage within host cell DNA. It seems unlikely that the HTLV-1 provirus per se can cause a chronic inflammatory disease or indeed the widespread immune activation seen in HTLV-1-infected individuals. It is more likely that proviral load acts as a surrogate marker for viral expression. What role does expression of the provirus, in particular the Tax protein, play in HAM/TSP pathogenesis?

Tax expression at either the mRNA or protein level is rarely detected by conventional techniques directly *ex vivo*; short-term culture is required to allow HTLV-1-infected cells to express Tax, and subsequently all other viral proteins. Yamano *et al.* found

that the ratio of Tax mRNA to Tax DNA (indicating the relative level of gene expression per HTLV-1 provirus) was higher in PBMCs from HAM/TSP patients than ACs [155]. They also found that the Tax mRNA load correlated with HAM/TSP disease severity, suggesting that increased HTLV-1 expression in infected cells plays an important role in the pathogenesis of HAM/TSP. Consistent with these observations, Asquith *et al.* showed that the rate of Tax expression in *ex vivo* $CD4^+$ cells was higher in HAM/TSP patients than ACs, at the same proviral load, and that rate of Tax expression was a significant predictor of HAM/TSP status [76]. However, these data are derived from studies of *ex vivo* PBMCs: is the situation the same for HTLV-1-infected T cells in the CNS?

The frequency of provirus+ cells is typically higher in the CSF than in the PBMCs of the same patient, indicating preferential concentration or proliferation (or both) of infected cells in the CNS. In situ hybridization studies have shown that Tax mRNA is present in infiltrating $CD4^+$ T cells in active HAM/TSP lesions [156]. Using a sensitive method of immunohistochemistry with tyramide signal amplification, Moritoyo *et al.* showed that Tax expression is more frequent, yet still below 1%, in uncultured cells from the CSF than in PBMCs from all HTLV-1-infected individuals, but the absolute frequency of Tax+ CSF cells was higher in HAM/TSP patients than in ACs [45].

A proportion of HTLV-1-infected cells is clearly capable of expressing Tax in both the peripheral blood and the CNS, so it may be assumed that Tax-driven T cell activation and cytokine production occurs in both environments; in turn, the cytokines are likely to contribute significantly to the neuroinflammatory damage seen in HAM/TSP. As well as driving $CD4^+$ cell activation, Tax expression will also render a productively infected cell susceptible to recognition and lysis by HTLV-1-specific $CD8^+$ cells. The relative absence of $CD8^+$ cells in the perivascular mononuclear cell infiltrate in early active HAM/TSP lesions [62,63] may allow Tax expression to proceed unhindered, in turn allowing Tax-driven cytokine production to occur at heightened levels. The presence of HTLV-1 antigens in the CNS and local glial cell activation would presumably then recruit $CD8^+$ cells from the periphery to control HTLV-1 infection in the microenvironment. In this scenario Tax expression within the CNS is a key determinant of HAM/TSP pathogenesis.

Is HAM/TSP an autoimmune disease?

HAM/TSP is an immune-mediated neuroinflammatory disease associated with the presence of $CD4^+$ and $CD8^+$ T cells, along with a degree of Tax expression and cytokine production, in the CNS. But are these factors sufficient to cause HAM/TSP? Multiple sclerosis (MS) shares similar symptomatic, histopathological, and MRI characteristics with HAM/TSP. The main pathomechanism in MS is considered autoimmunity, whereby, the adaptive immune response to exogenous antigens also recognizes CNS antigens, especially constituents of the axonal myelin sheath. In this way, MS is a disease primarily associated with immune-mediated demyelination and neuronal degeneration. In HAM/TSP however demyelination is present, but is not the chief pathology. Could HAM/TSP be an autoimmune disease?

There are three main ways by which an adaptive immune response can be autoimmune: epitope spreading, molecular mimicry, and breakdown of self-antigen tolerance. The evidence for an autoimmune component to HAM/TSP comes from the ability of certain HTLV-1-specific antibodies to cross-react with CNS-derived antigens (molecular mimicry).

Levin *et al.* have shown that IgG isolated from the sera of HAM/TSP patients, but importantly not ACs or uninfected individuals, is immunoreactive with uninfected neurons [157]. They further showed that this IgG recognized both the HTLV-1 protein Tax and the cellular heterogeneous nuclear ribonucleoprotein-A1 (hnRNP-A1) [158]. The hnRNP-A1 transports mRNA from the nucleus to the cytoplasm, and in the CNS it is expressed at higher levels in neurons than in glia. When hnRNP-A1-specific IgG was injected into rat brain sections,

neuronal firing was inhibited. In 2003, the same group then showed that naturally deposited IgG in autopsy tissue from HAM/TSP patients, but not controls, was found throughout the CNS correlating with areas of clinicopathological damage [159]. From this evidence, Levin *et al.* suggested that HAM/TSP patients develop autoimmune antibody responses [160]. A similar study has since been done in Colombia where antibodies were detected in the serum of HTLV-1-infected individuals that recognized proteins from normal human brain and cultured astrocytes [161]. The identity of these proteins has yet to be elucidated.

While the evidence for anti-hnRNP antibodies in HAM/TSP is strong, their role in pathogenesis remains unclear. HTLV-1-CNS antigen cross-reactive antibodies do not explain the initiation, the focal nature, or the distribution of HAM/TSP lesions in the CNS. It remains possible that exposure of CNS antigens and auto-antibody-mediated interference with neuronal function and integrity contribute to HAM/TSP progression. HAM/TSP might thus be more accurately referred to as a complex autoinflammatory, rather than an autoimmune disease [162].

HTLV-I and the central nervous system

HTLV-1 is not a neurotropic virus in that neural cells are not the natural host cells for HTLV-1. HTLV-1 is primarily carried by CD4 T cells *in vivo*, but *in vitro* HTLV-1 cell tropism is much broader. The reason for the strict T-lymphotropism of HTLV-1 *in vivo* is unknown, but is most likely to be due to the cell-cell mode of transmission, which appears to favor transmission to LFA-1^+ cells (i.e., predominantly T cells [163]). No neuropathic strain of HTLV-1 has been found, but, given the magnitude of T cell infiltration seen in histopathological studies of CNS lesions from HAM/TSP patients, it is possible that some cells of the CNS are infected by HTLV-1. Indeed, *in vitro* studies have shown that a human medulloblastoma cell line can be productively infected by HTLV-1, albeit transiently (<15 days) [164]. However, most studies have focused on HTLV-1 infection and the infectibility of the glia.

HTLV-I and glia

HTLV-1 is generally held to be present in the CNS only within the infiltrating lymphocytes. However, by in situ hybridization the presence of Tax mRNA deep within HAM/TSP neural tissue, not associated with the perivascular infiltrate, has been shown [165]: these infected neural cells were then found to be astrocytes. It is known that co-culture of HTLV-1-infected T cells and astrocytes alters the function of the astrocytes, inducing gliosis, impaired glutamate uptake, and increased pro-inflammatory cytokine production [166,167,168,169], but these effects are not necessarily caused by infection. Similarly, *in vitro* HTLV-1 infection and co-culture-mediated alterations in microglia have also been shown [29]. Oligodendrocytes, however, have thus far not been shown to support infection by HTLV-1. Also, HTLV-1 is able to productively infect primary cultures of sheep choroid plexus [27], further supporting the ability of HTLV-1 to directly interact with neural and glial cells.

The physiological and clinical importance of these findings, however, is limited without further *in vivo* studies of naturally HTLV-1-infected individuals. Also, given the rarity of direct infection of astrocytes in HAM/TSP, direct infection of neural tissue is unlikely to be a major pathomechanism of CNS degeneration.

HTLV-I entry into the CNS

The CNS is a specialized immune environment. That is, the CNS is secluded behind complex blood-CNS barriers, minimizing immune surveillance of the CNS to that required for normal homeostatic processes: few leukocytes are present at any one instant in the healthy CNS. In the event of an antigen-specific challenge, immune surveillance of the CNS is increased [170,171].

The blood-brain barrier (BBB) is a highly specialized barrier consisting of endothelial cells joined by an elaborate network of interendothelial cell tight junctions [172], which are in turn maintained and sealed by astrocytes [173]. In health the BBB is almost

impermeable to cells and molecules found in the serum. Because of the impermeability of the BBB, fewer T cells enter the CNS than any other organ. In addition, the degree and molecular requirements for T cell entry are different at different levels of the neuroaxis; leukocyte migration into the spinal cord is greater than entry into the cerebrum [174,175] and differs mechanistically in that it does not involve leukocyte rolling [176,177].

Leukocyte entry into the CNS in HTLV-1

Given the highly selective and tight control that exists over leukocyte entry into the CNS, how do HTLV-1-infected T cells enter? The lymphocyte infiltration seen in CNS lesions of HAM/TSP is often referred to as "aberrant" or "inappropriate," but perhaps HTLV-1-infected and HTLV-1-specific T cells are simply following a normal physiological process, just to an elevated degree.

T cells that are permitted to enter the CNS are usually $CD4^+$ $CD45RO^+$ memory cells [178], and the CD4:CD8 T cell ratio is also increased compared to PBMCs [179]. In an inflamed environment, either systemic or local to the CNS, activated T cells enter at random and only remain if they encounter their cognate antigen (reviewed in [170]). It appears that the cell's activation state (associated with the up-regulation of surface adhesion molecules) rather than its antigen specificity is the key determinant of passage of T cells through the BBB. Most work has been done on the migration and entry of $CD4^+$ T cells; very little is known about the entry of $CD8^+$ T cells specifically into the CNS, but it is likely to be distinct from, yet share much with, $CD4^+$ T cells.

It is known that the frequency of HTLV-1-infected cells is greater among cells recovered from the CSF than in PBMCs, suggesting preferential localization of HTLV-1-infected cells inside the CNS [55,180]. HTLV-1-infected cells share common provirus integration sites between the CSF and peripheral blood, suggesting that the HTLV-1-infected cells enter the CNS by migration rather than becoming infected after entering the CNS [181].

The ability of $CD4^+$ and $CD8^+$ T cells from HTLV-1-infected individuals to adhere to and migrate through an endothelial layer has been the subject of several studies *in vitro*. Using endothelial cells from different mammalian species and different anatomical sites, PBMCs from HAM/TSP patients have been shown to adhere to an endothelial monolayer to a greater extent than PBMCs from ACs or uninfected controls [182,183,184]. Increased transmigration across an endothelial monolayer [185] or a reconstituted basement membrane [186] has also been shown for PBMCs from HAM/TSP patients compared to controls, especially for $CD4^+$ T cells compared to $CD8^+$ T cells. Furthermore, the proviral load of transmigrating HAM/TSP PBMCs was found to be higher than the proviral load of non-transmigrating PBMCs [186]. This was not found to be the case for transmigrating and non-transmigrating PBMCs from ACs, but their proviral loads were low.

There is disagreement in the literature over the molecules that are required for PBMC or T cell adherence and/or transmigration in HTLV-1 infection. One study showed that HTLV-1-infected T cell lines up-regulate the expression of LFA-1 (the ligand for endothelial ICAM-1) but not VLA-4 (the ligand for endothelial VCAM-1) compared to uninfected T cell lines, suggesting that their adherence to rat brain endothelial cells might involve the LFA-1/ICAM-1 interaction [183]. Another study showed that the proportion of $CD4^+$ and $CD8^+$ T cells that expressed VLA-4 was increased in cells from HTLV-1-infected individuals [184]. Similarly, the proportion that expressed ICAM-1, which can interact homotypically as well as bind LFA-1, was also increased. Thus, it was suggested that VLA-4 and ICAM-1 were required for adhesion to human umbilical vein endothelial cells. Further, immunohistochemistry of HAM/TSP spinal cord samples suggests that endothelial cell VCAM-1 and infiltrating mononuclear cell VLA-4, LFA-1, and MAC-1 are all involved in migration into the diseased CNS [187].

Given the technical differences of approach and the anatomical distinction between different blood-CNS barriers, this confusion is not surprising.

In addition, it is not clear whether PBMCs from HAM/TSP patients are more migratory and adherent because the frequency of infected and/or activated cells is higher [182], or because HTLV-1 alters the migratory activity of uninfected ("bystander") T cells as well. Tax expression is known to up-regulate ICAM-1 [188,189] and VCAM-1 [190], and it has been shown that HTLV-1 can subvert T cell cytoskeletal polarization in response to ICAM signaling to aid cell–cell transmission [32,163,191], but it is not known if this interference also plays a role in increasing HTLV-1-infected T cell transmigration.

Chemokine recruitment of leukocytes into the CNS

A leukocyte may transmigrate across an endothelial cell layer after chance interaction with adhesion molecules, but it may also be actively recruited by chemokines. In the CNS, chemokines are produced by meningeal and perivascular macrophages, ependymal cells, activated astrocytes, and activated microglia. Exposure to IFN-γ, TNF-α, and IL-1 alters glial cell production of both chemokines and chemokine receptors [125]. In neuroinflammatory disease, IFN-γ, primarily released by invading T cells, could influence glial cell-mediated recruitment of additional leukocytes. This may represent an important mechanism by which an early focus of inflammation in HAM/TSP recruits additional $CD4^+$ and $CD8^+$ T cells to perpetuate disease.

Little is known specifically about chemokines and chemokine receptor expression in HTLV-1 infection. Cell lines derived from peripheral blood $CD8^+$ T cells from HAM/TSP patients have been found to secrete bioactive levels of the chemotactic MIP-1α, MIP-1β, and IL-16 [92]. Serum levels of the chemokines CXCL9 and CXCL10 were also found to be elevated in HAM/TSP patients compared to ACs and were positively correlated with PBMC IFN-γ production *ex vivo* [192]. And finally, a single nucleotide polymorphism in SDF-1β (*SDF-1β +801 A)* was found to be associated with a higher risk of HAM/TSP in southern Japan [82]. This polymorphism was not found to have an effect on HTLV-1 proviral load and is therefore likely to exert its effect through altered T lymphocyte recruitment into the CNS.

BBB restructuring

Systemic and local increases in IFN-γ and TNF-α during inflammatory responses affect the permeability of the BBB and prime the CNS for inflammation [193,194]. Cytokine-induced up-regulation of adhesion molecules on the endothelial cells increases the permeability of the BBB to activated T cells, and the BBB can also be breached, particularly by the actions of high concentrations of TNF-α. In HAM/TSP, the BBB is often referred to as damaged or "restructured" for three main reasons. First, the presence of a large number of infected T cells in the perivascular space of the CNS is assumed to indicate a BBB with reduced integrity. Second, HTLV-1-infected T cells co-cultured with endothelial cell monolayers have been found to result in an increased paracellular permeability [183]. This is thought to be caused by dissolution of interendothelial cell tight junctions and secretion of toxic cytokines (e.g., TNF-α). Third, the production of matrix metalloproteinases (MMPs) and their regulator proteins known as tissue inhibitors of MMP (TIMPs) has been shown to be elevated or altered in HTLV-1-infected cells and individuals [122,164,195,196].

MMP enzymes play a key role in the degradation of the extracellular matrix in cellular migration and tissue remodeling [197]. MMPs can also degrade myelin and myelin-associated proteins and may modulate TNF-α activity by converting an inactive TNF precursor to its active form [164]. T_H1 cells have been found to be more migratory, owing to preferential expression of MMPs, compared to T_H2 cells [198]. MMPs are thus directly implicated in the increased permeability of the BBB often seen in neurological diseases.

$CD8^+$ cells from HAM/TSP patients are known to secrete MMP-2 and MMP-9 [92], and HTLV-1-infected astrocytes or astrocytes transiently co-cultured with HTLV-1-infected T cells to increase production of MMP-2, MMP-3, MMP-9, TIMP-1, and TIMP-2 [199]. Further, MMP-9 and TIMP-3 concentration in the CSF of HAM/TSP patients is higher

than that of ACs and is associated with higher levels of the CNS inflammatory marker neopterin [122]. MMP-2 is found in the CSF of both HAM/TSP patients and ACs, but only MMP-9 is associated with HAM/TSP [122]. This is consistent with a recent study by Kodama *et al.* that showed an association between HAM/TSP and an MMP-9 gene promoter dinucleotide repeat length polymorphism; a larger dinucleotide repeat was found in HAM/TSP patients than ACs and was associated with an increase in Tax-driven transcription of the MMP-9 gene [200].

In the CNS tissue of HAM/TSP patients, MMP-9 has been detected in neural and mononuclear cells in the perivascular space [199]. Histochemical staining for collagen IV and decorin (basement membrane proteins) was found to be disrupted in the basement membrane of CNS parenchymal vessels at the site of mononuclear cell infiltrates in early active lesions [196], suggesting degradation and tissue restructuring. Indeed, MMP-9- and MMP-2-positive cells are more frequent in active than in inactive HAM/TSP lesions [196].

The evidence reviewed above suggests that increased MMP production – especially MMP-9 – by T cells or astrocytes contributes to destruction of the vascular endothelium and migration of T cells seen in the CNS of HAM/TSP patients.

In conclusion, HTLV-1-infected or HTLV-1-specific T cells gain access to the CNS by normal physiological routes; but as immune activation, inflammation, and tissue damage increase, access is also increased by active recruitment and disruption of both the BBB and presumably the blood-spinal cord barrier. Elevated levels of systemic and local pro-inflammatory cytokines are also likely to influence the progressive loss of blood-CNS barrier integrity by overcoming homeostatic control mechanisms and activating T cells and glia alike.

Do we need a new hypothesis for HAM/TSP pathogenesis?

The precise mechanisms underlying the neurological damage seen in HAM/TSP, especially demyelination and axonal loss, can only be inferred from limited immunohistochemistry, *ex vivo*, *in vitro*, immunogenetic, and clinical studies. Very few unequivocal risk factors for the development of HAM/TSP are known; a high proviral load, which is at least an order of magnitude higher in HAM/TSP patients than ACs, is the strongest correlate. In this section, we will highlight crucial factors associated with HAM/TSP and discuss the current hypothesis of HAM/TSP pathogenesis. In addition, we will ask "why do only a minority of HTLV-1-infected individuals develop HAM/TSP?" and explore a new hypothesis that may help us answer this central question.

A current hypothesis of HAM/TSP pathogenesis

From the evidence described and discussed in this chapter, there are five crucial factors associated with HAM/TSP. First, a high HTLV-1 proviral load: the odds of possessing HAM/TSP increase exponentially once proviral load is above 1% of PBMCs infected [41,72]. Second, a chronic and high level of immune activation, including both the humoral and cell-mediated adaptive immune responses. Third, the infiltration of a large number of $CD4^+$ and $CD8^+$ T cells into the CNS, especially the spinal cord. Fourth, the corollary of this infiltration is held to be inflammatory neurotoxicity. Finally, HAM/TSP develops after a long clinically asymptomatic phase, in the region of months to years after infection with HTLV-1.

The positive correlation seen between HTLV-1 proviral load and the level of the CNS-inflammatory marker neopterin in CSF suggests that HAM/TSP is a disease borne of two dynamic battles between the virus and the host immune response: one battle occurring in the circulatory and lymphatic systems (determining proviral load) and one in the CNS (causing neuronal degeneration). Thus, a plausible hypothesis of HAM/TSP pathogenesis is as follows [59,201,202,203,204].

If the peripheral immune response to HTLV-1 infection is poor or ineffective, viral expansion will occur relatively unabated; proviral and antigenic

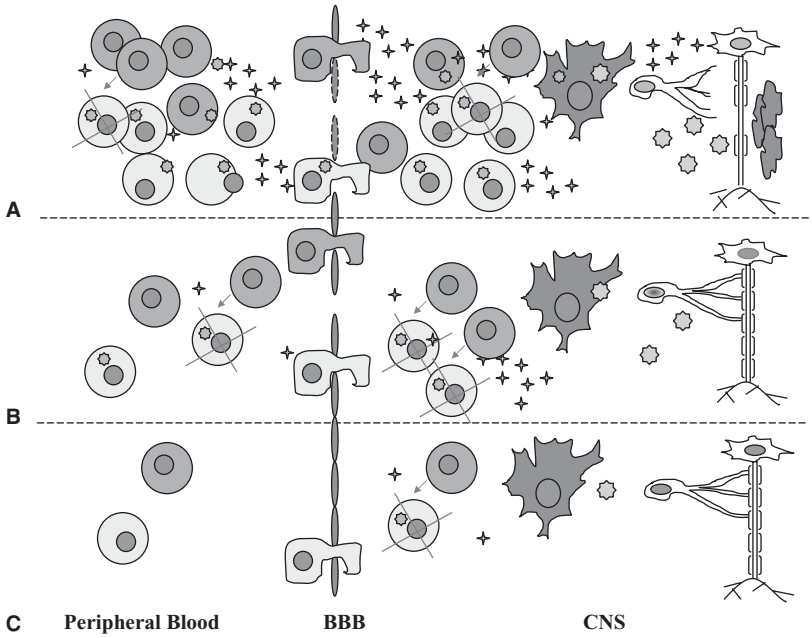

Figure 8.5. Hypothesis of CNS damage in HTLV-1 infection. (A) The pathogenic mechanism of HAM/TSP is unknown, but the current hypothesis suggests that inappropriate infiltration of T cells ($CD8^+$: cells, $CD4^+$: cells, Tax protein: star) into the CNS, across a damaged blood-brain barrier (BBB), produces an excess of proinflammatory cytokines (stars) that result in glial activation (cells), production of reactive oxygen species (stars), and neuronal damage (white cells). (B) At an early stage of HAM/TSP development, or in an AC with a high proviral load, we propose that T cell infiltration and neuronal damage occur, but at a lower level such that single neurological symptoms and clinical signs may be apparent. **C.** In ACs with a low HTLV-1 proviral load, T cell infiltration into the CNS occurs at a low level where HTLV-1 activity and inflammation are controlled efficiently and no neuronal damage results. (For figure in color, please see color plate section.)

loads will become high, chronically maintaining a large and active immune response. High frequencies of activated $CD4^+$ and $CD8^+$ T cells (infected and HTLV-1-specific) gain access to the CNS inappropriately. Within the CNS these activated T cells produce cytokines, either Tax-driven or in response to antigen recognition, that cause "bystander damage" to the CNS, by secreting high local concentrations of cytokines and thus precipitating the neurodegeneration and clinical symptoms observed. The disease progresses as the presence of HTLV-1 antigen in the CNS recruits more activated T cells, particularly HTLV-1-specific cytotoxic $CD8^+$ T cells, a large proportion of which may be infected at higher proviral loads, causing further damage to the CNS and the BBB, eventually leading to irreversible neurodegeneration, loss of BBB integrity, and a plateau in clinical symptoms (summarized in Figure 8.5A).

A new hypothesis of HAM/TSP pathogenesis

At first glance the hypothesis of HAM/TSP pathogenesis outlined above appears reasonable. However, many aspects remain unproved or unresolved. Most of what we know about the purportedly pathogenic T cell infiltration of the CNS in HTLV-1 infection is based solely on small-scale immunohistochemical studies of HAM/TSP lesions. Similarly, it is almost impossible to distinguish between the likely mechanisms of establishment of inflammatory

lesions and the mechanisms by which they are perpetuated.

From MRI studies, we know that HAM/TSP can be associated with multifocal lesions, some of which can resolve: Is the above hypothesis sufficient to explain these observations? Is each separate lesion the result of independent infiltration and inflammatory events? This hypothesis also requires an initiation event that occurs exclusively in HAM/TSP patients, but such an event has not been identified. Some groups state that the increased T cell infiltration into the CNS is sufficient to initiate HAM/TSP. However, here, we have established that entry of an increased number of activated T cells into the CNS is a normal physiological response to systemic infection – such as persistent HTLV-1 infection – and viral expression is likely to increase the level of CNS immune surveillance.

A new hypothesis of HAM/TSP pathogenesis is needed. Changing the question to "Why do the majority of HTLV-1-infected individuals *not* develop HAM/TSP?" offers a new approach to HAM/TSP pathology. This alternative perspective removes the need for a HAM/TSP-specific initiation event and suggests that there is a continuum of neuroinflammation across HTLV-1-infected individuals.

In all HTLV-1-infected individuals, activated T cells will enter the CNS and increase immune surveillance. In individuals with "good" immune-mediated control of viral replication, as indicated by a reduced proviral load, the absolute frequency of activated, and therefore infiltrating, T cells will be lower than in an individual with a less efficient immune response. A more efficient response to HTLV-1 replication in the periphery is likely to be associated with more effective control of HTLV-1-infected T cells in the CNS, limiting their opportunity to produce cytokines and precipitate neuroinflammation and damage. That is, infiltration of efficient $CD8^+$ T cells into the CNS may be beneficial at a low frequency. The number of cells in the CSF of HAM/TSP patients is higher than that in the CSF of ACs, but this parameter has yet to be corrected for – or correlated with – proviral load. In this way, the need for an initiation event is removed and the key determinant becomes the level of infiltration required to establish a self-perpetuating inflammatory lesion in the CNS. This model (summarized in Figure 8.5) also allows for multiple independent foci of inflammation to be established as a matter of course if infiltration is at a high frequency throughout the CNS, as would be the case in an HTLV-1-infected individual with a high proviral load. The threshold of T cell infiltration and inflammation required to establish a lesion in the CNS is likely to be high, in particular to overcome homeostatic glial responses to control CNS infection and maintain neuronal integrity simultaneously.

Under this new hypothesis, one would also predict that the key determinant of HAM/TSP is the rate of accumulation of damage in the CNS, reflecting alterations in the damage:healing ratio under inflammatory circumstances (Figure 8.6A). HAM/TSP only develops after a long clinically asymptomatic period; perhaps low-grade, subclinical inflammatory lesions arise continuously throughout this time period, especially in individuals with a high proviral load whose activated T cells are likely to enter the CNS more frequently than those of a low proviral load individual. The rate of accumulation of irreversible CNS damage is likely to correlate with HTLV-1 proviral load. The slow accumulation of damage with repeated minor insults (Figure 8.6B) is similar to the picture postulated in MS, where repeated systemic infections are associated with transient inflammatory and symptomatic relapses that eventually progress [205,206].

If accumulation of CNS damage occurs as a function of proviral load over time, then ACs, particularly those with a higher proviral load, would be expected to present with subclinical or other non-HAM/TSP neurological disorders. Although no systematic, large-scale study has been performed, neuorological signs have indeed been found in ACs [70,122,207,208,209,210,211,212,213,214]. Not all ACs with detectable neurological signs are likely to be pre-HAM/TSP patients, because the lifetime risk of HAM/TSP is low. Thus, the published cases referred to above are likely to underestimate the frequency of ACs with neurological signs, providing support for our new hypothesis of HAM/TSP.

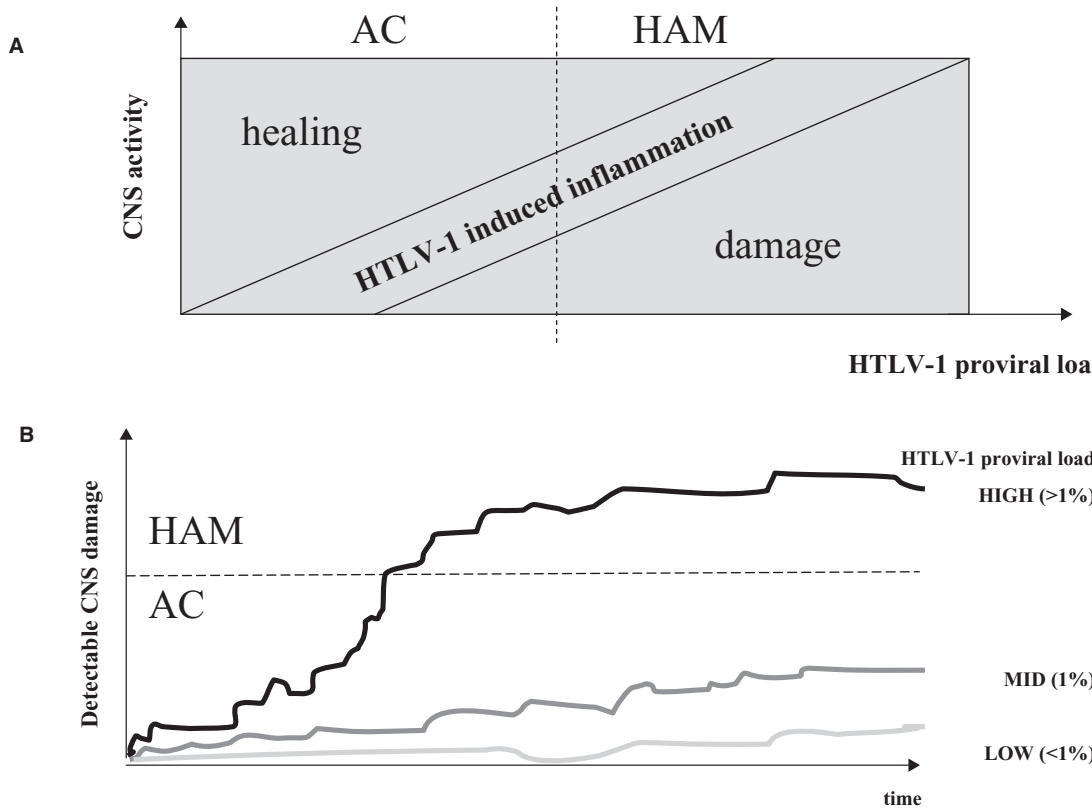

Figure 8.6. A new hypothesis of HAM/TSP pathogenesis. By asking the question "Why do the majority of HTLV-1 infected individuals not develop neuroinflammation?" we formulated a new hypothesis of HAM/TSP pathogenesis that centers on the homeostatic control of inflammation within the CNS. (A) We propose that the damage:healing ratio of inflammation driven by the presence of HTLV-1-infected or -specific T cells in the CNS is proportional to HTLV-1 proviral load and thus distinguishes between ACs and HAM/TSP patients. (B) Our new hypothesis of HAM/TSP pathogenesis also predicts that the rate of accumulation of CNS damage is proportional to proviral load and that HAM/TSP patients have a rate of accumulation of damage higher than that of ACs.

Conclusion

Here, we have described a hypothesis of the pathogenesis HAM/TSP. This hypothesis involves a large-scale infiltration of T cells into the CNS (induced by high proviral load and high immune response) that initiates and perpetuates an inflammatory cascade resulting in neurodegeneration and symptoms. We have expanded and refined this hypothesis to include all HTLV-1-infected individuals (ACs and HAM/TSP) and suggested that no specific initiation event is required for HAM/TSP. We also suggest that CNS infiltration by T cells is expected to be elevated in proportion to proviral load and immune efficiency and that the key determinant of HAM/TSP is the rate of accumulation of CNS damage. This hypothesis allows for the long clinically asymptomatic phase between infection and disease and a continuum of damage from ACs to HAM that correlates with proviral load. The exact mechanism by which the presence of infiltrating inflammatory T cells in the CNS results in neurodegeneration remains unclear,

but it appears extremely likely to be dependent on cytokines, both the concentration and the balance of their pro-inflammatory versus anti-inflammatory properties. In all cases the immune-mediated control of HTLV-1 expression and proviral load in the peripheral blood remains of prime importance.

While it is of great interest to elucidate fully the pathogenesis of HAM/TSP in order to improve clinical management and to identify ACs at high risk of developing disease, the most important factor we do not yet fully understand is the control of Tax expression. Tax expression maintains $CD4^+$ cell proliferation (and hence proviral load), stimulates the immune response (particularly Tax-specific $CD8^+$ T cells), drives the production of high levels of pro-inflammatory cytokines and is a strong independent indicator of HAM/TSP. Ultimately therefore, the best target for reducing the risk and/or progression of HAM/TSP, irrespective of the mechanism of pathogenesis, may not be HTLV-1 proviral load. Instead, the best approach may be to target the central regulator of HTLV-1 activity itself, the Tax protein.

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9

HIV infection of the central nervous system

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History, discovery, and epidemiology

On June 4, 1981, *The Morbidity and Mortality Weekly Report* from the Centers for Disease Control (CDC) in the United States published a report of five previously healthy young men with biopsy-confirmed pneumocystis carinii pneumonia (PCP) at three different hospitals in Los Angeles [1]. It is extremely rare for healthy young individuals to develop PCP without an underlying immunodeficiency. The single factor linking these five individuals was that they were all active homosexuals. One month later, a further report documented 26 cases of Kaposi's sarcoma, which until then had been an extremely rare tumor in the United States [2]. Again, all of the patients were young, previously healthy homosexual men. These were the first recorded reports of Acquired Immune Deficiency Syndrome (AIDS) and were quickly followed by reports of cases from other countries around the world. By September 1982, CDC had 593 reports of AIDS cases, 41% of whom were already dead. Seventy-five percent were known to be homosexual or bisexual males, and over half had PCP [3]. In 1983, workers at the Pasteur Institute identified a virus from the lymph node of an asymptomatic individual who presented with lymphadenopathy [4]. The virus replicated in culture releasing high titers of virions that contained magnesium-dependent reverse transcriptase activity and exhibited features of retroviruses on electron microscopy.

By 1984, the aetiological agent responsible for AIDS was identified as a novel RNA retrovirus previously designated lymphadenopathy-associated virus (LAV) or human T cell lymphotrophic virus type 3 (HTLV-III), both of which were superceded in 1986 by the common term human immunodeficiency virus (HIV) [5]. In 1986, a second related, but less pathogenic, HIV virus was discovered and designated HIV-2.

Discovery of the aetiological agent of AIDS enabled the mode of transmission to be established: sexual contact (homosexual and heterosexual), needle sharing (intravenous drug abusers) or needle stick injuries (medical workers), iatrogenic (blood transfusion with contaminated blood products), and mother to child. The virus is blood borne, primarily infecting CD4 T lymphocytes but also infecting cells of the macrophage/monocyte lineage, some dendritic cells, and certain classes of CD8 T lymphocytes. By directly targeting and killing cells that are critical for adaptive immune responses, the virus induces severe immune suppression which leaves the infected individual susceptible to otherwise innocuous pathogens and rare tumor formations, resulting in the clinical syndrome of AIDS.

Attachment of the virus to susceptible cells is normally via binding to the CD4 receptor on the cell surface, following which entry to the cell is mediated through binding of coreceptors, usually the chemokines receptors CXCR4 or CCR5.

Spread of the virus between individuals is normally through mucosal surfaces during sexual contact, with the exception of infections acquired through needle stick injuries, blood or blood product transfusion of unscreened blood, and intravenous

drug abuse, where the virus is introduced directly into the bloodstream.

Since the initial reports of AIDS cases, both the virus (HIV-1) and the disease have been found throughout the world with the disease quickly reaching pandemic status. While HIV-1 has spread from its original source in Africa to all regions of the world, HIV-2 remains predominantly in Western Africa, with more restricted global spread and far fewer global infections. Current estimates (December 2006) suggest that worldwide, 39.5 million people are living with HIV [6]. In 2006 alone, 2.9 million people are estimated to have died from AIDS and 4.3 million people acquired new infections [6]. The worst affected region of the world is sub-Saharan Africa where over half of the world's infected individuals live and over 70% of deaths occur (see Table 9.1) [6]. While the initial epidemic was identified in a cohort of homosexual men in the United States, AIDS is no longer a disease just affecting the homosexual community. In many countries, including those of sub-Saharan Africa, HIV is predominantly a heterosexual infection. It is estimated that worldwide 80% of transmissions are heterosexual, with transmission from homosexual men and injecting drug users (IDUs) accounting for between 5 and 10%, respectively, although this varies greatly in different regions and different countries of the world. In sub-Saharan Africa, nearly 100% of cases are heterosexual transmissions, while in Eastern Europe, particularly in countries of the former Soviet Union, drug abuse is the main recorded mode of transmission of the virus. In 2001, 54% of the 100 815 individuals in Eastern Europe who were newly diagnosed with HIV acquired their infection through injecting drug abuse. In some countries such as Estonia and Tajikistan, over 85% of new transmissions are through intravenous drug use. Reports from China suggest that almost half of the country's estimated 650 000 HIV infections were acquired through sharing of intravenous needles [7].

Although AIDS first came to prominence in the early 1980s, there were undoubtedly many cases that occurred before this time, but the lack of sophisticated disease recording, particularly in Africa, meant that AIDS cases in the 1960s and 1970s and probably earlier went undocumented.

Table 9.1. Number of individuals living with HIV infection by region of the world

Region	Current estimate of individuals living with HIV infection
Sub-Saharan Africa	24.7 million
South and Southeast Asia	7.9 million
Latin America	1.7 million
Eastern Europe and Central Asia	1.7 million
North America	1.4 million
East Asia	750 000
Western Europe	740 000
North Africa and the Middle East	460 000
Caribbean	250 000
Oceania	80 000
World Total	**36.9 million**

Data source: UNAIDS/WHO 2006.

Disease manifestation

In almost all untreated cases, infection with HIV leads eventually to AIDS. HIV infection has three phases: acute, asymptomatic, and symptomatic (or AIDS). Acute infection occurs in the first days after infection when virus is produced in large quantities by activated infected lymphocytes in lymph nodes leading to lymphadenopathy and, in some cases, a mononucleosis-like syndrome of fever, malaise, pharyngitis, and headache. Antibodies to HIV are detected between 2 weeks and 4 months after infection and are diagnostic for the infection. The initial acute viremia is normally controlled within a few weeks with the appearance of cytotoxic CD8 T lymphocytes and neutralizing antibodies to the virus. Productive infection in CD4 T lymphocytes results in death of infected cells and a transient decline in blood CD4 T lymphocyte counts (Figure 9.1).

In the asymptomatic phase of infection, the viremia resolves. However, the number of CD4

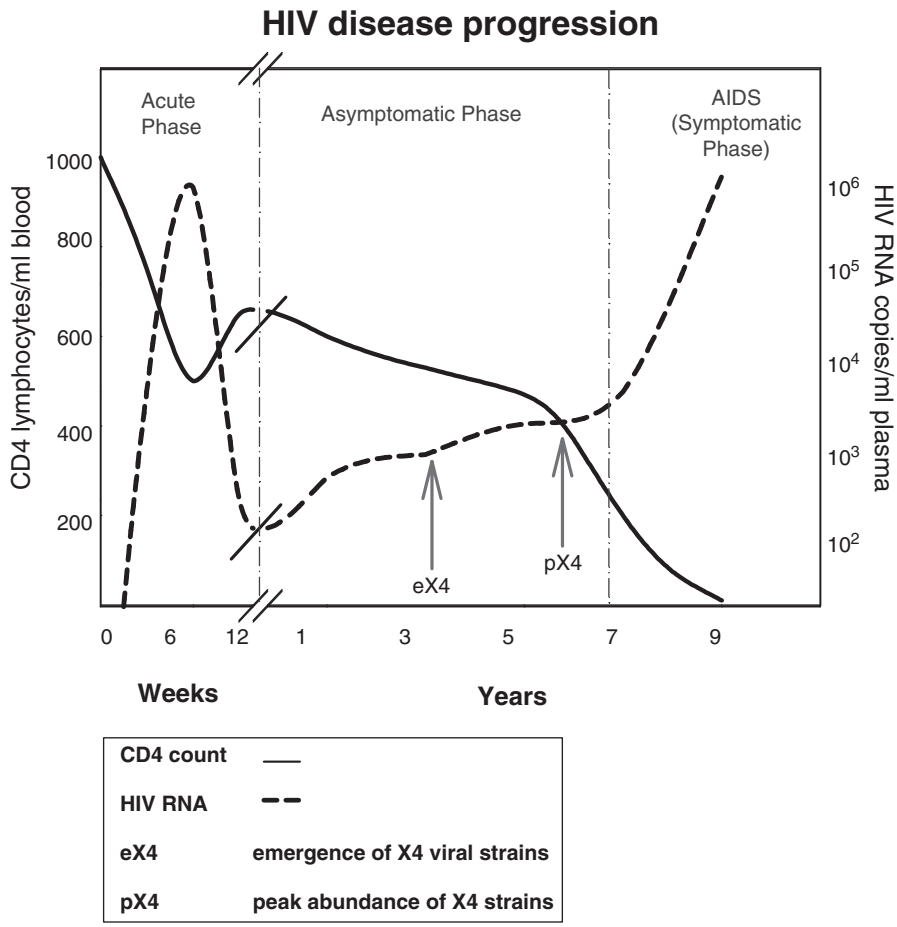


Figure 9.1. Diagram of disease phases: acute, asymptomatic, and symptomatic (AIDS).

T lymphocytes begins to decline over a variable number of years (3–10). During this phase, viral replication continues at a low level but kept in check by the elevated presence of CD8 cytotoxic T lymphocytes.

As CD4 T lymphocyte counts decline so does immune function, allowing both increased HIV viral replication and also increasing the susceptibility of individuals to opportunistic infections. The clinical transition from the presymptomatic HIV-infected stage to AIDS is based on guidelines issued by the CDC in 1993. These guidelines take account of the presence of opportunistic conditions and the lowest recorded CD4 T lymphocyte count for a patient. Table 9.2 gives the CDC classification system for HIV-infected adults while Table 9.3 lists common conditions encountered during HIV infection. These CDC guides have been used extensively in the past to classify HIV-infected subjects.

Approximately one-third of AIDS patients are diagnosed with neurological disorders at some time during the course of their disease. In the symptomatic phase of the disease, the central nervous system (CNS) can be affected by a number of opportunistic infections which usually lead to CNS dysfunction and ultimately death. HIV is capable of infecting some resident brain cells, including

Table 9.2. CDC classification system for HIV-infected adults

		Clinical categories		
		A	B	C
CD4 categories	CD4 count (cells/μl)	Asymptomatic acute HIV	Symptomatic conditions (not indicative AIDS)	AIDS indicator conditions
1	>500	A1	B1	**C1**
2	200–499	A2	B2	**C2**
3	<200	**A3**	**B3**	**C3**

Categories in bold indicate AIDS classification, i.e., CD4 count below 200 cells/μl and/or at least one AIDS-defining condition (see Table 9.3).

microglial cells, which are developmentally derived from monocyte lineage of cells. In many subjects, this infection of CNS cells appears limited, with only a low level of virus production occurring in the CNS. However, in approximately 10–20% of cases, productive HIV infection develops in the brain, resulting in a condition termed *HIV encephalitis* (HIVE). The common presence of opportunistic infections and/or HIVE and/or virus-induced CNS dysfunction results in a spectrum of neurocognitive disease ranging from minor mood disorders and depression to severe and debilitating HIV-associated dementia (HAD). HIV-associated dementia is a subcortical dementia affecting 10–20% of infected subjects.

Treatment

The first specific treatment for HIV was zidovudine (AZT), which was introduced in 1986. AZT is a nucleoside reverse transcriptase inhibitor (NRTI) and is a thymidine analog which is converted to the triphosphate (dTTP – deoxythymidine triphosphate) form by cellular enzymes. It is then incorporated by the viral reverse transcriptase into the growing viral DNA chain. However, the presence of a 3′-N_3 group inhibits further DNA chain elongation. Reverse transcriptase is more sensitive than DNA polymerase to AZT triphosphate, providing a degree of specificity for inhibition of viral replication. While cellular DNA polymerase is relatively insensitive to AZT, mitochondrial DNA polymerase γ is known to be inhibited by the drug, and this may account for many of the side effects of AZT [8]. Side effects of AZT include nausea, headache, changes in lipid distribution, anemia, and bone marrow suppression. AZT alone does provide some benefits including decreased incidence of HIVE encephalitis [9], although it does not eradicate the virus and merely slows down the disease process. Soon after AZT was introduced, other NRTIs were produced. These were often used in dual combination with AZT to maximize efficacy; however, success in limiting disease progress was minimal. The most significant step forward in therapy has been the introduction of highly active antiretroviral therapy (HAART) in 1996–1997. This therapy combines the use not only of several different drugs but several different classes of drugs, a combination that has proven highly effective in inhibiting viral replication in those compliant subjects able to tolerate the drug regime. Classes of drugs combined in HAART include: NRTI, non-nucleoside reverse transcriptase inhibitors (NNRTI), and protease inhibitors. The introduction of this therapy has arrested many infected individuals in the pre-symptomatic phase of the disease, with moderate CD4 T lymphocyte counts maintained and progression to AIDS halted. The partial restoration of the immune system with HAART has resulted in a decrease in the incidence of most opportunistic

Table 9.3. Common diseases in HIV-infected individuals

Disease	CDC clinical category (B or C)
Oral candidiasis	B
Oral hairy leukoplakia	B
Herpes zoster (Shingles)	B
Peripheral neuropathy	B
Pelvic inflammatory disease	B
Bacterial pneumonia	C
Candidiasis of the bronchi, trachea, or lungs	C
Cryptococcosis, extrapulmonary	C
Cytomegalovirus disease (other than liver, spleen, or nodes)	C
Encephalopathy, HIV-related	C
Herpes simplex: chronic ulcers (>1-month duration), or bronchitis, pneumonitis, or esophagitis	C
Kaposi's sarcoma	C
Lymphoma, Burkitt's lymphoma, immunoblastic, or primary central nervous system	C
Mycobacterium avium complex	C
Mycobacterium tuberculosis	C
Pneumocystis carinii pneumonia	C
Progressive multifocal leukoencephalopathy (PML)	C
Toxoplasmosis of brain	C
Wasting syndrome due to HIV (involuntary weight loss >10% of baseline body weight) associated with either chronic diarrhea (≥2 loose stools per day ≥1 month) or chronic weakness and documented fever ≥1 month	C

This is not a complete list of all conditions.

infections previously common in AIDS. However, severe side effects remain, and HAART is not a cure for HIV. Many of the drugs used in combination HAART regimes are highly toxic, resulting in a number of generalized side effects, including bone marrow suppression, gastrointestinal intolerance, nausea, diarrhea, pancreatitis, neutropenia, rashes, increased transaminases, and metabolic complications. Neurological side effects include headaches, insomnia, abnormal dreams, dizziness, confusion, and peripheral neuropathies.

Some HAART drugs have poor CNS penetration, but despite this, the incidence of both HIVE and HAD have declined in the HAART era. However, while the incidence of HAD is falling the prevalence is actually rising owing to longer life spans afforded by HAART and hence an increasing population living longer with HIV. Despite the success of HAART in lowering the incidence of HAD there are increasing reports of minor motor and cognitive disorders (MMCD) occurring in HAART-treated HIV-infected subjects. As life expectancies continue to rise, patients' quality of life continues to be diminished by milder residual neurocognitive impairment.

One of the major obstacles in the treatment of AIDS is the ability to accumulate significant amounts of antiviral drugs in the CNS. As HIV enters the CNS soon after infection, this organ is regarded as a sanctuary site where the virus persists. In this context, effective delivery of drugs to the brain compartment is very significant. Many of the drugs used in HIV therapy readily cross the blood-brain barrier (BBB), although several are known to be effectively removed by CNS efflux systems. This includes AZT and the protease inhibitor Saquinavir [10]. Saquinavir is thought to be excreted from the CNS via p-glycoprotein transporters that are constitutively expressed at the BBB. Protease inhibitors in general are highly bound to serum proteins, particularly α glycoproteins [11,12]. Drugs bound to serum proteins cannot effectively cross the BBB, resulting in poor CNS penetration.

NRTIs generally bind weakly to plasma proteins and have better CNS penetration than protease inhibitors. Studies of cerebral spinal fluid (CSF) viral load have shown that NRTIs and NNRTIs are effective in decreasing CSF viral load while protease inhibitors when used alone decrease plasma viral load but not CSF viral load [13,14,15].

In a study of two HAART regimes using either one or multiple CNS-penetrating drugs, Sacktor *et al.* (2001) reported no significant difference in psychomotor speed between the two treatment regimes

[16], suggesting that systemic control of the virus may be neuroprotective, at least in moderately immunosuppressed patients. There is often a considerable degree of BBB damage during HIV infection and this may also influence drug penetration of the CNS permitting access to the CNS compartment for drugs with supposedly low CNS penetration.

The high error rate of HIV reverse transcriptase and rapid turnover of the virus population contribute to the generation of extensive genetic variation in the HIV. This allows for the rapid emergence of drug-resistant strains in individuals who are ineffectively treated with HAART. Several studies have demonstrated a high prevalence of antiretroviral drug resistance [17,18]. Data from the CDC suggests that approximately 15.2% of new HIV diagnoses have strains resistant to at least one antiretroviral drug, with 3.2% being resistant to two or more drugs [19].

Virus classification, organization, and biology

Classification and organization

In 1986, the International Committee on Taxonomy of Viruses recommended that the virus causing AIDS be called HIV-1 and that it be classified as a lentivirus, a subgenus of the family *Retroviridae*. Lentiviruses, lenti being Latin for "slow," are retroviruses characterized by long incubation periods, persistence, and slowly progressive disease. Other lentiviruses have been identified in a range of other mammalian species, including sheep and goats, horses, cattle and cats, and a series of much more closely related viruses to HIV have been seen in a large number of African nonhuman primates (collectively referred to as simian immunodeficiency viruses [SIVs]). Lentiviruses cause immune deficiency and disorders of the hematopoietic and CNSs. Figure 9.2 shows the phylogenetic relationship between lentiviruses and other related viruses.

The genomes of lentivirus are relatively large, with additional genes compared to other retroviruses (Figure 9.3). All retroviruses contain genes for the polyproteins Gag (Group antigen gene: encoding structural proteins), Pol (Polymerase: encoding reverse transcriptase, protease, and integrase), and Env (Envelope: encoding retroviral coat proteins), which are subsequently processed by viral or cellular proteases into specific functional or structural proteins. The Gag precursor is cleaved into p17 matrix (MA), p24 capsid (CA), nucleocapsid (NC), and p6 proteins. Autocatalysis of the 160-kd Gag-Pol polyprotein, $Pr160^{Gag\text{-}Pol}$, gives rise to the protease (PR), the heterodimeric reverse transcriptase (RT), and integrase (IN) proteins, whereas proteolytic digestion by a cellular enzyme(s) converts the glycosylated 160-kd Env precursor, gp160, into the gp120 surface (SU) and gp41 transmembrane (TM) cleavage products. Complex retroviruses such as HIV also have additional encoded proteins including Vif, Vpr, Tat, Rev, Vpu, and Nef. Table 9.4 lists some of the key HIV proteins and their functions. Figure 9.3 schematically demonstrates the HIV genome and the polyprotein and mature processed proteins.

HIV is thought to have arisen from a cross-species infection of humans in West Africa by a related chimpanzee (cpz) virus termed simian immunodeficiency virus (SIV_{cpz}). There is strong evidence to suggest that HIV-2 in humans arose from a cross-species infection by a SIV that is genomically indistinguishable and phylogenetically closely related to the current human virus. The epicenter of the HIV-2 epidemic is in Western Africa and coincides with the natural habitat of the sooty mangabeys (Old World monkeys). A substantial number of sooty mangabeys are infected with SIV_{sm} and it is thought that close contact between these animals and humans, either through hunting of the animals or keeping them as pets, has resulted in transmissions of SIV_{sm} to humans [20]. The origin of HIV-1 is slightly more ambiguous than that of HIV-2. At least three distinct cross-species transmissions from chimpanzees to humans are thought to have occurred, giving rise to three divergent genetic lineages of HIV (groups M, N, and O). Current opinion suggests that HIV-1 originated from chimpanzees (*Pan troglodytes troglodytes* [ptt]), again, in West Africa. Wild ptt chimpanzees are infected with SIV_{cpz} and it is thought that zoonotic transfer of this virus to humans occurred via similar

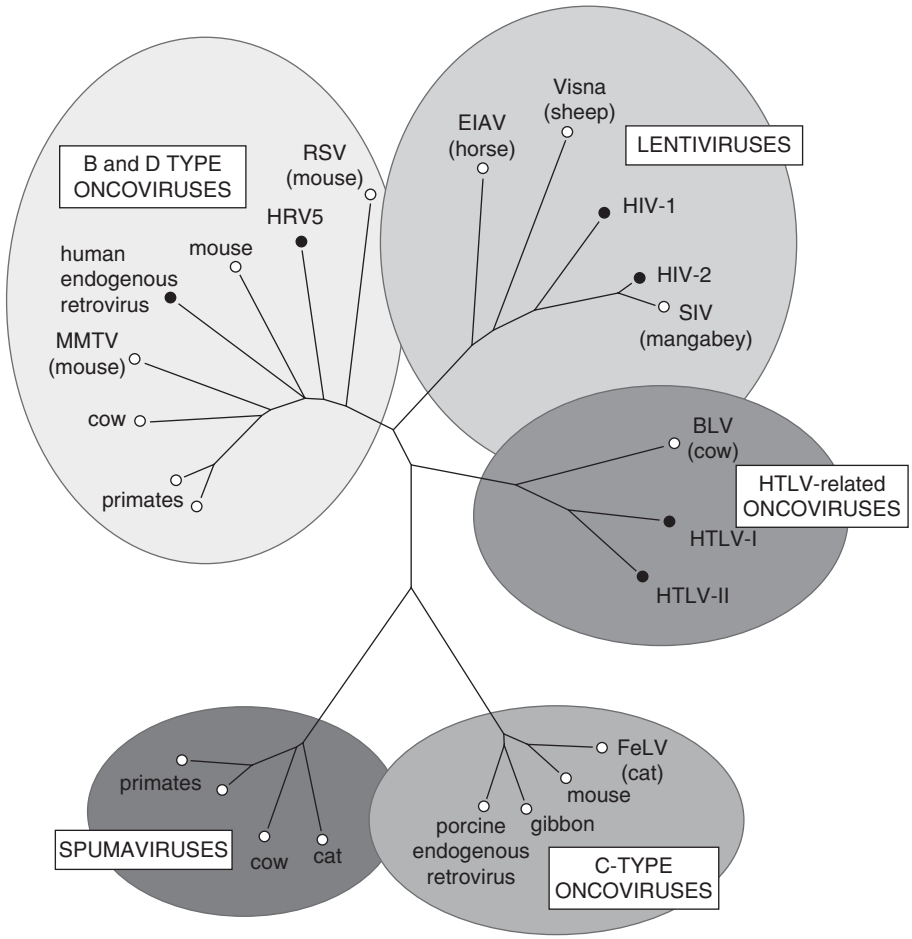


Figure 9.2. Phylogenetic relationship of lentiviruses.

mechanisms to HIV-2. The origin of SIV_{cpz} is also unclear; species-specific strains of SIV have been identified in more than 20 species of African primates, but all except SIV_{cpz} infect monkeys [20]. Data from Bailes *et al.* suggests that SIV_{cpz} resulted from coinfection of chimpanzees with two monkey strains of SIV; SIV_{gsm} from the greater spot nosed monkey and SIV_{rcm} from the red capped monkeys. They hypothesize that a recombination event occurred within the new chimpanzee host resulting in SIV_{cpz} [21,22]. Chimpanzees are known to hunt smaller monkey species and this may provide the transmission route between monkey and chimpanzee.

SIV is an asymptomatic infection in chimpanzees and as with other SIV infections does not cause disease in its natural hosts. Indeed, the development of AIDS seems to be a specific outcome of cross-species transmission into hosts by viruses not adapted to their new host. In addition to the example of AIDS development in humans from the original transmission of SIV_{cpz}, even more severe and rapid development of immunodeficiency occurs in the Asian macaques infected with the African SIV_{sm} variants. It should therefore always be borne in mind when considering the pathogenesis of AIDS in humans that HIV is not intrinsically pathogenic, nor does

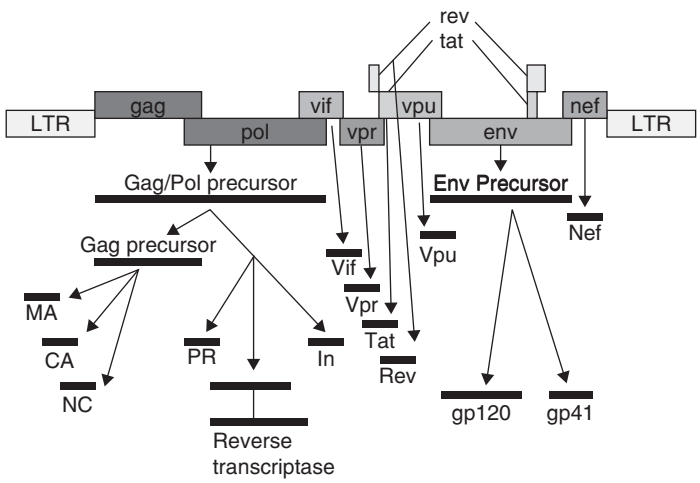


Figure 9.3. Schematic demonstration of the HIV genome, polyproteins, and mature processed proteins.

Key:
MA: p17 Matrix,
CA: p24 Capsid,
NC: p7 Nucleocapsid,
PR: Protease,
IN: Integrase

Table 9.4. HIV proteins and their functions

Type of protein	Protein name		Function
Structural proteins	Gag (group-specific antigens)	p17 Matrix	Membrane anchoring
		p24 Capsid	Nuclear transport of viral core protein
		p7 Nucleocapsid	Binds RNA
	Env (envelope)	gp120/gp41	Viral glycoprotein that binds to CD4 receptors mediating membrane binding
Enzymatic proteins	Pol	Protease	Cleaves viral polyproteins into their functional peptide components
		Reverse transcriptase	A DNA polymerase enzyme that transcribes single-stranded RNA into double-stranded DNA
		Integrase	Catalyzes the integration of viral genetic material into host cell DNA
Regulatory proteins	Tat (Transactivator of transcription)		Viral transcription transactivator
	Rev (Regulator for expression of viral proteins)		Regulator of structural gene expression
Accessory proteins	Vif (viral infectivity factor)		Promotes infectivity of cell-free virus
	Vpr (viral protein R)		Promotes infectivity, arrests growth in G2 phase of cell cycle
	Vpu (viral protein U)		Required for efficient virion budding
	Nef (Negative factor)		Downregulates CD4 and MHC class I

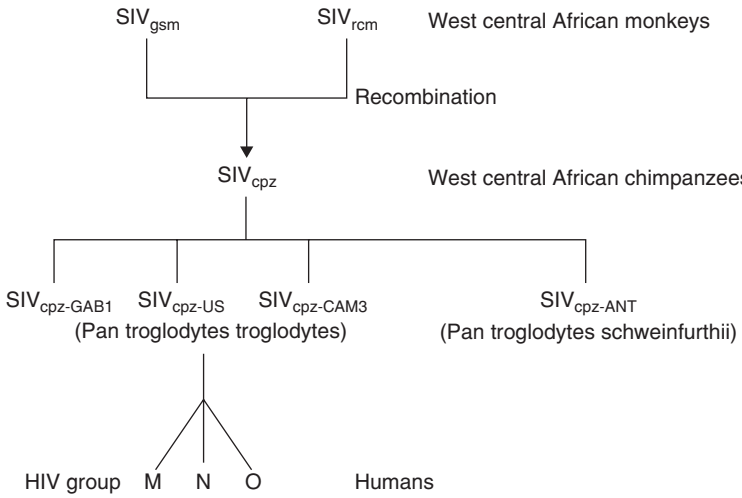


Figure 9.4. Diagram of HIV descent from SIV.

replication necessarily cause the destruction of immune function and neuropathology observed in human and other maladapted hosts.

Phylogenetic analyses of HIV-1 identifies discrete clusters or clades described as the M (major); O (outlier); and N (non-M or O) groups. These correspond to descendants of HIV variants descended from separate cross-species transmissions of SIV_{cpz} into humans (Figure 9.4). The M group contains over 95% of global virus isolates and consists of at least eight clades (A,B,C,D,F,G,H,J) (Figure 9.5) [23,24,25]. Clade B is the most common in Western Europe, North America, Australia, and New Zealand. Interclade viral recombinants are also commonly found in multiple regions of the world containing genomes that are mosaics of two or more subtypes [26,27,28].

Analysis of proviral isolates from different geographical regions demonstrates extensive genetic heterogeneity [29]. Nucleotide sequence analysis of HIV samples recovered from even a single individual exhibit significant variability. Although nucleotide changes are distributed throughout the HIV genome, the greatest number of substitutions occurs in the gene encoding the envelope (Env) glycoprotein gp160. Several factors contribute to the genetic heterogeneity of HIV including the error rate of HIV reverse transcriptase enzyme, high frequency of recombination, and high levels of virus production (10.3×10^9 virions/day) [30,31,32].

Biology

The defining feature of HIV infection is impaired immune cell function with loss of CD4 T lymphocytes. Initially, the body is able to compensate for loss of crucial CD4 T lymphocytes by increasing production of these cells in the bone marrow. An anti-viral response is mounted early after infection, which controls the initial viremia. However, the virus is never eradicated from the body and eventually CD4 T lymphocyte loss can not be compensated for leading to impairment of the immune system. Impaired immunity results in difficulty in controlling pathogens, many of which would be otherwise innocuous to a healthy individual. At the end stages of disease, the immune system capitulates, with most AIDS patients dying from opportunistic infections.

Immune response to HIV

There is both a humoral and cellular immune response to HIV. Antibodies to HIV are detected shortly after acute infection. In the acute phase and

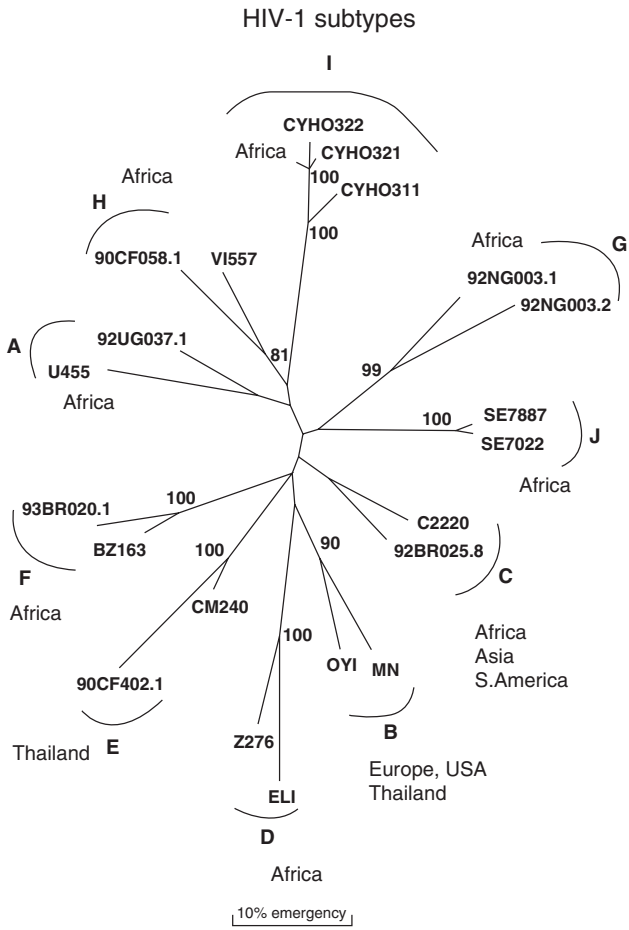


Figure 9.5. Phylogenetic tree of HIV-1 showing different clades.

presymptomatic phase neutralizing antibodies may play a role in limiting viral replication. However, the titers of these antibodies are generally low and viral escape occurs quickly. HIV infection disrupts both arms of the adaptive immune response. Although HIV does not replicate in B lymphocytes, infection does give rise to severe B lymphocyte abnormalities compromising humoral immunity. Abnormalities include increased production of nonspecific immunoglobulins (Ig) IgG, IgA, and IgM, while production of specific antibodies to both new and recall antigens is inhibited.

There are several factors that may be involved in the aberrant immune responses observed during HIV infection. The most obvious are the direct viral cytopathic effects on infected cells; however, other factors are also central in inducing immune dysregulation. These include aberrant cytokine production within the body. It has been proposed that the normal cellular/humoral T helper 1 and T helper 2 (TH1/TH2) immune balance within the body becomes skewed during HIV infection in favor of the humoral TH2-promoting cytokines. Defects in production of interleukin-2 (IL-2) and IL-12, both important mediators of T lymphocyte activation, have been reported and may be at least partially responsible for the failure of immune response to recall antigens observed in AIDS [33–35]. Increased expression of the TH2 cytokines IL-4 and IL-10 have also been described, suggesting that a switch does occur from the more prominent TH1 to TH2 [36]. The exact mechanism through which this immunological switching occurs is unclear, but there is evidence to suggest that viral proteins may play a role. The viral protein Nef has been reported to decrease production of both IL-2 and IFNγ [37]. Although numerous reports provide evidence of TH1/TH2 shift, other studies have suggested that a TH1/TH2 switch does not occur in HIV [38]. Regardless of whether a switch does occur there is clear evidence of aberrant cytokine expression both from *in vivo* and *in vitro* studies which undoubtedly influences the immune response to the virus. Increased production of proinflammatory cytokines IL-1ß, IL-6, and TNFα is reported, however, expression of antiviral interferon (IFN)γ is reportedly decreased *in vitro*, yet *in vivo* reports suggest that it is actually elevated in AIDS [39,40,41]. Cytokine cascades are complex and our understanding of these in different disease settings is confounded by the bifunctionality of many cytokines in different settings.

Reasons for immune failure

Large numbers of HIV-specific immune cells are detected during the course of HIV infection, yet despite this the immune system is unable to fully

control virus replication. There is increasing evidence that the number of antiviral cells is not the problem but rather their ability to function. Chronic antigenic stimulation by persistent viruses can lead to exhaustion of CD8 T lymphocytes by preventing the formation of renewable memory cells [42,43,44]. Aberrant cytokine production is a feature of HIV infection and may also influence CD8 T lymphocyte function [45]. Although HIV-1 primarily infects memory CD4 T cells, a number of other cellular reservoirs exist; one of which is CD8 T cells [46,47,48]. Activation of CD8 lymphocytes induces expression of CD4 and thus renders these cells susceptible to HIV-1 infection [49,50]. The generalized immune activation seen during HIV-1 infection leads to increased numbers of cells with an activated phenotype, a state which contributes to the pathogenesis [51]. In infected subjects who abuse illicit drugs, particularly opiates, there may be further mechanisms through which CD8 lymphocyte function is disrupted as a direct result of opiate interaction [52].

Viral entry and replication

The primary target cells for HIV are those carrying the CD4 surface receptor. These are primarily T lymphocytes, but other cells including macrophages and microglia also express CD4. CD4-positive T lymphocytes are the major reservoir of HIV. While both naïve and memory CD4 T lymphocytes can be infected with HIV, the virus preferentially replicates in activated memory cells [53]. The CD4 molecule is a member of the Ig superfamily, and its normal function is to stabilize the interaction between the T-cell receptor on the surface of T lymphocytes and class II major histocompatibility complex (MHCII) molecules on antigen-presenting cells.

In the CNS, the primary cell type infected is the resident brain microglial cell (monocyte/macrophage-derived cells), although a restricted, non-productive, CD4-independent infection of astrocytes has also been reported [54]. Figure 9.6 shows the main steps in HIV replication.

In addition to the role of CD4, HIV coreceptors also play an important part in cell infection. The primary coreceptors are the chemokine receptor CXCR4 and CCR5; however, a number of other chemokine receptors can serve as co-receptors, at least *in vitro*, and these include CCR2b, CCR3, and CCR8. It has also been suggested that certain isolates of HIV and SIV can infect cells in a CD4-independent manner utilizing just coreceptors [55,56]. Coreceptors may also play a role in resistance to HIV infection. Some individuals who are at high risk of HIV infection through their behavior patterns remain uninfected despite repeated exposure to the virus [57]. A mutant allele of the CCR5 gene has been found with a 32bp deletion which in homozygotes confers protection against HIV infection, highlighting the role of coreceptors in the infection process [58].

The surface of the viral envelope contains glycoprotein spikes consisting of a transmembrane viral glycoprotein (gp41) and a surface viral glycoprotein (gp120). The glycoprotein spikes exist as a trimmer of three gp120 molecules held together by three gp41 molecules. They mediate attachment of the virus and fusion with the cell membrane. The interaction of CD4 and a coreceptor with gp120 mediates a rearrangement of gp41, exposing the hydrophobic fusion domain. This then embeds into the cell membrane leading to viral uncoating and entry. Once inside the cell the viral capsid proteins are shed prior to initiation of reverse transcription. Viral RNA is then reverse transcribed in the cytoplasm into double-stranded DNA. The viral DNA and replication complex are then transported to the nucleus where viral integrase catalyzes the insertion of the viral DNA into the cellular chromosomal DNA forming the provirus. Thereafter host cell apparatus is responsible for transcription and translation of the virus, with transcription being dependent on the host cell's state of activation and being regulated by binding of cellular transcription factors such as NF-κB to the 5′ long terminal repeat (LTR) of the viral genome. Free unintegrated viral DNA can also be translated, but this is far less efficient than provirus transcription.

The function of host cell transcriptional apparatus is closely regulated by cytokines, and hence these also influence HIV replication in infected cells. Persistent immune activation due to release of cytokines

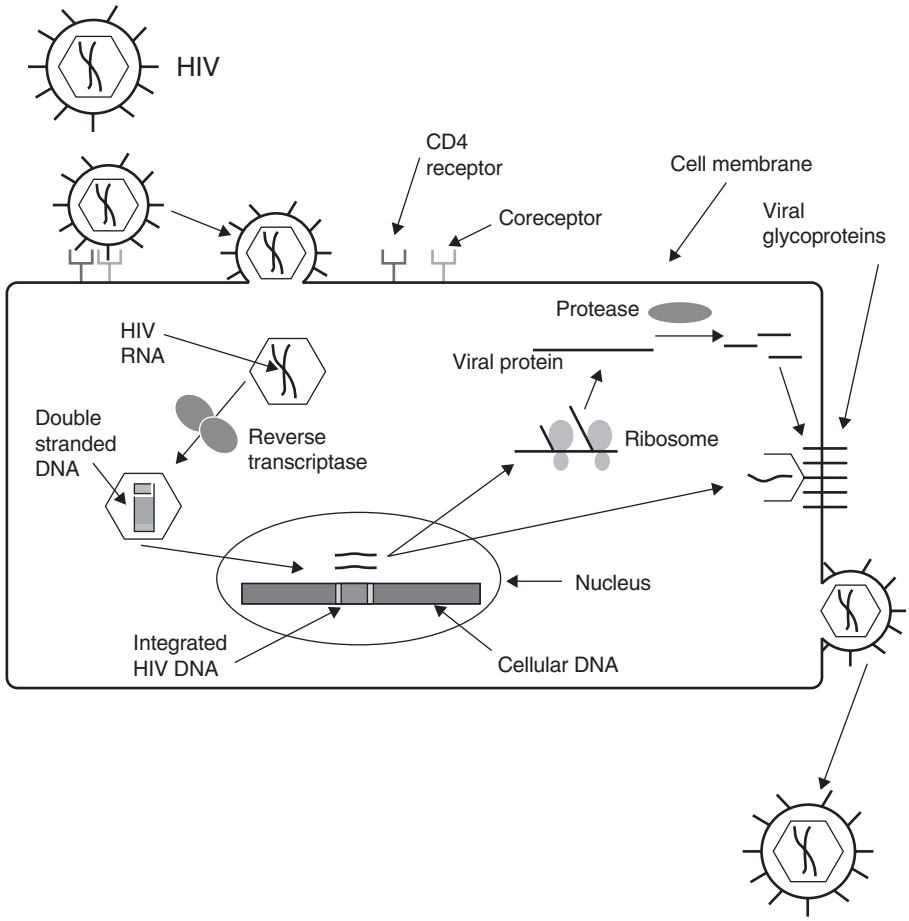


Figure 9.6. Major steps in HIV replication.

can drive HIV replication, with cytokines including IL-1α, IL-1β, IL-2, IL-6, TNF-α, and TNF-β promoting replication. IL-13, IL-15, IL-16, IFN- α, and IFN-β exhibit inhibitory effects. Other cytokines including TGF-β, IL-4, IL-10, and IFN-γ can either suppress or activate replication depending on conditions within the cell. Hence, the immune response to both HIV and associated opportunistic infections can actually influence viral replication.

Chemokines also play a role in influencing viral replication. *In vitro* viral entry can be blocked by several chemokines through competitive binding of cellular receptors. ß chemokines MIP-1 α, MIP-1β, and RANTES, the natural ligands for CCR5, inhibit R5 strain entry *in vitro*, while SDF-1, the natural ligand for CXCR4, inhibits X4 strain entry.

Virus evolution within the body

Viruses isolated from individuals shortly after they have been infected with HIV are frequently macrophage tropic (M-tropic) utilizing the CCR5 coreceptor and are usually nonsyncytium forming [59,60]. Isolates recovered late in the disease course more often utilize CXCR4, are syncytium forming and are T lymphocyte tropic (T-tropic). Somewhat paradoxically, studies of pairs of individuals in which one chronically infected subject is known to have

recently infected a second subject show that the transmitter of the virus has a diverse blood viral population capable of infecting both macrophages and T lymphocytes and capable of syncytium formation. In contrast, the recently infected subjects, who are in the acute phase of the disease, show only M-tropic virus which are unable to form syncytia. Surprisingly, the transmitted virus represents only a minor component of the virus population in the blood of the transmitter [60]. The reason that only minor populations of M-tropic virus are transferred is unclear. It is possible that selection occurs at the point of entry to the body, with M-tropic strains being favored as these can readily infect macrophages in the submucosal spaces.

CCR5 is expressed on memory CD4 T lymphocytes, while in contrast CXCR4 is expressed on both naive and memory CD4 T lymphocytes. In the circulating blood only 10% of CD4 T lymphocytes express CCR5, while up to 95% express CXCR4. Early emergence of syncytium-forming X4 strains or dual tropic R5/X4 strains is associated with faster disease progression [61,62], while peak production of X4 strains is associated with failure of T lymphocyte homeostasis and decline in CD4 T lymphocyte count below 200 cells/μl (Figure 9.1) [63].

HIV reservoirs and viral sequestration in the brain

Therapeutic strategies face two major problems in eradicating HIV from the body. First, the high rate of viral evolution and diversity within a single infected subject permits development of drug-resistant strains. Second, at an early stage in infection, a latent viral reservoir is established which has the potential to persist for the life of an individual. During acute infection, long-lived memory CD4 T lymphocytes are infected with provirus stably integrating into the host cell genome. In a latent state, no viral proteins are produced within the cell, hence protecting the latent virus from both the immune system and from antiretroviral drugs.

Other reservoirs of latent HIV infection have been proposed, including the CNS, where both microglia and astrocytes may harbor latently infected cells. Virus sequestered in the brain is likely to be an even greater challenge to eradicate from the body than isolates in the periphery due to the presence of the blood-brain barrier (BBB) and the immune-privileged status of the CNS. The BBB consists of tightly packed specialized endothelial cells with the interface between these cells sealed by tight junction proteins such as occluden and zonnula occludens 1 and 2 (Figure 9.7). Additional components of the BBB include the presence of astrocyte foot processes on the abluminal side of brain microvascular capillary cells. The result of these modifications to the microvasculature of the brain is a BBB that blocks diffusion of most molecules into the brain. The exceptions are molecules such as oxygen and carbon dioxide and those substances that are actively transported such as glucose. Substances with a molecular weight higher than 500 daltons cannot freely cross the blood-brain barrier while smaller molecules usually can. Through these mechanisms, the BBB controls the microenvironment of the brain, maintaining a constant homeostasis for the delicate neurons. The BBB also regulates the entry of immune cells to the brain ensuring that peripheral systemic infections do not result in large immune responses within the brain that could be damaging to neurons. Entry of immune cells to the healthy brain is normally highly restricted, although

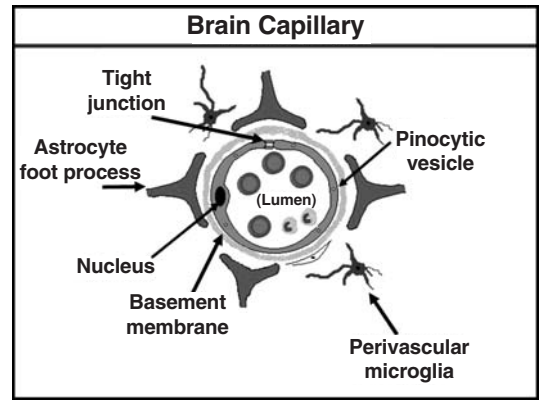


Figure 9.7. Constituent components of the blood-brain barrier.

there is a low level of circulating T lymphocytes and very occasional B lymphocytes which do patrol the normal brain. In addition to lymphocytes, there is a low level influx of macrophage/monocytes into the brain. These cells migrate to the brain and differentiate *in situ* into perivascular microglia. Brain microglia cells fall into two categories; parenchymal and perivascular microglia. Microglial cells within the brain are derived from monocyte lineage cells which migrate to the brain during fetal development and take up lifelong residence. The parenchymal microglia lie deep within the tissue and are rarely replaced by new cells from the blood. In contrast, the perivascular microglia surrounding brain blood vessels are occasionally replaced by new cells from the blood. The long half life of parenchymal microglia ensures that it would be nearly impossible for either the immune system or current therapy to eradicate all latent virus present in these cells within the lifetime of an individual.

Phylogenetic reconstructions have shown clustering of sequences according to the tissue from which they originate including the brain, suggesting that segregation and sequestration of viral strains does occur. It is unclear whether this segregation is a result of the unique replication conditions within the CNS. Thus, the cells capable of supporting infection in the CNS may not be as permissive as peripheral equivalents and furthermore the virus may be under less pressure from the immune system as both neutralizing antibody titers and cytotoxic T cell responses are weaker in the CNS than in the periphery.

HIV neuroinvasion

Early in the HIV epidemic, the CNS was recognized as a major target site for the virus. The virus enters the brain within days of infection. Evidence for this comes from the iatrogenic infection of a 68-year-old man who died 15 days later from another condition unrelated to HIV. At autopsy, HIV DNA was recovered from several sites in his brain [64].

There are several potential mechanisms through which HIV could enter the brain. The most likely route for brain invasion by HIV is across the BBB [65], although the choroid plexus and CSF pathway may also be implicated [66]. It is possible that free virus could enter the brain via adsorptive endocytosis into endothelial cells, with the virus then infecting the juxtaposed resident perivascular microglia. A more likely explanation is that the virus is carried in by infiltrating immune cells. There are two possibilities that would permit this to occur. First, the virus could be carried in by circulating memory CD4 T lymphocytes with resultant infection of resident CNS cells. Alternatively, the virus could enter via infected macrophages which occasionally migrate into the brain to replace the perivascular microglia. The latter option seems the most plausible given that viral isolates recovered from the brain are almost invariably M-tropic. Once within the brain, the primary target for the virus is the resident microglial cells. These monocyte lineage cells express the primary HIV receptor, CD4, albeit at a low level, together with the chemokine coreceptor CCR5 [67]. They are capable of supporting productive HIV infection resulting in HIV encephalitis (HIVE). Infection of microglia by HIV has two potentially CNS-damaging effects: first, production of new virions and viral proteins and, second, induction of aberrant cytokine expression within the CNS [68]. Apart from microglia, astrocytes are also a possible target but are thought to be capable of only a restricted form of HIV infection *in vivo* [69]. The mechanism by which astrocyte infection occurs is unclear as these cells are not thought to express CD4, although they do express the chemokine receptors CCR5 and CXCR4. Regardless of whether astrocytes are infected or not, it is likely that these cells play a key role in the neuropathogenesis of HIV infection if their crucial functions in supporting neurons are disrupted. The function of astrocytes includes the production of neurotrophic cytokines and buffering of substances that are neurotoxic in high concentration such as glutamate.

There have also been occasional reports of endothelial cells, oligodendrocytes, and neurons being infected with HIV. However, these reports have not been widely reproduced, and the consensus opinion is that infection of these cell types is either

extremely rare or does not occur at all and is unlikely to contribute significantly to HIV-related CNS disorders. Given the lack of evidence for significant neuronal infection, it is assumed that neuronal damage and death in AIDS is an indirect result of HIV infection of other cells in the brain [70].

Neuropathology of HIV infection

Opportunities for neuropathological study of early HIV infection, when subjects are still in the pre-symptomatic phase of the disease, are rare because most infected subjects do not die until they reach the symptomatic (AIDS) phase of the disease. However, a unique cohort of HIV-infected intravenous drug abusers in Edinburgh (UK) has afforded a rare insight into CNS involvement in pre-symptomatic HIV. Within this large cohort lethal drug overdoses were common, resulting in the death of many HIV-infected subjects before they progressed to AIDS. Study of this cohort has shown that the brains of pre-symptomatic HIV-infected drug abusers show relatively minor changes in comparison with those seen in AIDS [71]. There is no evidence at this stage of HIV encephalitis (HIVE) or of CNS opportunistic infections or lymphomas, all of which represent AIDS-defining illnesses in their own right. However presymptomatic individuals are likely to show a low grade lymphocytic leptomeningitis and perivascular lymphocytic cuffing particularly in the central white matter [71,72]. The primary cell type found in these infiltrates is the CD8 positive lymphocyte, but a significant proportion of CD20 positive B lymphocytes are also present [73]. It has been suggested that these lymphoid cells may be responsible for controlling the initial viral infection in the CNS [74].

Other phenomena reported in early infection include subtle gliosis and microglial activation, which may represent part of an immune response to early CNS entry of the virus [75]. Alternatively, activation of CNS microglia and astrocytes may simply be an indirect effect of the more vigorous systemic response to infection, due to systemically released cytokines. Evidence of mild axonal damage can sometimes be observed in the early stages of the disease [76]. This is revealed by focal swellings and accumulation of normal axonal molecules such as β amyloid precursor protein (βAPP) due to disrupted transport within the affected axon. Axonal damage can be caused by a number of insults including trauma, inflammation, and hypoxia and is also seen in the brains of HIV-negative drug abusers [77,78].

In pre-symptomatic subjects, there is no evidence of productive infection in any cell type. However, polymerase chain reaction (PCR) studies have confirmed low levels of HIV in the brains of some presymptomatic subjects [72]. While it is presumed that HIV is present in the microglial population based on analysis of recovered virus which is normally macrophage (CCR5) trophic, there is still no conclusive evidence as to which brain cells are harboring the virus in the early stages of infection.

As HIV-infected subjects progress into symptomatic AIDS, they become vulnerable to CNS complications, which are reflected in conspicuous neuropathological changes observed at autopsy. The most significant of these are the emergence of opportunistic infections, primary CNS lymphomas and/or HIVE [79]. These conditions may be found in isolation or together, but there is no convincing evidence to date of synergy between them. Common opportunistic infections seen in the brain in AIDS include meningitis resulting from Cryptococcus neoformans (2–30% of cases), cytomegalovirus (CMV), encephalitis (9%), toxoplasmosis (4%), herpes simplex virus encephalitis (4%), progressive multifocal leukoencephalopathy (PML) associated with JC virus infection of oligodendrocytes (2%), and primary central nervous system lymphomas (PCNSL) driven by Epstein-Barr virus (EBV) (5–10%) [80,81,82,83] (Figure 9.8). The prevalence of opportunistic conditions varies somewhat depending on the population and geographic exposure risk. Many of the agents responsible for these infections commonly infect healthy humans but rarely cause disease in immune-competent individuals. For instance, JC virus is estimated to infect 90% while EBV is thought to infect 95% of the population. In the setting of HIV-induced immunosuppression, JC virus infects and destroys

oligodendrocytes, the cells responsible for myelination of axons in the CNS. This may be a result of reactivation of the virus due to immunosuppression or alternatively the virus may be reactivated by HIV gene products, such as Tat, which may be able to transactivate the JC viral promoter directly [84]. PML manifests itself as demyelinating lesions which may be necrotic and which are associated with inclusion-bearing oligodendrocytes and enlarged, often bizarre astrocytes. CMV is promiscuous in its cellular targets for infection, and viral particles may be identified in endothelial cells, neurons, and glial cells. Typically, the infected cell shows enlargement of the nucleus and/or the cytoplasm and viral inclusions may be identified in both. Two major forms of CMV encephalitis are described. These take the form of a microglial nodular encephalitis in which CMV inclusions may be quite hard to find. The other form is necrotizing, and CMV inclusion-bearing cells are found relatively frequently in association with polymorphonuclear leukocytic infiltration and foci of necrosis. Toxoplasma may give rise to a similar

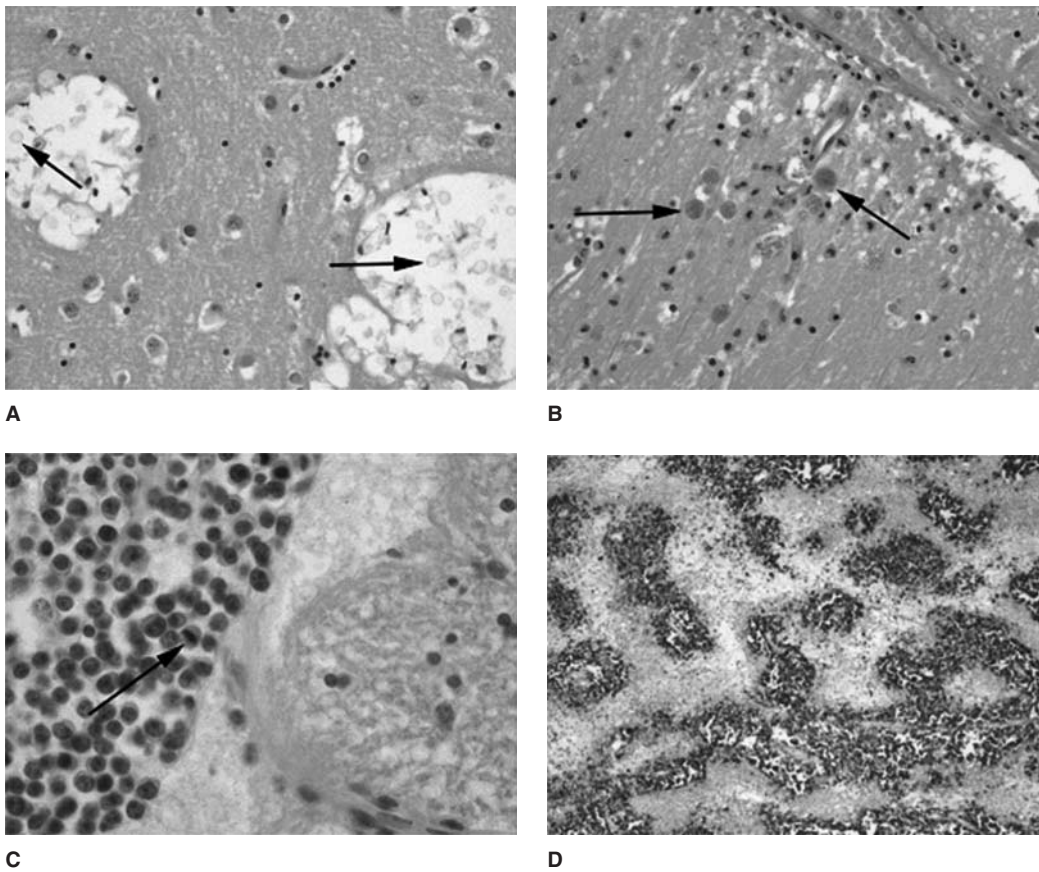
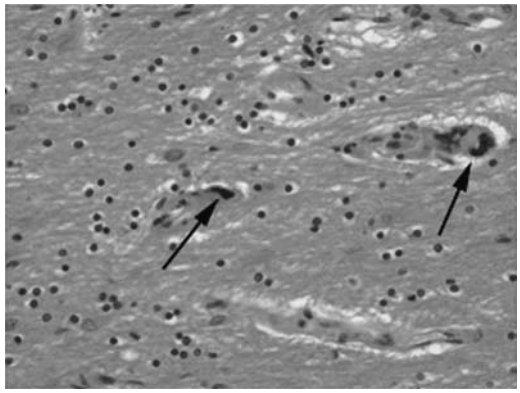


Figure 9.8. Neuropathological findings in HIV-infected subjects. (A) Cryptococcus (×200). The arrows indicate two of many cryptococcus organisms in cystic cavities within the basal ganglia. (B) Toxoplasma (×200). Showing many cysts containing organisms in the cerebellar cortex. (C) Primary central nervous system lymphoma (PCNSL) (×400). Showing malignant B lymphocytes abutting on white matter in the basal ganglia. A mitotic cell is arrowed. (D) Primary central nervous system lymphoma (PCNSL) (×400). Showing *in situ* hybridization for Epstein-Barr virus. (Continued)



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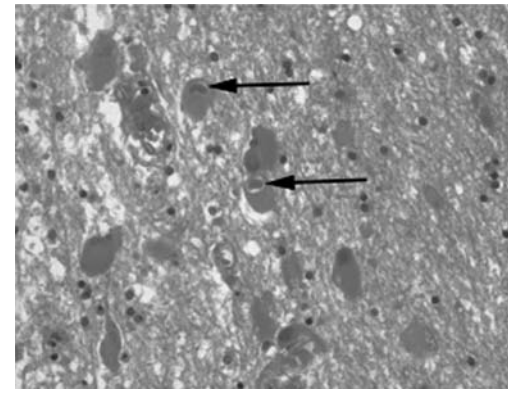

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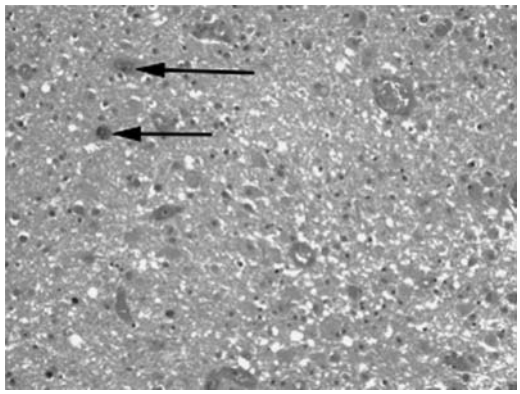

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Figure 9.8 (Continued). (E) HIVE (×200). Showing perivascular giant cells in the white matter. (F) CMV (×200) showing enlarged cells with intranuclear viral inclusions. (G) PML (×100) showing necrotizing demyelination of the white matter with enlarged bizarre glial cells. (For figure in color, please see color plate section.)

necrotizing encephalitis, particularly in the periventricular regions, and the acute inflammatory exudate may spread to involve the ventricular cavities. Toxoplasma is a protozoan that can exist in the brain parenchyma as numerous free organisms or as characteristic encysted forms containing numerous organisms.

EBV is ubiquitously present in PCNSL in the setting of HIV. These high-grade lymphomas are B lymphocytic in origin and are usually monoclonal. EBV is the aetiological agent driving proliferation of B lymphocytes and eventual neoplastic transformation [82,85,86]. In nearly all instances there is expression of two key EBV oncogenes LMP-1 and EBNA-2. Expression of LMP-1 leads to upregulation of anti-apoptotic genes such as BCL-2 in the infected B lymphocyte, while EBNA-2 is responsible for driving the infected cell into S-phase of the cell cycle [87,88]. The expression of these two proteins plays a key role in the immortalization of B lymphocytes. In most instances, analysis of B lymphocyte immunoglobulin receptors shows that when these tumours occur in AIDS they are monoclonal, resulting from the outgrowth of just one infected cell. PCNSL display a predominantly perivascular distribution and can be found in almost any

location in the brain including the brain stem and spinal cord.

In most subjects with CNS opportunistic infections, microglial activation and focal infiltrates of CD8 lymphocytes are present particularly in the vicinity of focal pathology.

In some AIDS patients, even at advanced stages of immunosuppression, there is little evidence of significant CNS disease, and HIV-related disorders may not be evident in the brain at autopsy. However, the brain is rarely entirely normal and at least minor, nonspecific neuropathological changes are present in the vast majority of subjects with AIDS.

Other common CNS complications include HIVE and cognitive dysfunctions. The pathognomonic histological feature of HIVE is the presence of giant cells [89,90] together with immunopositivity for HIV antigens in microglia/macrophages, signaling the presence of productive viral infection [91,92]. Infection is detected predominantly in the perivascular microglia/macrophages, although parenchymal microglia are also frequently immunopositive [93]. Before the advent of HAART, HIVE occurred in 20–50% of cases with wide variation between different cohorts [9,94]. Productive HIV infection induces activation of surrounding microglia, which show increased expression of a variety of cell surface antigens including CD14, CD16, CD68, and MHC class [68,95,96,97]. In addition to the microglial activation, there is also a prominent CD8 T lymphocytic response, although it is unclear how effective these cytotoxic T cells are in late-stage AIDS when the immune system is severely disrupted by HIV. Prominent astrocytic hyperplasia is also common in HIVE [9,91] often co-present with macrophage-predominant inflammation and microglial nodules.

HIVE may be present in any area of the brain, particularly the basal ganglia and central white matter, but the neocortical gray matter and to a lesser extent the brain stem and cerebellum are sometimes involved. The severity of HIVE also varies from mildly affected cases in which only a few productively infected cells and/or giant cells are seen to very severe states with widespread inflammation and damage and numerous giant cells. These variations are likely to contribute to the observed range of clinical symptoms.

The introduction of HAART has led to the emergence of a new pathological phenomenon in HIV called immune reconstitution syndrome (IRIS). In these cases, extensive demyelination and white matter damage is found in HIV-infected individuals following the institution of HAART. The myelin damage is accompanied by marked lymphocytic infiltrate of brain parenchyma suggesting that the pathogenesis of the white matter damage may be immunologic following a HAART-induced upturn in the numbers of circulating CD4 and CD8 lymphocytes and sudden massive influx of these cells into the brain [98]. No information is available with regard to the viral load in brain tissue in these cases. Although there is an assumption that the observed demyelination is caused by the influx of autoimmune lymphocytes into the brain explanation, it should be noted that lymphocytic infiltrate of the brain is also prominent in some presymptomatic individuals without obvious myelin damage.

Other generalized CNS findings in AIDS include myelin pallor [99] and dendritic [100], synaptic [101], and axonal [102] damage. Damage to myelin likely results from the initial viral infection or may represent an indirect effect of the immune reaction to the virus. Axonal damage is highly variable between cases, ranging from a few focal deficits to widespread disruption in the central white matter [92].

The clinical manifestations of CNS disorder in HIV/AIDS include depression and various degrees of cognitive impairment up to and including HIV-associated dementia (HAD). Symptoms and signs of HAD include tremor, gait ataxia, loss of fine motor movement, mental slowing, forgetfulness, poor concentration, and behavioral abnormalities. This subcortical dementia affects 10–20% of infected subjects. Other significant complications include peripheral neuropathies and long tract signs [103]. The exact causes of HAD are not clearly established, but it is likely that neuronal dysfunction is a result of indirect viral toxicity with microglial activation [68,104].

The presence of HIVE shows a degree of correlation with HAD, but this is not an absolute association. The best correlate of AIDS dementia is reportedly not viral replication or viral load but rather the degree of monocyte infiltration and the level of microglial activation in the brain [95]. Both infected and non-infected activated microglia have been suggested as pathogenic factors in the development of HAD. There is a range of mechanisms through which microglia may induce neuronal damage, including the release of potentially neurotoxic levels of oxidative radicals, nitric oxide, and/or the cytokines TNF α and IL-1 [68,105,106,107]. Aberrant cytokine release can also affect astrocyte function leading to a loss of glutamate buffering and subsequent neurotoxicity [68].

Microglial activation and infiltration of monocytes into the CNS have been widely purported to be the driving force behind the development of HAD [108]. The mechanisms involved in recruitment of monocytes and activation of microglia in HAD are still unclear. The most obvious explanation is that this is a response to viral production in the CNS, but evidence of productive infection is not always present in subjects with HAD, and there are occasional cases with evidence of HIVE at autopsy who had no clinical history of dementia [9,109]. These anomalies undermine the notion of a simple link between viral presence in the brain and the disturbance of higher functions.

Neuroimaging of HAD patients reveals generalized white matter reduction, with additional gray matter loss particularly in the basal ganglia and posterior cortex [110,111]. These findings fit with the general neuropathological findings in these cases. Neuronal loss has been described in HAD, and apoptotic cells are commonly found in the basal ganglia and to a lesser extent in other regions of the brain including the hippocampus and frontal cortex [112,113].

Mechanisms of neurodegeneration

The paucity of evidence for HIV infection of neurons suggests that neuronal damage is mediated via indirect mechanisms. That is to say that the virus itself does not infect neurons but causes neuronal damage and death by stimulating inflammatory reactions in the brain and by inducing the release of toxic substances.

Infected cells, primarily microglia, can release not only HIV virions but also HIV proteins, several of which are neurotoxic, including gp120 and Tat. HIV gp120 can cause neuronal damage by activating macrophages and microglia to secrete inflammatory cytokines and arachidonic acid [114]. It can also potentially act directly on neurons to induce apoptosis by altering Ca^{2+} metabolism, though the concentrations required for this direct effect are unlikely to be achieved *in vivo*, suggesting an indirect role for gp120. Gp41 can reportedly induce production of nitric oxide (NO) by increasing production of inducible nitric oxide synthase (iNOS, NOS-2) (see also Chapter 14) [115]. iNOS reacts with arginine to produce citrulline and NO. NO in turn can react with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), a potent neurotoxin [116]. Peroxynitrite stimulates an increase in neuronal Ca^{2+} resulting in neuronal apoptosis [117]. iNOS is reportedly increased in HIV dementia subjects at autopsy and its presence correlates with the degree of neurocognitive impairment [118,119].

HIV Tat is thought to increase Ca^{2+} in neurons, impair glutamate uptake by astrocytes, and induce iNOS resulting in increased NO production, all of which can lead to neuronal apoptosis. Tat is also thought to increase astrocyte expression of MCP-1 which may promote the recruitment of further monocyte lineage cells into the brain, enhancing neuroinflammation.

Neuroinflammation can result in neuronal damage and death via a variety of mechanisms. Activation of microglia and gliosis are common in HIV particularly in those with dementia. Aberrant cytokine release, including IL-1, TNFα, and IL-6, is also reported, and this can impact on normal glial function. Both microglia and astrocytes can be induced to express iNOS, while upregulation of the cytokine IL-1β can also upregulate iNOS. TNFα impairs glutamate uptake by astrocytes from the extracellular

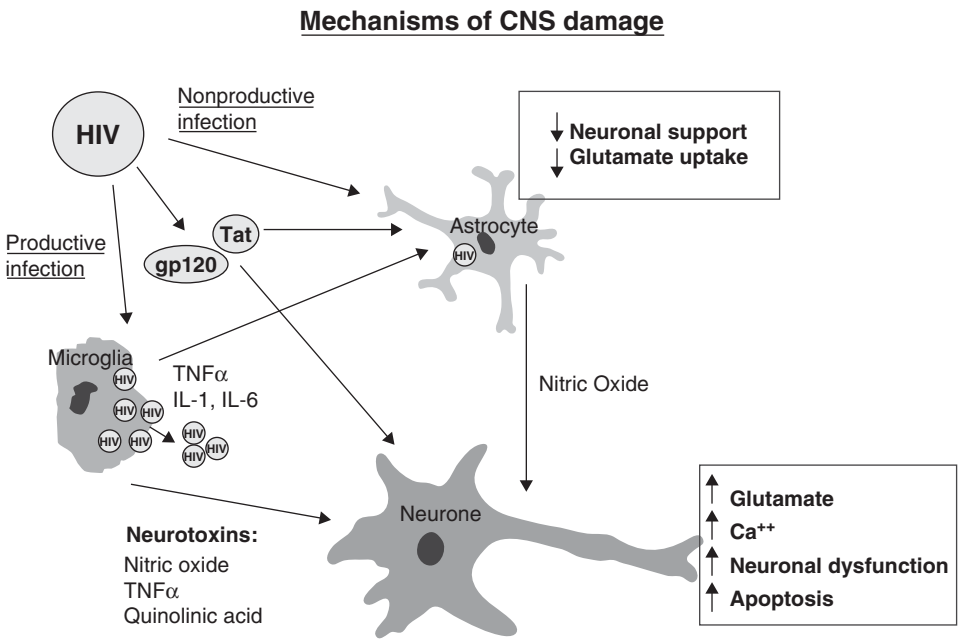

Figure 9.9. Mechanisms of CNS damage during HIV infection.

milieu, resulting in neuronal damge. In combination with IL-6, TNFα can stimulate HIV replication by inducing expression of nuclear factors that are able to act on the HIV LTR, further adding to CNS disruption. TNFα inhibits the astrocytes' ability to remove excess glutamate from the extracellular milieu, resulting in neuronal damage. Figure 9.9 shows the interaction of some of these factors in neuronal damage and CNS dysfunction.

Neuropathogenesis of HIV

The original proposals to explain the pathogenesis of HAD centered on the "Trojan horse" model. This suggested that the virus entered the brain early in infection carried by infiltrating immune cells, either T lymphocytes or macrophages. The virus then set up a latent infection in the resident microglia cells and remained dormant in the brain until the later stages of the disease when CD4 T cell counts fell and the immune system failed, leading to viral production and associated brain damage. There are several problems with this model. While there is evidence of HIV DNA in the brains of presymptomatic subjects, the viral load is generally extremely low and productive infection is never observed, raising questions as to how well-seeded the brain becomes in the early phase of the disease. Second, not all subjects with clinical dementia have evidence of productive infection at autopsy. This suggests that while HIVE may contribute to the development of HAD, it is neither necessary nor probably sufficient to cause HAD.

Recently, a new model for the pathogenesis of HAD has been proposed that suggests critical events in the pathogenesis of HAD actually occur outside the CNS. The model suggests that aberrant cytokine production in late-stage AIDS results in increased macrophage colony stimulating factor (M-CSF) production in the bone marrow [108]. Increased M-CSF results in altered monocyte production within the bone marrow with an increase

in the proportion of $CD14^+$ $CD16^+$ monocytes produced. In healthy individuals, this phenotype forms approximately 6% of total blood monocytes. However, in late-stage AIDS this increases to around 16% and in AIDS subjects with dementia it is increased further to 37%. These cells exhibit features of tissue macrophages, are more phagocytic than $CD14^+CD16^-$ cells, express higher levels of TNFα, IL-1, and major histocompatibility complex (MHC) class II antigens [120], and are perceived to be highly neuroinvasive. It is suggested that an influx of these cells into the CNS late in AIDS may cause the damage associated with HAD. It may be that these cells also carry virus into the brain, reseeding the brain before the onset of HIVE, but the most likely cause of damage is through the induction of neuroinflammation. There is some neuropathological evidence to support this model. Fischer-Smith *et al.* have reported an increase of $CD14^+$ $CD16^+$ monocytes in the brains of AIDS subjects with HIVE and dementia. It is suggested that these $CD14^+$ $CD16^+$ monocytes have recently entered the brain from the blood; this assumption is based on the expression of both CD14 and CD45 by these cells. CD14 and CD45 are normally detected by standard immunohistochemistry only on perivascular macrophages and not on resident microglia. This implies that any observed increase in these cells results from an influx from the blood across the BBB. Further evidence to support this concept comes from the absence of significant cell proliferation amongst cells of this phenotype in HAD. However, other studies have shown that the use of signal amplification techniques reveals low level antigen expression of CD14, CD16, and CD45 on resident parenchymal microglia, not only in HAD but also in control brains. These findings suggest that resident cells may simply upregulate expression of these particular cell surface markers in response to certain stimuli rather than CD14/16/45 positivity representing only recently imported cells. Microglia *in vitro* can be induced to express these macrophage antigens, which is unsurprising given that both microglia and macrophages originate from the same bone marrow progenitor cell lineage. If upregulation of CD14 and CD45 does occur on resident cells then this may inflate estimates of the influx of monocyte/macrophages in HAD. The most probable explanation is that some influx does occur and that this then stimulates resident cells, driving phenotypic and morphological change.

Future perspectives

The advent of HAART therapy has greatly extended the life expectancy of infected subjects and improved the clinical scenario for many individuals. However, two major concerns remain. First, the toxicity of these drugs is high and as a result many individuals are unable to comply with therapeutic regimes. Second, the virus is not eradicated from the body, which leaves subjects with a chronic persistent viral infection the long-term consequences of which are at present unclear. There has always been concern that because of the major CNS involvement in HIV that infected subjects would be at high risk of developing neurocognitive disorders. In the post-HAART era, these concerns remain, and given the increased life expectancy and quality of life afforded by HAART, concerns over future cognitive functioning are perhaps more important now than ever. There are several studies which suggest that premature neuroaging may be a feature of those maintained long term on HAART. Green *et al.* have shown increased deposition of beta amyloid in HAART-treated subjects, while Anthony *et al.* have demonstrated elevated levels of hyperphosphorylated Tau in the hippocampus [121,122]. These are the two key proteins involved in CNS dysfunction in Alzheimer's disease, while hyperphosphorylated Tau is also associated with a number of other dementing disorders. Gelman *et al.* have also shown increased high-molecular-weight ubiquitin-protein conjugates, which are associated with aging in the CNS [123]. In conjunction with these neurodegenerative findings, ongoing neuroinflammation has also been reported particularly in the hippocampus [124]. This data points toward premature ageing of the CNS in HIV-positive HAART-treated

subjects, though it remains to be seen what impact this will have on individuals as they age with HIV and HAART.

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10

JC virus molecular biology and the human demyelinating disease, progressive multifocal leukoencephalopathy

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Introduction

JC virus (JCV) is a human neurotropic polyomavirus that causes the fatal demyelinating disease of the central nervous system (CNS) known as progressive multifocal leukoencephalopathy (PML). JCV has also been found in association with various tumors of the CNS and other tissues. This virus requires strong suppression of the immune system in order to thrive and is usually present in a latent state in immunocompetent individuals. With the emergence of AIDS in the 1980s, the incidence of PML surged. Recently, there has been renewed interest in JCV due to the occurrence of PML in patients receiving treatment with novel classes of immunosuppressive monoclonal antibody drugs such as natalizumab, which inhibits extravasation of T-lymphocytes, and rituximab, which targets mature B-lymphocytes. Here we review the molecular biology of JCV Section (1) and the pathophysiology of PML Section (2) with special emphasis on exciting aspects of current research into this increasingly important pathogen.

The molecular biology of JCV

Introduction to JC virus (JCV)

JCV is a small human DNA virus and contains a double-stranded covalently linked circular genome, 5130 base pairs in size. It is the causative agent of a demyelinating disease in the CNS known as progressive multifocal leukoencephalopathy (PML). It has an icosahedral capsid approximately 45 nm in diameter. Originally JCV was classified in the *Papovaviridae* family within the polyomavirus genus [1], but later the polyomaviruses were recognized as a viral family separate from the papovaviruses by the International Committee on the Taxonomy of Viruses. Antigenic and structural studies have indicated that JCV is closely related to the polyomaviruses BK virus (BKV) and SV40. This family of viruses has been under intense investigation for many years for two main reasons. First, their genome serves as a miniature model system to study many aspects of DNA structure, replication, and transcription of more complex mammalian genomes. Second, under certain conditions, their tumorigenic proteins, large T antigen and small t antigen, transform cells both in tissue culture and experimental animals. Such a transformation process provides a model system to understand the progression of human tumors. Furthermore, the detection of the JCV genome in a variety of human tumors raises the possibility that JCV may play a role in induction of human tumors [reviewed by 2,3,4]. As a result, the study of these viruses, particularly SV40, has greatly increased our understanding of many facets of the molecular biology of mammalian systems including gene transcription and regulation, DNA replication, DNA structure, and DNA-protein interactions [5]. JCV was first isolated from brain tissue of a PML patient by Padgett *et al.* in 1971 [6]. The brain tissue was used as a source of inoculum to infect primary cultures derived from human fetal brain. The

virus was then successfully isolated from these long-term cultures mainly consisting of glial cells [6]. This was the first direct evidence suggesting that a neurotropic virus was associated with the incidence of PML. Soon after its isolation, the oncogenic potential of the virus was tested both in tissue culture and experimental animals. In particular, animal model studies showed that it induces tumors in tissues of neural crest origin [7,8,9,10,11]. Recent findings regarding the detection of JCV genome in a variety of human tumors raise the possibility that JCV may be associated with the induction of human tumors [3,12,13,14,15,16,17,18].

JCV lytically infects oligodendrocytes, the myelin-producing cells of the CNS, and leads to the neurodegenerative disease PML, which develops mostly in patients with underlying immunosuppressive conditions, including lymphoproliferative diseases, AIDS, and Hodgkin's lymphoma [19,20,21]. In a small number of cases, however, PML may also be found to affect individuals with no underlying disease [19,21]. PML used to be a rare complication of middle-aged and elderly patients with lymphoproliferative diseases. However, due to the AIDS epidemic, it is now a commonly encountered disease of the CNS in patients of different age groups. This noticeable increase in the incidence of PML in HIV patients suggests that human immunodeficiency virus (HIV) infection may directly or indirectly participate in the reactivation of JCV and the induction of PML. Recent estimates indicate that the incidence of PML in HIV-seropositive patients reached 5% [19,22,23,24]. Furthermore, reactivation of JC virus in multiple sclerosis (MS) or Crohn's disease patients, who were treated with both interferon β-1A and natalizumab, a selective adhesion-molecule blocker, suggests a possibility that such a treatment would be a risk factor in induction of PML in MS and Crohn's disease patients [25,26,27,28,29,30].

Seroepidemiological data indicates that a majority (70–80%) of the world's population is infected with JCV [19,21,31] and JCV establishes a persistent infection in the kidneys (latent infection) after a subclinical primary infection. Recent reports indicate that peripheral blood B lymphocytes, hematopoietic progenitor cells, and tonsillar stromal cells could also harbor JCV and therefore these sites can be considered additional potential sites for JCV latent infection [32,33,34,35,36,37].

Genomic organization of JCV

The JCV genome is composed of a bidirectional regulatory region and two coding regions (Figure 10.1) [1]. The regulatory region contains the origin of DNA replication and promoter/enhancer elements for viral early and late genes. The coding regions can be divided into early and late regions. The early coding region primarily encodes two regulatory proteins, small and large T antigen, although recent findings indicate that this region also encodes three additional small peptides called T' proteins [38]. The late coding region encodes structural capsid proteins (VP1, VP2, and VP3) and a small regulatory protein known as agnoprotein. Structural studies demonstrated that the genome of JCV exhibits substantial sequence homology to two closely related polyomaviruses, BKV and SV40 in coding regions. However, the sequences within the regulatory region of JCV significantly diverge from those of BK virus and SV40 [1].

Studies over the years have shown that the regulatory region of JCV mainly confers the tissue-specific expression of JCV genome, although large T antigen (LT-Ag) also contributes to this specific expression [39,40,41,42,43,44,45,46,47,48]. In addition, tissue-specific cellular transcription factors have been shown to contribute to the neurotropic expression of JCV [40,41,46,47,49,50,51,52]. *In vivo* and *in vitro* transcription assays as well as cell-fusion experiments have clearly indicated that there are positively and negatively acting transcription factors involved in regulating the expression of the JCV genome in glial and nonglial cells, respectively [40,41,49,53]. For instance, studies conducted by Beggs *et al*. showed the suppression of JCV early promoter in glial cells when fused with fibroblasts to form heterokaryons. This suppression suggests the presence of positively and negatively acting factors in glial cells and in non-glial cells, respectively [53].

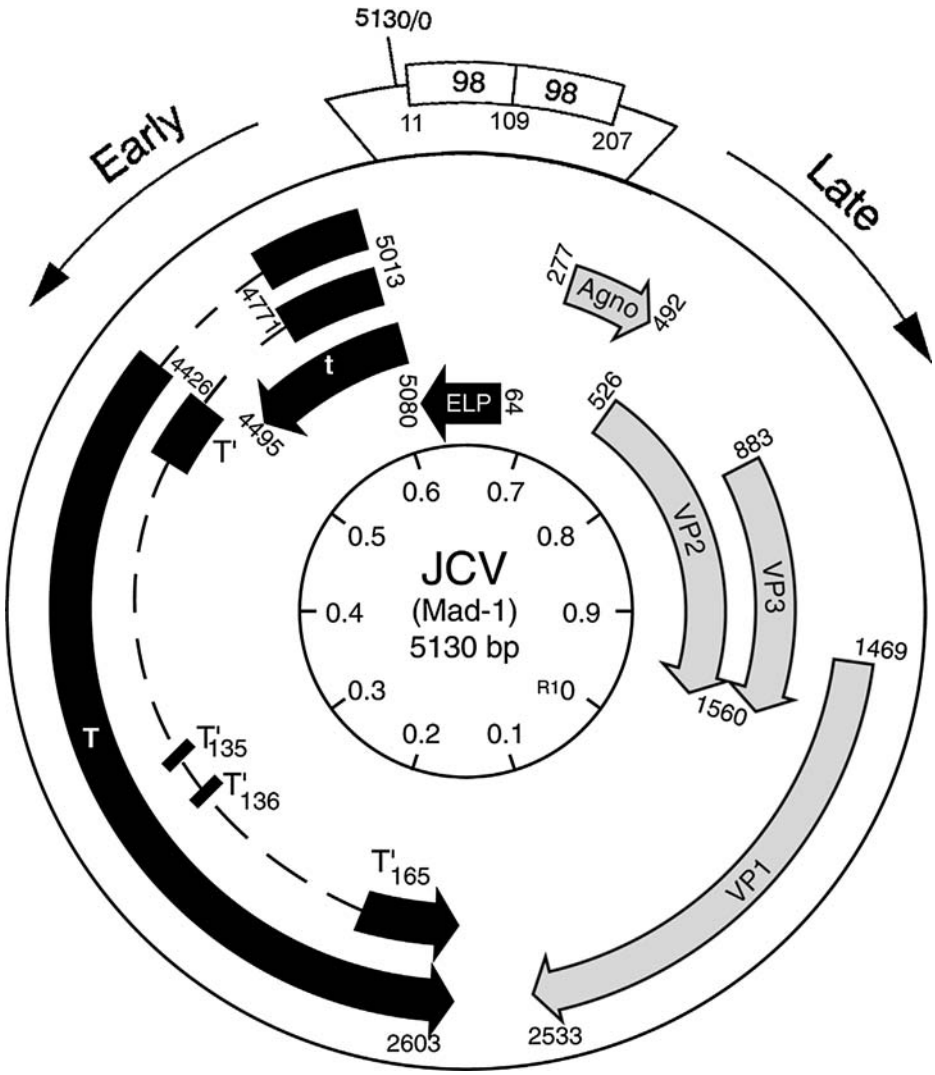

Figure 10.1. Genomic organization of JCV. The JCV genome is expressed bidirectionally. The early coding region encodes LT-Ag, Sm t-Ag, and the T' proteins (T'_{135}, T'_{136}, and T'_{165}). The late coding region encodes agnoprotein and the three capsid structural proteins VP1, VP2, and VP3. The noncoding regulatory region is located between the two coding regions and contains origin of replication (*ori*) and promoter/enhancer elements for early and late promoters.

The noncoding regulatory region of JCV

The regulatory region of JCV is hypervariable in nature and yet largely confers the tissue-specific expression of viral early and late genes [54,55,56]. Comparison of the regulatory sequences among a number of JCV isolates revealed that the hypervariability is mostly confined to the 98 base pair tandem repeat region (Figure 10.2). Based on the variations including deletions and duplications, JCV isolates are classified into two classes or groups [31,54,55,56]. The class I viruses are characterized by the presence

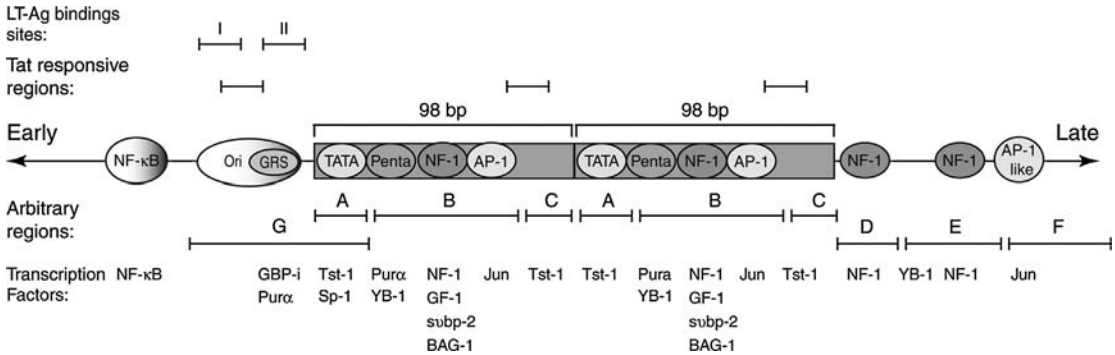

Figure 10.2. Control region of JCV. The control region of JCV contains the origin of replication (*ori*) and promoter/enhancer elements as indicated. Two 98-bp tandem repeats are characteristic of the JCV control region of Mad-1 strain isolated from a PML patient. Promoter/enhancer elements (nuclear factor kappa b binding element [NF-κB], pentanucleotide element [Penta], nuclear factor 1 binding element [NF-1], GC-rich element [GRS], activating protein 1 binding element [AP-1]) serve as binding sites for many transcription factors as illustrated at the bottom of the schematic. The approximate location of the LT-Ag antigen-binding sites and that of Tat-responsive elements are also indicated.

of the 98 base pair tandem repeat within its regulatory region. The prototype strain of JCV, Mad-1, belongs to class I. The class II viruses contain strains that exhibit variations from the regulatory region of the class I with deletions and insertions. The 98 base pair repeats appear to vary in size, and distal repeat with respect to the origin of replication lacks the TATA box sequences. There tends to be a full-length or partial duplication of the 23 bp sequence element usually occurring at nucleotide 36 of the first tandem repeat [31]. Although the mechanism by which these deletions and duplications occur and give rise to new strains of JCV remains unknown, it is generally accepted that both classes are derived from a common ancestral form such as JCV archetype strain, mainly detected in the kidney [57]. It is postulated that alterations within the regulatory region of JCV archetype result in a new strain with new features. The new strain of JCV is then capable of replicating in new cells and tissues [31].

The control region of JCV encompasses the origin of viral DNA replication and multiple *cis*-acting regulatory motifs that regulate transcription of the viral early and late genomes. The origin of DNA replication is a 68-bp element located between the NF-κB motif and the first tandem repeat of JCV Mad-1 strain. It exhibits substantial homology with those from SV40 [31,58]. The transcriptional regulatory regions are composed of two complete 98-bp tandem repeats (Mad-1 strain), each containing a TATA box located in the early side of the individual repeat. The first TATA box with respect to origin of DNA replication is involved in the positioning of the transcription start sites for viral early genes [31,58,59,60]. The second TATA box does not appear to have a similar function for the viral late genes. The remaining regions within the 98-bp repeats were shown to confer crucial *cis*-acting elements that serve as binding sites for several transcription factors and contribute to tissue tropism [21,31,52,58]. The major *cis*-acting elements and transcription factors that bind to the control region are illustrated in Figure 10.2.

JCV regulatory and structural proteins

The JCV genome encodes both regulatory and structural proteins. The early coding region encodes only regulatory proteins, including large T antigen (LT-Ag), small t antigen (Sm t-Ag), and the T' spice variants (Figure 10.1). The open reading frames for these proteins are located on the early side of the viral promoter with respect to origin of DNA replication and are transcribed counterclockwise from the opposite strand relative to the late genes (Figure 10.1). Viral

transcripts of LT-Ag and Sm t-Ag are produced as a result of differential splicing of the early pre-mRNA and share a common amino terminus [31]. An additional small predicted protein is also thought to be expressed from the early transcripts termed JCV early leader protein (ELP) [61]. However, the identity and the function of this protein have yet to be determined. T' protein transcripts are also produced as a result of alternative splicing [31,62] of the early pre-mRNA. In contrast to early coding region, the late coding region of JCV encodes both regulatory (agnoprotein) and structural (VP1, VP1, and VP3) proteins [1].

Large T-Antigen

Large T-Ag is a large multifunctional phosphoprotein involved in transactivation of viral late gene and suppression of its own gene expression by an autoregulatory loop [63,64]. It is also required for initiation of JCV DNA replication [59,60,65]. It is a 688-amino-acid-long protein and shows 70–80% sequence identity to the LT antigens of SV40 and BKV. Studies with SV40 LT-Ag showed that this protein harbors many functional domains that are critical for viral life cycle as well as for the cell transformation [66,67]. Figure 10.3 schematically illustrates different functional domains of SV40 LT-Ag. The amino terminus of LT-Ag contains two distinct domains important in cell transformation. The amino terminus of LT-Ag contains the J domain, which functions as a chaperone for proper folding of protein complexes. LT-Ag and Sm t-Ag share first 82 amino acids of their amino terminus. The next functional region at the amino terminus of LT-Ag is the binding region to pRb and the pRb family members, p107 and p130 [68,69]. By sequestering these proteins, LT-Ag influences the cell cycle progression [70]. Although the function of p107 and p130 in cell cycle regulation remains unclear, the mechanism of action of tumor suppressor protein pRb at the G1 checkpoint has been well-demonstrated. pRb forms an inactive complex with a transcription factor E2F and arrests cells at the G1 phase of cell cycle. When specific cyclin-dependent kinases phosphorylate Rb, it releases transcription factor E2F. E2F in turn transactivates S phase specific gene promoters and causes the cell to progress into S phase. When bound to Rb, LT-Ag inactivates the regulatory function of pRb, which allows unscheduled S-phase entry, thereby establishing favorable conditions for cellular transformation [70]. LT-Ag also targets another tumor suppressor protein, p53. p53 like pRb is a tumor suppressor protein and plays a critical role in cell cycle progression at the G1 checkpoint. p53 induces apoptosis when over-expressed in cultured cells [71,72]. p53 is found mutated or lost in up to 50% of all human cancers, which emphasizes the

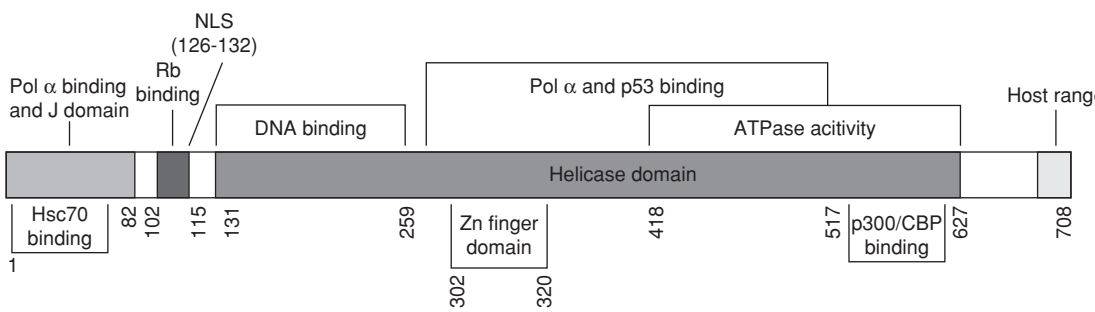

Figure 10.3. Schematic representation of functional domains of SV40 LT-Ag. Indicated are the approximate minimal regions of LT-Ag that retain binding activity to polymerase α-primase (Pol α), tumor-suppressor proteins Rb and p53, human heat shock protein 70 (hsc70), and coactivators p300 and CBP. The DNA-binding domain, ATPase activity domain, nuclear localization signal (NLS) domain, helicase domain, host range domain, Zn finger domain, and J domain are also depicted.

importance of its functional loss in induction of tumors [73,74]. SV40 LT-Ag possesses p53 binding sites near its carboxy-terminal end. By binding to p53 at these sites, LT-Ag inhibits p53-mediated activities, including arresting cells that have mild DNA damage in G1 or G2/M phases of the cell cycle for DNA repair and eliminating the cells that have extensive DNA damage by apoptosis. Under such circumstances, the cells with damaged DNA go through the cell cycle stages without DNA repair. This may result in accumulation of cellular mutations and increased genomic instability, which may subsequently lead to tumor induction.

LT-Ag, in addition to targeting cellular tumor suppressor proteins, also targets nuclear acetylases including CREB-binding protein (CBP), P/CAF, and p300. These regulatory proteins function as cofactors and play important roles in transcription and post-translational modification of cellular tumor suppressor proteins, including pRb and p53. LT-Ag interacts with these proteins through multiple regions [75,76] and inactivates their important cellular functions. This is also thought to contribute to deregulation of cell cycle progression and tumor induction.

Furthermore, one of the essential functions of LT-Ag is to initiate the viral DNA replication. The helicase domain of LT-Ag is critical for this function (Figure 10.3) and is located at the center of the protein and makes up a large portion of the protein. It binds to the *ori* at LT-Ag binding site II in a double hexameric form and unwinds it in an ATP-dependent manner [77,78]. The DNA template is then more accessible to other factors that are required for DNA replication, such as the DNA polymerase α/DNA primase complex and single-stranded DNA binding protein [77,78,79]. Since JCV LT-Ag shows significant sequence homology (70%) to SV40 LT-Ag, it is reasonable to suggest that JCV LT-Ag functions in a similar manner and contains similar functional domains.

Besides these activities, LT-Ag is also an oncogenic protein. Mechanistically, this activity appears to be, at least in part, mediated by the inactivation of tumor suppressor proteins, including p53, pRb, and p130. Co-immunoprecipitation assays using cellular extracts from JCV-transformed glial cells show LT-Ag complex formation with pRb, p53, and p107 [80]. A report by Rencic *et al.* also [16] suggests a role for LT-Ag in the induction of oligoastrocytomas in an immunocompetent patient. JCV LT-Ag has also been shown to interact with cellular and viral proteins including YB-1, Purα, JCV agnoprotein, and insulin receptor substrate 1 (IRS-1) [81,82,83,84]. IRS-1 is the major signaling molecule for the type I insulin-like growth factor receptor (IGF-IR) [85]. In addition, recent reports also indicate a possible communication between JCV LT-Ag and the Wnt-signaling pathway in induction of tumor progression because cells expressing LT-Ag were also found to be expressing β-catenin and its partner LEF-1 at higher levels. These two proteins were previously shown to be important in tumor progression. Induction of these proteins by LT-Ag expression further emphasizes the critical role of LT-Ag in tumor formation [86].

Small t antigen

JCV Sm t-Ag is a small protein consisting of 172 amino acids, 82 amino acids of which are shared with the N-terminal sequence of LT-Ag. Sm t-Ag is produced by the alternative splicing of the JCV early pre-mRNA. Sm t-Ag shows 30% and 18% sequence homology to SV40 and BKV Sm t-Ags, respectively. Amino acid alignment of all three proteins shows that most of the divergent sequences are localized toward the central portion of the proteins

Unlike LT-Ag, the function of Sm t-Ag in JCV life cycle and in cellular transformation is largely unknown. However, functional studies with SV40 Sm t-Ag showed that it plays an important role in permissive and nonpermissive infections and enhances the ability of SV40 LT-Ag to transform cells [87,88,89,90,91,92]. It is also believed that Sm t-Ag antagonizes LT-Ag-induced cellular apoptosis and thereby contributes to more efficient transformation of rat embryo fibroblasts [93]. In addition, transgenic animals created with a Sm t-Ag deletion mutant of SV40 genome developed tumors almost exclusively in highly mitotic tissues such as lymphoid organs [94,95], suggesting that Sm t-Ag plays a significant role in tumor induction in nonproliferative tissues.

This tumorigenic potential of Sm t-Ag is linked to the inhibition of protein phosphatase 2A (PP2A), a member of an abundant family of serine/threonine phosphatases [96,97,98]. Recent reports indicate that this interaction may lead to a change in the priority in substrate selection for PP2A and this may be involved in the mechanism whereby Sm t-Ag contributes to cell transformation [99]. In addition, several growth-promoting pathways have been found to be altered in the presence of Sm t-Ag [89,92,98], including activation of phosphatidylinositol 3-kinase [100], protein kinase Cζ [100], and mitogen-activated protein kinase [98]. Furthermore, expression of Sm t-Ag in cells was observed to be associated with the phosphorylation states of Akt [101] and Shc [102] and with stimulation of AP-1 [103].

T′ proteins

In addition to LT-Ag and Sm t-Ag, the JCV early coding region also encodes three additional variants, T'_{135}, T'_{136}, and T'_{165} [62], which were originally assumed to be LT-Ag degradation products. These three T′ proteins share their N-terminal 132 amino acids with LT-Ag (Figure 10.1). While the C-termini of T'_{135} and T'_{136} are unique, T'_{165} also shares its C-terminal 33 amino acids with LT-Ag. It appears that the unique nature of the C-termini of these T′ proteins renders them so as to possess a different set of activities. Recent studies on this phenomenon suggested that T'_{136}, for example, exhibits a different phosphate turnover rate than LT-Ag [99]. In addition, these variants differentially enhance LT-Ag-mediated DNA replication [104] and differ in their ability to interact with human pRb, p107, and p130 *in vitro*. These findings suggest that the T′ proteins may make unique contributions to the viral life cycle as well as to cell cycle progression and transformation [38].

Agnoprotein

Agnoprotein is a small (71-amino-acid long), basic, and multifunctional phosphoprotein, which is encoded by the leader sequences of the viral late transcript. It is a cytoplasmic protein with high concentrations accumulated in the perinuclear area of infected cells [84]. Agnoprotein, like Sm t-Ag and LT-Ag, shows significant homology to its counterpart in BKV and SV40 (approximately 70%), but divergent sequences are clustered toward the C-terminus of each protein. Although there has been significant progress in understanding the functions of this protein, the complete picture with respect to its role in the JCV life cycle remains elusive. It has been shown that agnoprotein is involved in JCV gene transcription and replication through the interaction with JCV LT-Ag and the cellular factor, YB-1 [84,105]. In addition, it has also been shown that this protein exhibits the ability to dysregulate cell cycle progression in that cells that stably express agnoprotein largely accumulate at the G2/M phase of the cell cycle [106]. Furthermore, it has been suggested that agnoprotein is involved in the transport of virions from nucleus to cytoplasm through the interaction with cellular proteins, FEZ1 and HP1α [107] and thereby facilitates the propagation of JCV [108]. Moreover, this protein appears to be target of cellular kinases, including protein kinase C (PKC) during the infection cycle. PKC phosphorylates Ser7, Ser11, and Thr21 on agnoprotein and these sites are critical for its function because mutational analysis of these three sites showed that JCV cannot continue its life cycle when these sites are mutated to alanine [109]. Recent studies also suggest that agnoprotein may be involved in cross-regulation of HIV-1 transcription [110].

Structural capsid proteins (VP1, VP2, and VP3)

JCV structural proteins are, like agnoprotein, encoded by the late transcripts by alternative splicing [111]. These three proteins form the viral capsids and function in the attachment, adsorption, and penetration of the virus to the host cells. Studies from SV40 capsid proteins showed that VP1 forms the outer shell of the virion and VP2/VP3 are located in the inner layer. The viral DNA genome and histone proteins form the core of the virions [112]. It is thought that the assembly of these virions takes place in two phases [112]. In the first phase, pentamerized VP1 associates with VP2/VP3 in the cytoplasm soon after the proteins are synthesized and

are then transported into nucleus. The second phase of the virion assembly begins once the capsid proteins enter the nucleus. In a stepwise model of virion formation, the capsid proteins are sequentially added to and arranged on the viral genome through interactions between the capsid protein and viral genome. This results in the condensation and packaging of the viral genome into the capsids. Studies from SV40 also showed that capsid proteins specifically interact with the viral genome with the help of the cellular transcription factor Sp1 [113,114] and the viral packaging signal (ses) of the SV40 genome, which contains the encapsidation signal of the SV40 DNA and is located within the regulatory region of the viral genome [115,116]. These findings suggest that specific DNA binding activity of capsid proteins as well as specific protein-protein interactions between individual capsid proteins contribute to the virion assembly. VP2 and VP3 contain overlapping regions. The last 225 amino acids of VP2 completely overlap with VP3. The VP1 coding region starts with the VP2/VP3 C-terminal region but does not overlap with them at the amino acid level (Figure 10.1).

The life cycle of JCV

As observed with other small nonenveloped DNA viruses, the infection process of JCV begins with the attachment of the viral particles to cell surface receptors. Recent reports indicate that JCV enters the susceptible cells through clathrin-dependent endocytosis. It appears that the serotonin receptor $5HT_{2A}$ as well as α(2–6)-linked sialic acid is critical for the attachment of JCV to the cell surface [117,118,119,120,121,122]. Figure 10.4 highlights the events of the life cycle of a polyomavirus. The next step in viral infection is the transport of viral particles into the nucleus and uncoating of capsid proteins to give exposed viral nucleosomes, which subsequently leads to the expression of the viral early genome within the nucleus. Viral early transcripts undergo splicing in the nucleus before they are transported to the cytoplasm for translation. Translation of the early transcripts leads to the production of the early regulatory proteins, LT-Ag, Sm t-Ag, and the T' proteins. LT-Ag is then transported to the nucleus where it initiates viral DNA replication and transactivates the viral late promoter. The splicing and translation of late transcripts result in the production of the regulatory protein, agnoprotein, and three structural capsid proteins; VP1, VP2, and VP3. Capsid proteins are then transported back to nucleus for virus assembly. In this process, the capsid proteins are sequentially added to and arranged on the viral genome through interaction between the capsid protein and viral DNA, which leads to the packaging of the viral DNA into the capsids. The final step in the viral life cycle is the release of the virions from infected cells. The process of how virions are released from the infected cells is not known; however, it is likely that this takes place upon the destruction of infected cells by the cytopathic effect of the virus.

Expression of viral genes

Promoter-swapping experiments and somatic cell hybridization studies have indicated that *cis*-acting elements present within the control region of JCV as well as the tissue-specific cellular factors that are critical for the narrow host range and neurotropism of JCV. Studies in tissue culture and promoter-swapping experiments using transgenic mice models [123] have indicated that restriction of expression of the early JCV LT-Ag may largely contribute to this narrow host range and cell-specific expression of JCV genome [31,46,65,124]. Reporter gene assays using the regulatory region of the JCV in *in vivo* and *in vitro* transcription experiments [39,49] have shown that expression of the viral early promoter is significantly higher in glial cells than in nonglial cells. In addition, studies have also shown that viral DNA replication and induced late gene expression can only occur when the JCV regulatory protein LT-Ag is provided to the system [64]. Somatic cell hybridization studies between mouse fibroblast cells and transformed hamster glial cells expressing the viral LT-Ag have suggested the presence of positive regulatory factors in glial cells and negative regulatory factors in nonglial cells [53].

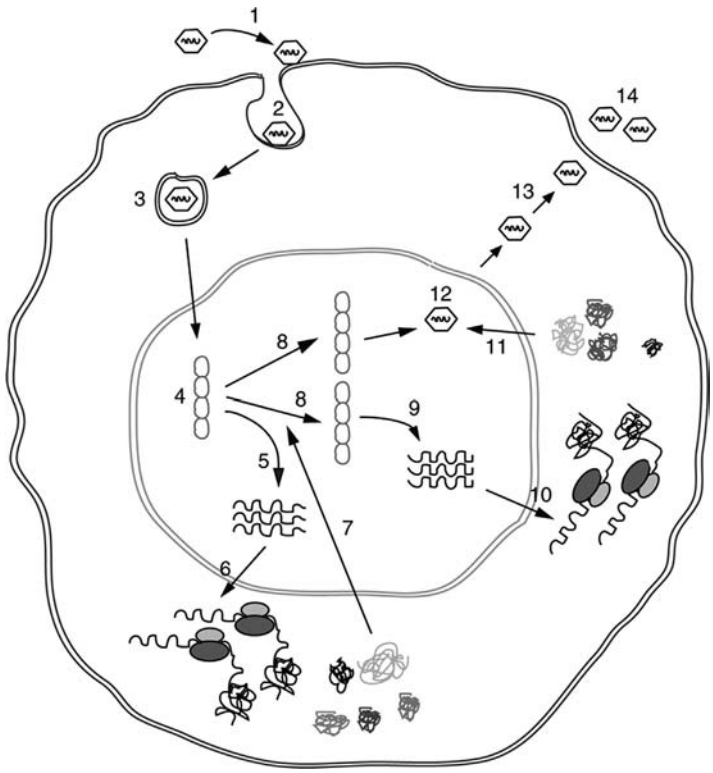

Figure 10.4. Life cycle of JCV. Steps in the life cycle of JCV are indicated by numbers as follows: (1) adsorption of virus to cell surface receptors; (2) entry by clathrin-dependent endocytosis; (3) transport to the nucleus; (4) uncoating; (5) transcription of the early coding region; (6) translation of early mRNAs to produce the early regulatory proteins LT-Ag, Sm t-Ag, and T'_{135}, T'_{136}, and T'_{165}; (7) nuclear localization of LT-Ag; (8) replication of viral genomes; (9) transcription of the viral late genes; (10) translation of viral late transcript to produce agnoprotein and the capsid proteins (VP1, VP2, and VP3); (11) nuclear localization of capsids; (12) assembly of viral progeny in the nucleus; (13) release of virions by an unknown mechanism; (14) released virions.

The position of the transcription start sites for JCV early and late gene transcription varies depending on the position of the TATA box element in the 98 base pair repeats and the stage of viral gene expression [63,125,126,127]. Results from S1 nuclease and primer extension assays have shown that the major early mRNA start site is mapped to approximately 25 nucleotides downstream from the first TATA box. The minor transcription start sites are, however, initiated with respect to near the first TATA box. In addition, a shift in the early transcription start site from major to minor sites was observed as the viral lytic cycle progresses toward late gene expression. These observations suggest that the first TATA box is important for positioning the transcription start sites for the early gene [125,126], but the second TATA box appears to play a negligible role in this regard. Additional studies with respect to localizing the transcription start sites for late gene were also performed [125], and four major and three minor sites were identified spanning the regulatory region of JCV. Although the TATA boxes do not seem to have a role in positioning the transcription start sites for the late gene, an ATACCTA sequence located within the late region of the regulatory sequences appears to be involved in specifying the transcription start site for the late gene.

Cellular factors that are involved in JCV gene expression

A number of ubiquitous and cell-specific factors as well as viral regulatory proteins, including LT-Ag and agnoprotein, were shown to be involved in the expression of the viral promoters. These factors directly or indirectly interact with the *cis*-acting elements present within the viral control region. As stated earlier, LT-Ag is a multifunctional phosphoprotein, involved in both late gene expression and in LT-Ag-dependent viral DNA replication. Along with the cellular factors that are required for DNA replication, LT-Ag binds to the origin of DNA replication, unwinds it, and initiates DNA replication in a bidirectional manner [59,128]. Studies also show that LT-Ag regulates its own transcription by an autoregulatory loop and plays a major role in the transcriptional switch from viral early to late gene expression [63,64].

Computer-aided predictions and experimental analysis of the regulatory region have revealed the presence of multiple potential and authentic *cis*-acting elements to which transcription factors bind and regulate JCV expression. These elements include NF-κB, GRS, penta, NF-1, and AP-1. In addition, regulatory region also contains Tat-responsive elements and LT-Ag-binding regions. Furthermore, for the simplicity of the description of JCV regulation by transcription factors, the control region of JCV is divided into arbitrary regions including A, B, C, D, E, F, and G. NF-κB, an inducible transcription factor, binds to NF-κB elements located on the left side with respect to the origin of DNA replication and modulates gene expression from viral early and late promoters [129]. NF-κB represents a large family of transcription factors that are inducible in response to a wide variety of extracellular stimuli including phorbol ester and cytokines. While constitutively expressed subunits p50 and p52 activate transcription from D domain [130], subunit p65 activates transcription from the NF-κB motif [129]. NF-κB family members p50/p65 were also shown to indirectly influence JCV gene transcription through a 23-bp element present within the regulatory regions of many JCV variants [131]. GBP-I, another inducible factor, interacts with GRS sequences present within the G region of the viral promoter and modulates viral late promoter activity [51]. Tst-1, a member of the well-characterized tissue-specific and developmentally regulated POU family of transcription factors, has also been shown to regulate JCV transcription [132,133]. Tst-1 was found to have distinct binding sites at the A and C regions and was shown to transactivate viral early and late gene promoters by interacting with the A region. In addition, the physical interaction of Tst-1 with JCV T-Ag was shown to lead to synergistic activation from both viral early and late promoters [132]. A ubiquitously expressed and GC-rich binding transcription factor, Sp-1, was also found to stimulate JCV gene expression, particularly from the early promoter [134,135]. The control region of JCV also contains several binding sites for a transcription factor named nuclear factor 1 (NF-1) [52], and these binding sites are scattered and localized to regions B, D, and E. NF-1 has been shown to be involved in both viral transcription and replication [136,137,138,139]. The extensively studied cellular factors that interact with the B region include a 45-kDa protein [45], GF-1 [140], YB-1, Purα [141], and AP-1 [41,142]. GF-1 is a partial recombinant protein cloned by Kerr and Khalili [140] from a human fetal brain expression library and represents a human homolog of the cloned murine Sμbp-2 protein [143]. The binding specificity of GF-1 is similar to that of NF-1. GF-1 was found to transactivate viral early and late promoters [140]. YB-1 and Purα were also found to interact with this region and are involved in both viral gene expression and replication through interaction with LT-Ag [81,83,144]. The control region of JCV also contains binding sites for YB-1 localized to region E [130]. Recently, a novel Bcl-1-interacting protein, BAG-1, was cloned from p19 embryonic carcinoma cells using the JCV-NF-1 binding site as a probe. BAG-1 is expressed ubiquitously in neuronal and nonneuronal cells and regulates JCV early and late promoters through the NF-1 binding site [145]. Another cellular factor that interacts with the B region is c-Jun, which is a member of the AP-1 family of transcription factors. The members of this family are known as the immediate

early inducible proto-oncogenes. c-Jun's binding activity with the B region appears to be modulated by the NF-1 transcription factor. NF-1 binds to adjacent NF-1-like sequences [41] and activates transcription from JCV gene promoters [41,142]. It was also recently shown that c-Jun is phosphorylated during JCV replication cycle and functionally interacts with JCV LT-Ag [142,146]. In addition to its own regulatory proteins, the JCV genome was also shown to be cross-regulated by the regulatory proteins that are encoded by other viruses including Tat from HIV-1 and immediate-early transactivator 2 (IE2) from cytomegalovirus (CMV) [47,147].

JCV DNA replication

SV40 genome has been used as a model system to understand viral as well as eukaryotic genome replication in *in vivo* and *in vitro* systems. Most of the work done on SV40 DNA replication is likely to be applicable to JCV because there is little difference among the sequences of both viral origins. In addition, LT-Ag of both viruses exhibits approximately 70% homology at the amino acid level. The functional organization of both virus DNA replication elements are similar in that they consist of three functional elements: (1) the core origin (*ori*), which is necessary and sufficient for viral DNA replication, (2) auxiliary element 1, *aux*-1 (LT-Ag binding site I, BSI), and (3) auxiliary element 2, *aux*-2 (a GC-rich segment containing six copies of the binding site for the cellular transcription factor Sp1 and the 72-bp tandem repeats that represent the SV40 enhancer). JCV uses the proximal part of its first 98 base pair repeat as *aux*-2 [31,58,59,137,148]. The core sequences in both viruses are highly conserved [58,59,137], but the sequences in the auxiliary elements diverge considerably.

The core origin is composed of LT-Ag binding site II (BSII), imperfect palindrome (IP), and A+T-rich region (AT) (Figure 10.5). The sequences within BSII, nucleotides 30 to 38 on the SV40 genome, appear to adopt a bent DNA structure. It is thought that this structure facilitates the helicase activity of LT-Ag to enlarge the replication bubble, which is initiated in the inverted repeats [77]. In contrast, the corresponding sequences on JCV seem to adopt a non-B conformation, which is thought to contribute to an

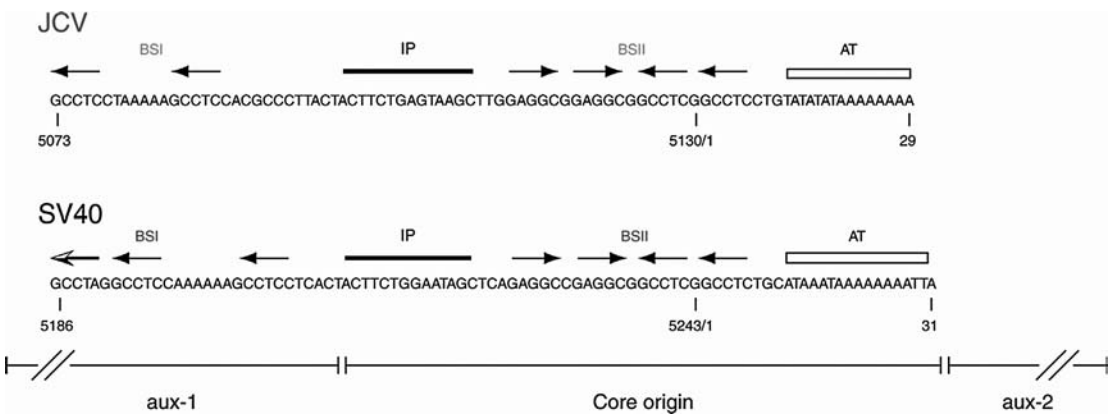

Figure 10.5. Comparison of the JCV (Mad-1) and SV40 (776) origins of replication. The nucleotide sequences of each viral core origin and a portion of *aux*-1 are shown. The binding sites (BSI and BSII) for LT-Ag contain multiple copies of the pentanucleotide sequence indicated by arrows. A variant consensus sequence in SV40 is represented by a hatched arrow. The imperfect palindrome (IP) and AT-rich (AT) regions of *ori* are denoted by an open and closed bar, respectively. The nucleotide positions are indicated under each sequence. Numbering begins at nucleotide 1 at the center of BSII and continues around the circular genome and ends at nucleotide 5130 (JCV) or 5243 (SV40).

inefficient interaction of LT-Ag with its own origin of replication. Deletion mutational analysis of this region showed that it is responsible for the reduced replication function of JCV LT-Ag [59,137].

Three components are necessary for viral DNA replication: (1) the origin of viral DNA replication, *ori*, (2) LT-Ag, and (3) host replication factors. The SV40 origin of DNA replication was identified as a 88-bp segment between nucleotides 5186 and 31 on the viral genome [77], and several known transcription factors bind to sequences within these elements and stimulate viral DNA replication as well as transcription [149]. The process of viral DNA replication is initiated by LT-Ag, which binds to *ori* at BSII in a hexameric form and unwinds the DNA with its intrinsic ATP-dependent DNA helicase activity [77,78]. The DNA template is then more accessible to other factors required for DNA replication, such as the DNA polymerase α/DNA primase complex and others [77,78,79]. Subsequently, the replication process proceeds bidirectionally. In addition, beside LT-Ag, three JCV early proteins, T'_{135}, T'_{136}, and T'_{165}, which are produced due to the alternative splicing of the early precursor mRNA, have also been shown to be involved in modulation of LT-Ag-mediated viral DNA replication [58,62,104].

JCV molecular biology: Overview, currrent research, unanswered questions, and future directions

Since the first cultivation of JCV from a PML patient in 1971 [6], we have learned much about the biology and neurotropic features of this virus. However, the molecular mechanisms of important aspects of its life cycle (viral entry, transport of the viral particles into nucleus, transcription, replication, and assembly and release of virions) continue to be elusive. The more that our knowledge of these processes expands, the more new complexities of JCV biology are revealed.

Although JCV shows significant sequence homology to human polyomavirus, BKV, and simian virus, SV40, it exhibits unique and differing features with respect to its biology. First, JCV is the only polyomavirus that displays neurotropic features, although its genome has been also detected in B lymphocytes [34]. In addition, it is the only polyomavirus that induces solid tumors in nonhuman primates [9,150]. Furthermore, JCV is a slow-growing virus compared to SV40. It takes several weeks to grow an equivalent amount virus in tissue culture compared to 2 to 3 days for SV40. JCV also exhibits marked divergence from SV40 with respect to the mode of viral entry into cells. SV40 enters cells through a caveola-dependent endocytosis pathway [151,152], whereas JCV enters by a clathrin-dependent receptor-mediated endocytosis pathway [122]. It appears that JCV uses the serotonin receptor $5HT_{2A}$ as well as α(2–6)-linked sialic acid to attach on the cell surface, and both receptors are critical for viral entry [117,118,119,120,122].

Understanding the transformation efficiency of LT-Ag is important to study the JCV-induced tumors in experimental animals and perhaps in humans. Recent reports indicate that Sm t-Ag of SV40 is critically important for the SV40 LT-Ag-mediated transformation of the human cells [99,115]. Similarly, JCV Sm t-Ag might be equally important for this reason in cell transformation, and we are engaged in further studies to explore this notion. Equally important are studies to further elaborate the functions of another JCV regulatory protein, agnoprotein. Agnoprotein expression occurs not only during acute JCV infection but also in some tumors that are associated with JCV [13,153]. Our recent analysis of Sm t-Ag phosphorylation mutants indicated that it is a critical protein for the progression of JCV infection. Conversion of Ser7, Ser11, and Thr21 of this protein to Ala by single mutation or in combination renders the virus incapable of sustaining its replication cycle [109]. This illustrates new aspects of agnoprotein molecular function and emphasizes the importance of agnoprotein in the JCV life cycle and possibly in JCV-associated tumors by regulating viral and cellular processes. We are undertaking further analysis of agnoprotein function to elucidate these regulatory events.

As we discussed earlier, JCV establishes a lifelong latent infection in early childhood in a majority of

the human population. The kidney appears to be the primary site for latent infection [21], although B lymphocytes and tonsillar tissue have been also considered as additional sites for latency [35,36,37]. The mechanisms of how JCV is reactivated from latent infection and how the regulatory region of JCV undergoes genetic rearrangement to yield a more virulent virus remain largely unknown and are actively under investigation. Another important research question to be addressed by investigators is to elucidate how virulent virus is transported to CNS to infect oligodendrocytes. Since JCV infections *in vivo* are restricted to specific cell types, including oligodendrocytes and astrocytes, it is critical to investigate whether there is a cell type-specific transcription factor or factors that permit or restrict JCV expression. It is also interesting to investigate whether these events involve specific functional interactions between viral regulatory proteins and host factors. Answers to such relevant questions will considerably enhance our understanding of the complex molecular biology of JCV and would shed more light on the molecular mechanisms governing the JCV infection cycle. This, in turn, would allow us to design effective therapeutics to intervene the infection cycle at an early stage before it causes more advanced disease.

The pathology and pathogenesis of progressive multifocal leukoencephalopathy

Historical notes

Although the first observations on cases of demyelination, which seem to correspond to PML, can be traced to a monograph on neurological diseases difficult to classify published in 1930 by Hallervorden [154], the characterization of the disease as a clinical and neuropathological entity was not done until 1958 by Åström and Richardson [155]. Their manuscript describes the clinical and histopathological aspects of the first three well-documented cases of PML and is still considered the classical description of the disease. At the time, the etiology of the disease was a mystery, and it was Richardson himself who first suggested the possibility of PML being an infectious disease [156]. The infectious hypothesis was later confirmed by elegant electron microscopy studies performed almost simultaneously by Zu-Rhein and Rubinstein, who showed viral particles in the nuclei of oligodendrocytes from PML cases [157,158,159]. However, the final confirmation proved difficult to demonstrate, since the virus would not replicate in conventional cultures due to its high neurotropism. The isolation and characterization of JCV was finally achieved from a human fetal brain culture of spongioblasts [6], opening a new chapter in the study of neurovirology.

Clinical and epidemiological aspects

Infection with JCV seems to be widespread in the adult population worldwide, as demonstrated by seroepidemiological studies in which as many as 92% of people exhibit hemagglutinating antibodies for JCV [160,161]. The same studies have proven that the primary infection occurs in early childhood, since the percentage of children exhibiting antibodies jumps from 10% between the ages of 1 and 5 to 65% between the ages of 10 and 14 [160]. Infection by JCV is subclinical and two ways of transmission have been proposed. The finding of JCV DNA sequences in B-lymphocytes from lymph nodes and tonsils of the Waldeyer ring first suggested the mode of transmission was through the respiratory tract [35]. In recent years, however, a second theory has emerged. JCV has been identified in urban sewage by nested polymerase chain reaction (PCR) [24], suggesting a possible fecal-oral transmission or at least infection through the digestive tract [162]. Detection of JCV genomic sequences in epithelial cells from the upper [17] and lower digestive tract [15] and in tumors from the esophagus and colon [14,163] further reinforces the hypothesis of infection through the digestive tract; however, both modalities of transmission are not mutually exclusive.

Once the primary infection has occurred, the virus remains in latent state in the kidney of healthy individuals. Under certain conditions it can be reactivated, as demonstrated by the recovery of viral particles from the urine of pregnant women or patients

undergoing therapy for renal transplant, indicating viral replication without any overt signs of disease. Once these conditions are resolved, viral replication stops and the urine is clear of viral load [164,165]. However, immunosuppressive conditions will result in the reactivation of JCV in the brain and in the development of PML. Before the AIDS pandemic, PML was considered a rarity, accounting for less than 250 cases reported in the literature, and mostly associated with other immunosuppressive conditions such as leukemias and lymphomas, carcinomas, and inflammatory and granulomatous diseases including tuberculosis, sarcoidosis, and lupus [166]. The first case of PML associated with AIDS was reported by Miller in 1982 [167]. In the present times, HIV infection is by far the most common underlying condition associated with PML, which is considered an AIDS-defining disease [168]. Several epidemiological studies have estimated the prevalence of PML among AIDS patients between 1–5% and even up to 10% [169,170]. The common factor shared by all of these predisposing conditions seems to be a T-cell deficiency.

The clinical signs and symptoms of patients with PML depend on the location of the demyelinated lesions. Since the most frequently affected location is the frontal lobe, cognitive impairments, including dementia, are the predominant symptoms [22]. Lesions that cause interruption of the corticospinal tract result in paresias, paresthesias, sensory loss, and motor dysfunctions. Plaques located in proximity to the angular gyrus, the Wernicke area, or the Broca area will result in dysarthria and speech aphasias [166]. Seizures, usually focal, have been reported in as many as 10% of the patients, resulting from subcortical affection predominantly of the temporal lobe [171]. Involvement of the basal ganglia and cerebellum results in gait disorders. Demyelination affecting the visual tract explains visual disturbances ranging from scotomas to homonymous hemianopsia to cortical blindness in cases of bilateral involvement. Lesions affecting the medial longitudinal fasciculus in the brain stem result in diplopia and oculomotor palsies, due to interruption of the pathways for the III, IV, and VI cranial nerves [172].

In a large clinical study of 154 patients with AIDS-associated PML ranging in age from 5 to 68 years, the most common signs and symptoms were headaches, limb weakness, and cognitive impairments [166].

Histopathological aspects

Gross examination of the brain from patients with PML characteristically reveals areas of softening and discoloration of the subcortical white matter. These plaques are irregular in shape, well-defined, with inconspicuous borders and a brown-yellowish color and soft in consistency. Lesions are usually multifocal, and in advanced cases they can become confluent, eventually leading to cavitation. It is not unusual to find lesions in different stages of progression in the same patient, from small to big and coalescent, to even cavitations in advanced cases. The demyelinated plaques exclusively affect the subcortical white matter, and the most frequently affected places are the frontal lobe, followed by parietal, temporal, and occipital. In severe cases plaques of demyelination can also be found in the cerebellum, basal ganglia, and even the brain stem, always affecting white matter tracts and respecting neuronal bodies in deep nuclei [173,174]. PML does not affect the optic nerve or the peripheral nervous system.

Histologically, PML is a unique entity, characterized by three prominent features, plaques of demyelination, enlarged oligodendrocytes harboring intranuclear inclusion bodies, and bizarre astrocytes. In early lesions, it is possible to find small foci of exclusively JCV-infected oligodendrocytes, which are two to three times larger than normal oligodendrocytes and display an intranuclear eosinophilic inclusion body. The inclusion body represents the site of active viral replication, as demonstrated by the presence of the characteristic icosahedral JCV particles by electron microscopy. In the more characteristic lesions of later stages, the predominant features are the plaques of demyelination, in which foamy macrophages are present. The function of these macrophages is to phagocyte the myelin breakdown products released by the lysis of oligodendrocytes. In the margins of the plaques, enlarged

oligodendrocytes hosting inclusion bodies can be identified. The second type of cell characteristic of PML is the bizarre astrocyte, present throughout the plaques and characterized by atypical, hyperchromatic nuclei, frequently multinucleated and pleomorphic cytoplasm, which confers them with the atypical and bizarre appearance. Other nonspecific features of PML, which may be prominent or conspicuous depending on the immune status of the patient, are perivascular cuffs of lymphocytes and parenchymal microglial nodules, common to most viral infections of the CNS [Figures 10.6 and 10.7].

The multifocal nature of the demyelinated lesions suggests that the virus reaches the brain via hematogenous spread, carried perhaps by B-lymphocytes, which have been shown to carry and allow JC viral replication in PML patients [175,176]. By immunohistochemistry, viral proteins can be detected in both types of cells infected by JCV, oligodendrocytes, and bizarre astrocytes. The early product T-antigen is found in the nuclear compartment, and the capsid protein, an indicator of active viral replication, can be found in both the cytoplasm and predominantly in the nuclei of infected cells. Finally, the accessory late product agnoprotein is located exclusively in the cytoplasm of both cell phenotypes [177]. Overall, histologically and immunohistochemical comparative studies have shown no significant differences between AIDS and non-AIDS associated PML, with only a slight tendency to more severe demyelination and a higher rate of infected cells in the AIDS-related cases reported [23].

Prognosis and treatment

Despite significant advances in antiretroviral therapy, thus far no effective treatment has been developed for PML, which remains a fatal disease with a poor survival, ranging from 4 to 6 months after the onset of symptoms [178] (see also Chapter 19). The longest survival reported for a patient with PML is 92 months [166]. Several factors can impact the gravity of the disease and the length of survival, including a CD4 count above 300 cells/mm^3 and low levels of JC viral DNA in the CSF [179,180]. The current strategies aimed to increase the survival of patients with PML include aggressive antiretroviral therapy that includes protease inhibitors [181,182] and treatment with alpha-interferon, which has only been shown to delay the progression of the disease [183]. A glimpse of hope was provided by *in vitro* experiments demonstrating the effective inhibition of JCV replication by cytosine arabinoside, a nucleoside analog that interferes with DNA synthesis in an immortalized human neuroglial cell line [184]; however, clinical trials failed to yield effective results in the course of PML [185,186]. Other failed studies have included the use of cidofovir, an acyclic nucleoside phosphonate with antiviral effects against DNA viruses [187]. Clinical trials currently underway include topotecan, an inhibitor of DNA topoisomerase that has been shown to inhibit JCV replication *in vitro* [188].

Physiopathology

Despite extensive molecular studies on the structure and function of JCV and a good understanding of its life cycle, several key physiopathological questions remain unanswered. Among these questions are the way in which the virus reaches the brain and infects glial cells in particular, the mechanism of rearrangement that results in neurovirulent forms, and the mechanisms by which the virus induces demyelination once it has been reactivated in oligodendrocytes. Unraveling of these points could provide suitable targets for the development of future therapies.

One of these questions is why patients with AIDS develop PML with a much higher incidence than patients with other immunosuppressive conditions, suggesting that the presence of HIV-1 in the brain participates, directly or indirectly, in the pathogenesis of this disease [189]. It is possible that both viruses communicate at the molecular level. One likely candidate to orchestrate the molecular events involved in the activation of JCV is the HIV-1 transactivator protein Tat, a 14-kD protein transcribed early in the HIV-1 infection cycle and important for transcription and replication through interaction with the HIV-1 LTR [190,191]. It has been demonstrated that Tat has the capacity of being

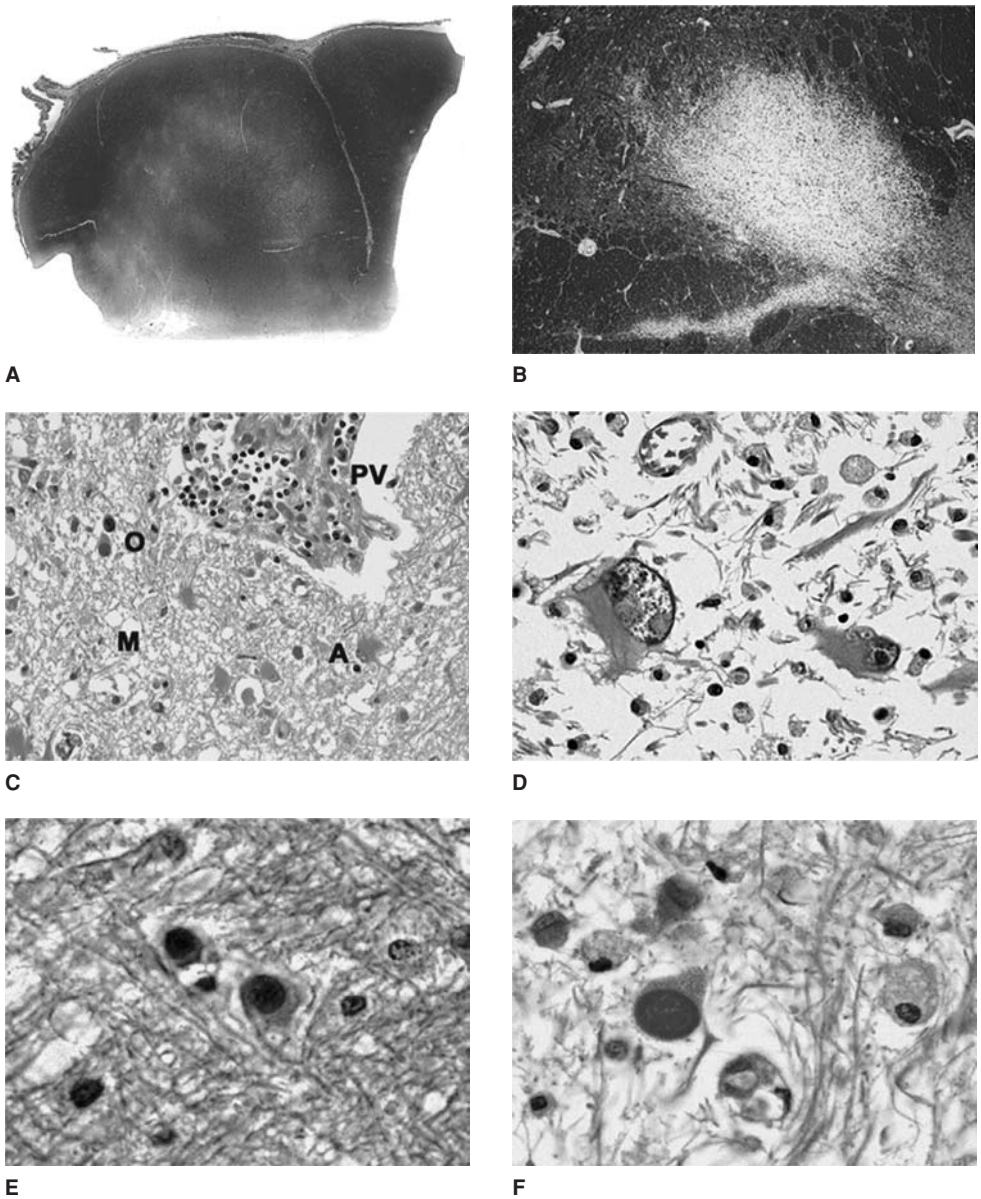

Figure 10.6. Histological characteristics of progressive multifocal leukoencephalopathy. Montage of the frontal lobe of a patient with PML demonstrating multiple confluent plaques of demyelination in the subcortical white matter (Panel A). The center of a demyelinated plaque is shown in a low magnification view (Panel B, Luxol Fast Blue, original magnification × 20). The most prominent features of PML are shown in this view of a demyelinated plaque, oligodendrocytes harboring intranuclear inclusion bodies (O), bizarre astrocytes (A), and foamy macrophages (M), adjacent to a blood vessel infiltrated by perivascular cuffs of lymphocytes (PV) (Panel C, original magnification × 200). Bizarre astrocytes (Panel D) and enlarged oligodendrocytes with intranuclear inclusion bodies in plaque at early (Panel E) and late stages (Panel F) of demyelination are shown (H&E, original magnification × 1000). (For figure in color, please see color plate section.)

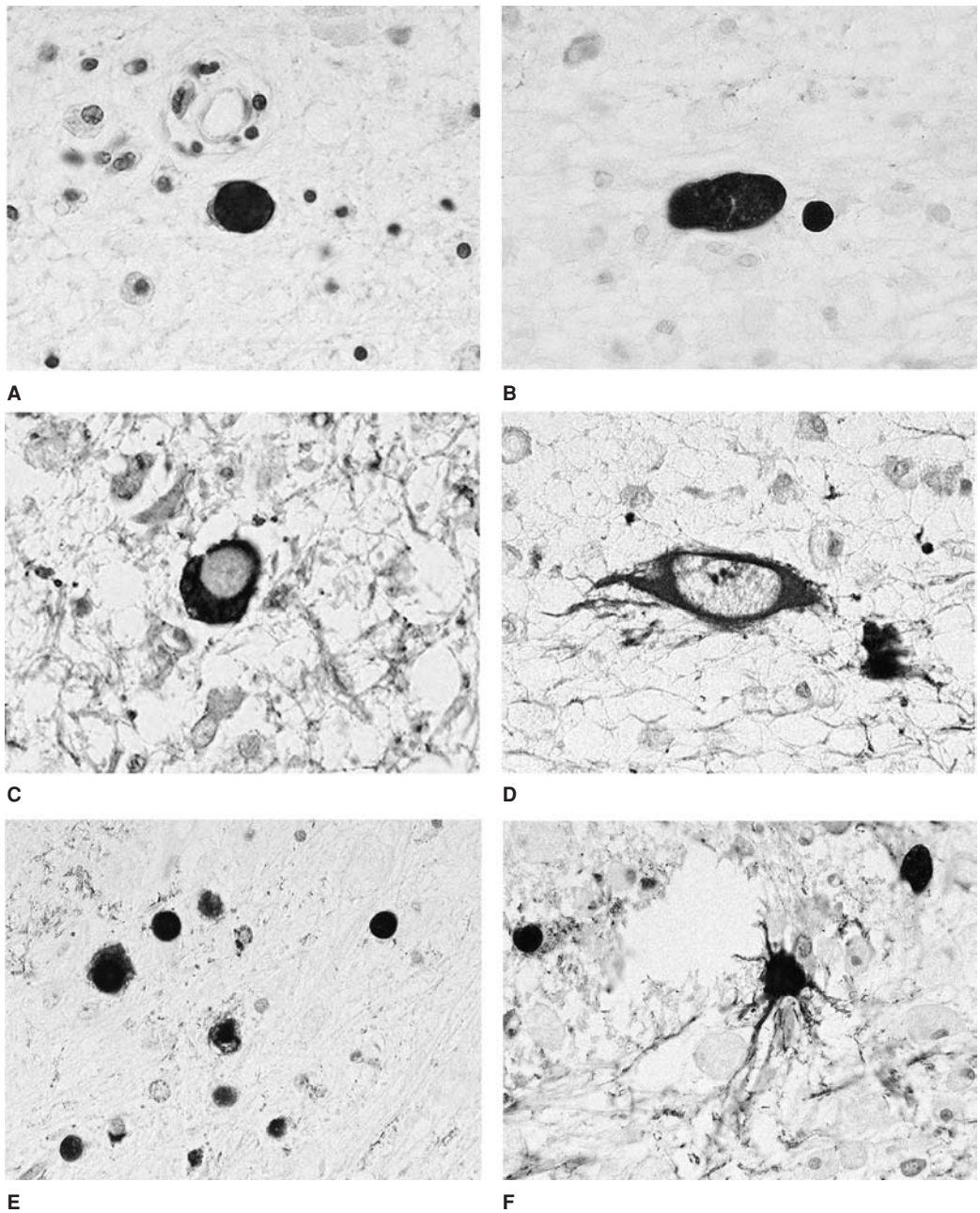

Figure 10.7. Detection of JCV proteins in PML. Immunohistochemistry with a specific T-antigen antibody shows its location in the nuclei of enlarged oligodendrocytes (Panel A) and bizarre astrocytes (Panel B). In contrast, the late accessory product agnoprotein is detected in the cytoplasm of oligodendrocytes (Panel C) and bizarre astrocytes (Panel D). The JCV capsid protein VP-1 is robustly expressed in the nuclei and cytoplasm of both phenotypes of cells, oligodendrocytes harboring inclusion bodies (Panel E), and bizarre astrocytes (Panel F), indicating active viral replication. Panels A to D original magnification 1000×, E, and F 400×.

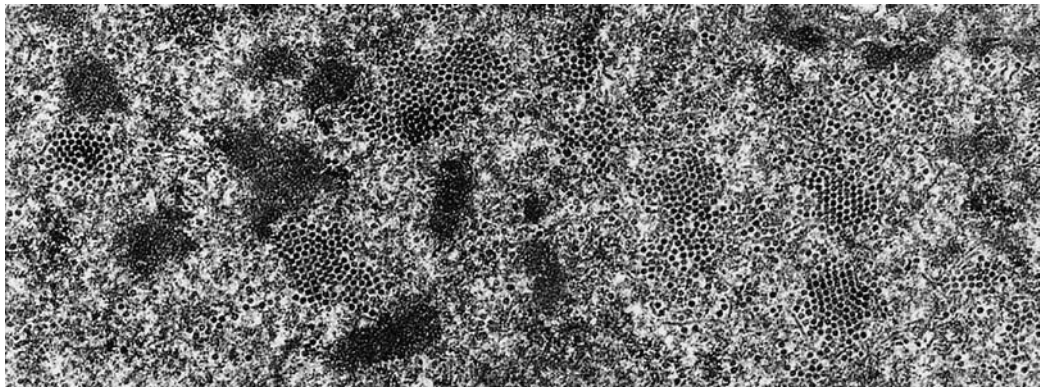

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Figure 10.7 (Continued). Electron microscopy of the inclusion bodies shows the characteristic hecosahedric viral particles (Panel G). (For figure in color, please see color plate section.)

secreted by HIV-1 infected cells, such as endothelial cells, macrophages, microglial cells, and astrocytes and absorbed by neighboring noninfected cells [177,192], proving that reactivation of JCV may not require coinfection by both viruses. It has been shown that Tat has the ability to bind to specific sequences within the JCV control region, to result in enhancement of JCV promoter transcription and enhancement of viral DNA replication as demonstrated *in vitro* [42,47]. Immunohistochemical studies have shown an alternative but not exclusive mechanism of JCV promoter activation mediated by Tat, which includes stimulation of intermediary proteins, such as TGF-β1, which binds to the TGF-β1 receptor of JCV-infected oligodendrocytes, resulting in transactivation of the JCV promoter by Smad 3 and Smad 4 [177]. In both cases, the transactivation of the JCV promoter by HIV-1 Tat in oligodendrocytes will result in active viral replication, lytic destruction, and eventually the development of PML.

Another intriguing question regards the mechanism of cell death upon JCV infection. Do enlarged oligodendrocytes die of a cellular process such as necrosis or do they literally burst when unable to contain a large amount of viral particles? Furthermore, why astrocytes, also capable of sustaining active viral replication, do not die but acquire a transformed and bizarre phenotype? A clue to answer these questions was found in our laboratory, when immunohistochemical experiments revealed the presence of survivin, an antiapoptotic protein in both the JCV-infected oligodendrocytes and the bizarre astrocytes [193]. Although apoptosis has been demonstrated in a variety of viral infections of the CNS, including HIV-encephalitis [194,195], there is a noticeable lack of reports linking apoptosis to PML, suggesting that the presence of JCV disrupts the mechanisms that control apoptosis. Survivin, a member of the inhibitors of apoptosis family, is normally expressed during development in proliferating tissues but should be completely silenced in adult, fully differentiated tissues [196]. The surprising finding of survivin in JCV-infected cells leads to the hypothesis that upon infection, JCV is capable of activating the normally silent survivin promoter in order to prevent apoptosis and allow completion of its life cycle. *In vitro* experiments in JCV-infected glial cultures later corroborated this hypothesis and further showed lower apoptosis when compared to noninfected cultures and dramatically higher apoptosis upon siRNA inhibition of survivin. The JCV-survivin-mediated inhibition of apoptosis also helps to explain the fate of infected astrocytes, which have time to acquire the transformed phenotype. This pathway could be a target for future therapeutic approaches.

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11

The herpes simplex viruses

David C. Bloom and Nicole V. Giordani

Introduction

Overview

The virus family *Herpesviridae* consists of a number of double-stranded DNA (dsDNA) viruses that share patterns of gene expression and the ability to undergo latency. Herpesvirus family members naturally infect a wide range of hosts, from mollusks to mammals. In humans, there are eight known herpesviruses with different primary sites of infection and cell types in which they can become latent. Data suggests that HSVs have persisted in the hominid population for millions of years. HSVs' lengthy coexistence with humans likely led to the these viruses' ability to maintain a latent state for the life of the host, with periodic stress-induced reactivations that produce progeny viruses. Members of the alphaherpesvirus subfamily, which includes herpes simplex virus (HSV) and Varicella-zoster virus (VZV) (see also Chapter 12), have a tropism for neuronal cells during latency. HSV exists as two distinct serotypes (HSV-1 and HSV-2) that cause similar clinical infections of the mucosal epithelia, although HSV-1 is typically associated with infections of the oral mucosa while HSV-2 is more frequently attributed to genital mucosa infections. This chapter will focus on the biology of HSV to understand how these viruses can persist in the nervous system and be shed periodically. We will also survey the diseases caused by these viruses, which are generally subclinical. Because this chapter provides a broad overview, we include citations for recent literature reviews of specific areas in addition to key primary references for the interested reader.

Structural features

An infectious HSV virion consists of a glycoprotein-spiked envelope surrounding the amorphous tegument, an icosahedral capsid, and the genome-containing core (Figure 11.1). Twelve virally encoded glycoproteins are present in the envelope, a number of which play essential roles in binding and entry of the virus into target cells [1]. The tegument consists of several viral proteins, including VP16, which play critical roles in facilitating transcription of the lytic genes [2]. Finally, the viral genome is packaged within the capsid as a histone-free structure [3].

The HSV genomes are large in comparison to other viruses, with approximate sizes of 152 kb for HSV-1 and 155 kb for HSV-2 [4,5,6]. A key feature of the HSV genomes is that they contain two sets of repeats (R_L and R_S) that flank two unique regions of the genome (U_L and U_S). The functional significance of the repeats is not known, though they contain a number of essential regulatory genes. While the genomes are similar in structure and share greater than 50% homology at the nucleotide level, there are several differences, including a truncation in one HSV-1 glycoprotein-encoding gene that decreases the size of its genome by approximately 2 kb [7]. The HSV genomes encode over 80 gene products, and the order and position of these genes are conserved between HSV-1 and HSV-2. During the replicative

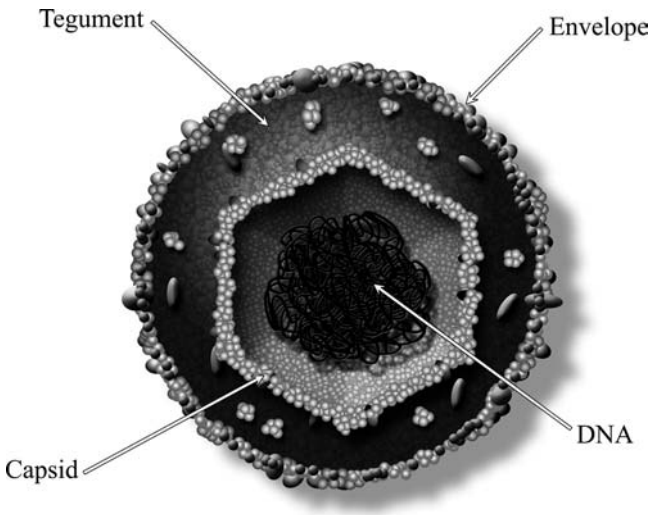


Figure 11.1. Diagram of the HSV virion. The DNA genome is packaged within an icosahedral capsid. The capsid is surrounded by a lipid envelope obtained as the capsid passes through the transgolgi apparatus. The envelope contains 12 virally encoded glycoproteins, including gB, gC, and gD, which play critical roles in binding to cellular receptors. The space between the capsid and envelope, referred to as the tegument, contains a number of viral proteins, such as VP16, that play a role in initiating viral transcription after the virus enters a cell.

phase of the infection, the viral genome exists in a linear form while persisting as a circular episome during latency [8,9] (Figure 11.2).

Viral life cycle

In vivo infection cycle

The HSV virion's sensitivity to desiccation dictates that the only efficient means of transmission is direct person-to-person contact. For HSV-1 the most common route of transmission is via the oral mucosa. For HSV-2 the most common route is via the genitalia. The moist mucosal surfaces probably help maintain the HSV virions in a viable state as well as provide access to the mucosal epithelium efficiently infected by HSV. Infection through fomites such as drinking glasses or toilet seats rarely, if ever, occurs, since HSV does not remain viable for long on these surfaces. HSV cannot infect through cornified epithelial surfaces unless there is a cut or abrasion exposing the underlying epidermis.

Once on the surface of the epithelium, the virion enters the cell through a fusion mechanism facilitated by several viral-encoded glycoproteins located within the viral envelope. HSV replicates productively at the site of infection and spreads to neighboring cells primarily by cell-to-cell fusion. This local replication may result in a vesicular lesion such as the classic HSV-1 cold sore; however, the most common outcome of this primary infection is subclinical epithelial disease, with only 10% of seropositive individuals ever aware of a primary lesion. During local replication, some virions are released from the infected cells and bind to sensory nerve termini located within the mucosal epithelium. HSV is taken up by these nerve termini and transported via fast axonal transport to the cell body, or soma, of the neuron, where it enters the nucleus (Figure 11.3). Within the nuclei of the sensory neurons, the virus either initiates a productive infection, or lytic gene transcription is silenced and the virus enters latency. The primary site of HSV-1 latency following an infection of the oral mucosa is the trigeminal ganglia.

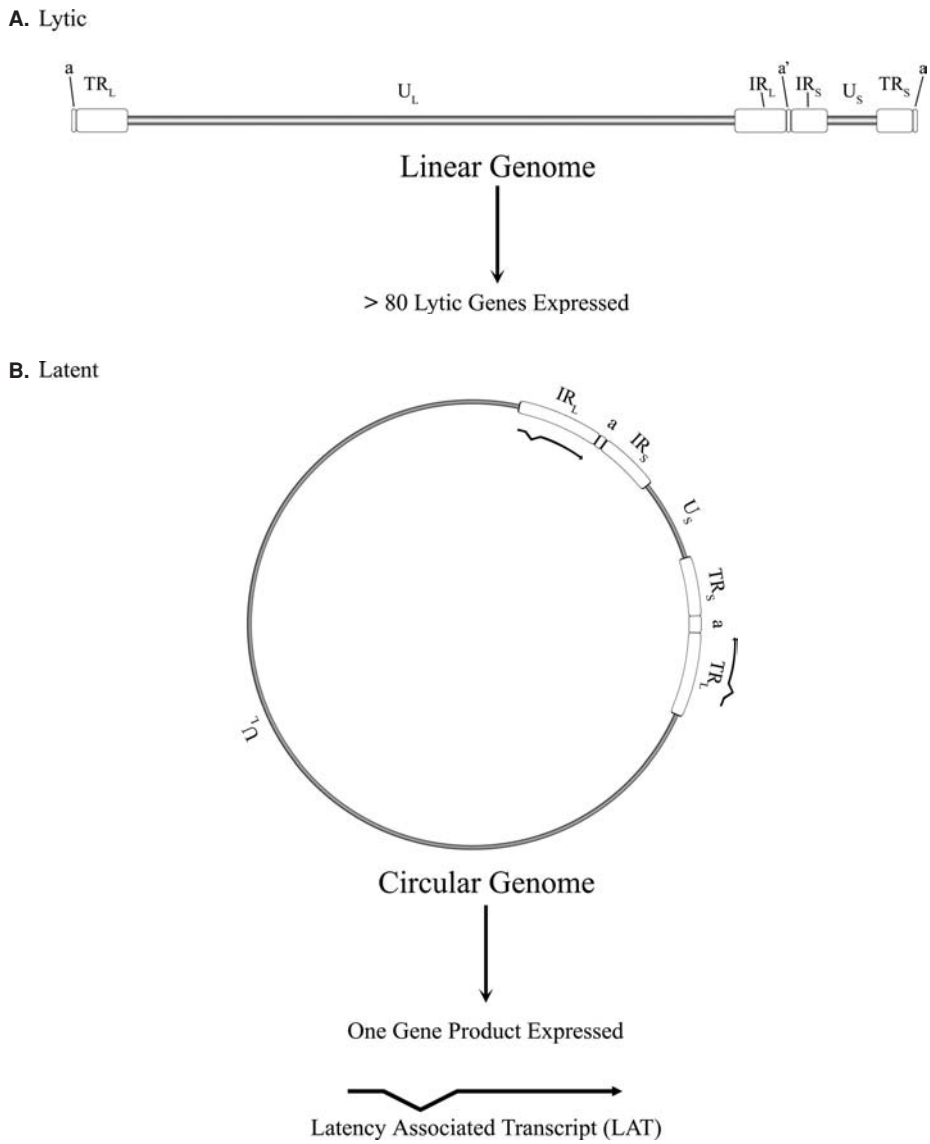


Figure 11.2. Diagram of the HSV genome. (A) Depiction of the HSV-1 linearized genome, the predominant form during lytic replication, during which over 80 viral lytic genes are expressed. The genome consists of four regions: unique long (U_L) and short (U_S) regions flanked by the terminal repeat long (TR_L) and internal repeat long (IR_L), and the internal repeat short (IR_S) and terminal repeat short (TR_S), respectively. A cluster of reiterated elements, termed the "a" sequences, are located at the ends of the R_L and R_S. These elements play a role in genome circularization and genome packaging, as well as contain sequence elements important for cleavage of the replication intermediates, which exist as concatemers. (B) During latency, the HSV genome exists as a circular episome. Lytic gene expression is repressed, and only the latency associated transcript (LAT) is abundantly expressed.

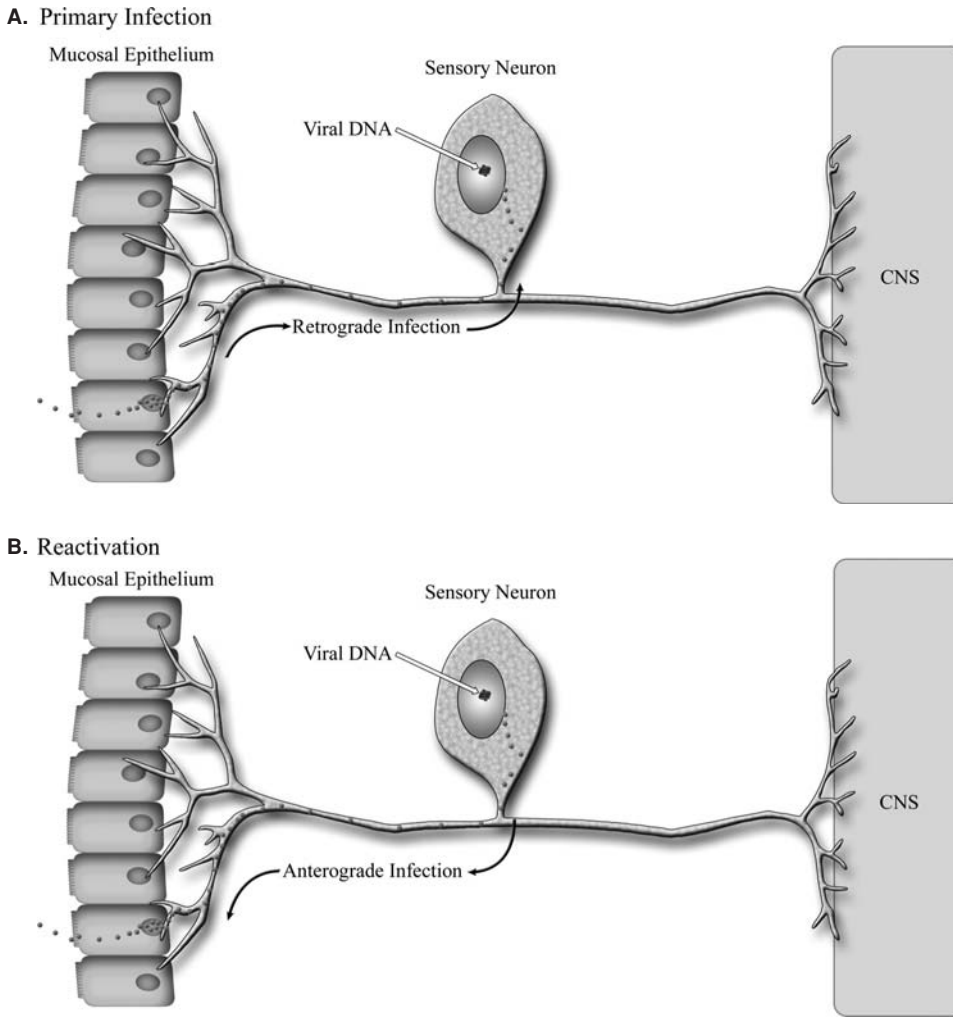


Figure 11.3. Diagram of the HSV lytic/latent infection cycle. (A) During the typical course of the initial (primary) infection, HSV infects the mucosal epithelium and gains access to sensory nerve termini. After entry into the termini, the capsid is transported by retrograde fast axonal transport to the neuron's cell body, where the viral DNA enters the nucleus. Here, the virus can become latent. (B) Periodically, the latent genome can reactivate. When this occurs, progeny virions are transported in an anterograde manner to the site of the primary infection, where the virus can reinfect the mucosal cells and shed virus. (For figure in color, please see color plate section.)

In HSV-2, the primary site of latency is the sacral ganglia enervating the genital region.

Periodically, physiological stress can cause reactivation of the latent infection. When this occurs, lytic viral transcription occurs, followed by genome replication and the production of infectious virions. These virions are delivered by anterograde transport down the axons of the sensory neurons to the epithelial site of the primary infection (Figure 11.3). The reactivation can be asymptomatic or can result

in a lesion; however, in either case, the infectious virus at the epithelial surface is available for transmission to a new host.

Transmission and epidemiology

Epidemiological studies have shown that HSV-1 is typically acquired early in life, with a large proportion of children in the United States possessing antibodies to HSV-1 by age 5 (see also Chapter 17). By age 70, seropositivity approaches 90% [10,11]. Consistent with sexual transmission, the incidence of HSV-2 is lower and the mean age of seroconversion is higher than with HSV-1. Additionally, the overall seroprevalence rate of HSV-2 in the United States is only 5% by age 19 and 28% by age 70. It should be noted, however, that incidence rates among some socioeconomic and ethnic groups approaches 70% [10]. In addition, recent studies suggest that overall incidence rates for HSV-1 and HSV-2 might be slightly understated due to a small but significant portion of the population in which shed virus is detectable by polymerase chain reaction (PCR), but concurrent antibody titers are below the levels of seropositivity, as assayed by currently available tests. As much as 15% of the population may fall into this group.

While HSV-1 and HSV-2 are serologically distinct, their genomes are colinear and share about 65% identity overall. Clinically, HSV-1 is associated with 85% of orofacial HSV infections, while HSV-2 is associated with the other 15%. Similarly, HSV-2 is found to be the cause of between 60–70% of genital infections with the remainder caused by HSV-1 [12,13,14]. While the increased incidence of HSV-2 as a cause of orofacial disease has increased in the United States in recent years due to changes in sexual practices, recent studies suggest that the anatomical basis for these two infections is based, at least partly, on a preferred tropism of these viruses for the sensory ganglia that enervate the sites of primary infection [15,16]. While it is difficult to separate the roles of infection route vs. differences in viral tropism in establishing HSV-1 and HSV-2 into their respective anatomical latency sites, it seems likely that these viruses have adapted their infection program to be most efficient at sites where they have the greatest chance of initiating a primary infection.

The fact that the majority of the US population is persistently infected with HSV indicates that the virus must reactivate frequently to be efficiently transmitted to such a large percentage of the population. In studies where HSV-2 seropositive individuals performed genital swabs several times a day over several months, viral DNA measured by sensitive PCR analysis was present in approximately 72% of the subjects who reported no genital lesions [17]. While actual infectious virus was only detected 30% of the time in those studies, animal studies suggest infection can occur even when levels of infectious virus are below detectable levels in swabs. This is supported by documented cases in which HSV-2 infections occurred in sexual partners of seropositive individuals without lesions or detectable HSV-2 infectious virus. However, the mean threshold level of detectable viral DNA that corresponds with the ability to cause infection is still unclear. Nonetheless, it appears likely that over the range of a month, HSV-2 is shed the majority of the time.

PCR analyses to detect HSV-1 DNA in oral mucosa have yielded similar results. Twenty percent of patients visiting dental clinics had detectable HSV-1 in their saliva [18]. Again, while it is not known whether these patients were infectious at the time, it is clear that HSV is shed frequently, corresponding with its high seroprevalence in the population.

Human disease

While the usual outcome of HSV-1 and HSV-2 infections is a subclinical primary infection followed by lifelong periodic shedding, an estimated 1–5% of the U.S. population suffers from symptoms [19]. The most common clinical diseases caused by HSV-1 and HSV-2 are herpes labialis and herpes genitalis, respectively. However, these viruses can cause more severe and occasionally life-threatening complications, particularly in the immunocompromised. The sections below discuss these diseases

from the perspective of the organ system(s) that are affected.

Diseases of the skin/mucosa

As mentioned previously, the majority of herpes labialis cases are caused by HSV-1. The most common presentation is an ulcerated lesion (cold sore), often on the lip, which usually resolves within 4 to 7 days (Figure 11.4). In individuals who experience recurrent disease, various types of stress including UV exposure, neurological stress, and trauma can result in recurrence. A hallmark of herpes labialis is that recurrent lesions occur in the same anatomic spot as the primarily lesion, a consequence of the virus reactivating from the sensory neurons directly connected to that region of the skin. Occasionally, more severe forms of orolabial disease, such as herpes gingivostomatitis, are seen. They can involve the gums and other soft tissues of the mouth. Such cases can be particularly painful and make eating and drinking difficult. Fortunately, these manifestations are rare and often associated with immunodeficiency caused by chemotherapy or HIV infection.

Genital herpes lesions are similar to those of herpes labialis and are most often the result of HSV-2 infection [20]. It should be noted that while HSV-1 can infect the genitalia (and be shed), it rarely produces clinical lesions at this anatomic site. In general, herpes genitalia differs from herpes labialis in that lesions occur more frequently and are more painful, an effect likely due to the higher density of sensory nerves in the genital region. As noted previously, in patients with herpes genitalia, virus is shed even in the absence of clinical lesions, and it has been estimated that virus is present 20 out of 30 days a given month [17,21].

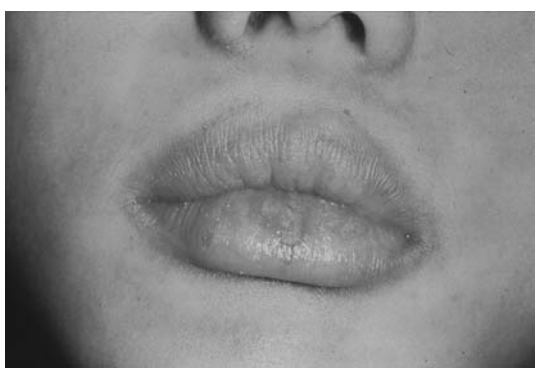


Figure 11.4. Recurrent herpes simplex labialis. Common recurrent lesion (cold sore) on the lip produced by HSV-1 reactivation. (For figure in color, please see color plate section.)

While HSV is most efficiently transmitted via mucosal surfaces, inoculation through keratinized epithelium via abrasion or cuts can occur. In these types of infections, the latent virus can reactivate, resulting in a lesion at the original site of infection. Occasionally, wrestlers become infected on their limbs or torso, resulting in lesions known as herpes gladiatorum [20]. Herpes whitlow, usually on the hands or fingers, is seen most often in health care workers, especially dentists and dental hygienists, who become infected with HSV after contact with saliva on chapped hands or from injection via a dental instrument [22]. Reactivation episodes of herpes whitlow are sometimes associated with arthritic-like pain in addition to lesions.

Disseminated herpes simplex

A rare but almost always fatal disease caused by HSV is a disseminated infection often accompanied by a viremia that can affect multiple organ systems [23]. This disease is seen primarily in individuals with severe combined immunodeficiency, women who receive a primary HSV infection – either HSV-1 or HSV-2 – in their second trimester, or newborn infants. Inter-utero infections or infections from a mother reactivating during natural childbirth are almost always caused by HSV-2. In addition, there are a few documented cases of transmission of HSV-1 to newborns by saliva contact or through breast lesions [24,25].

Herpes encephalitis

Herpes simplex encephalitis resulting from HSV-1 or HSV-2 can be caused by either a disseminated infection or by neuroinvasive spread from the trigeminal

ganglia (TG) to the central nervous system (CNS) as a result of an uncontrolled primary infection or reactivation of a pre-existing latent infection [26]. In either case, the ability of the virus to replicate within the CNS is usually the result of an immunological deficit. These two etiologies can often be differentiated by the patterns of infectious foci in the brain, with the neuroinvasive spread tending to be more discrete and localized to the root entry of the sensory ganglia, whereas the disseminated infection results in multiple foci as a result of hematogenous, or blood-borne, spread. The prognosis of herpes encephalitis is generally poor, with a greater than 30% lethality rate, even following antiviral therapy. Of the survivors, at least a third suffer from significant neurological sequelae.

Ocular disease

HSV-1 ocular infections are not uncommon and are likely the result of transmission from the mouth to the eye via saliva. Initial replication occurs in the soft tissue of the eye as well as in the cornea, where classic dendrites can often be observed (Figure 11.5). During the acute replication period, which typically lasts 6 to 10 days, virus can be detected in tears either by infectious plaque assay or by PCR for viral DNA. Latency can be detected in the trigeminal ganglia as well as in the pterygopalatine and superior cervical ganglia. Reactivation of the latent virus back to the eye results in local replication detectable in the tears with or without clinical manifestations.

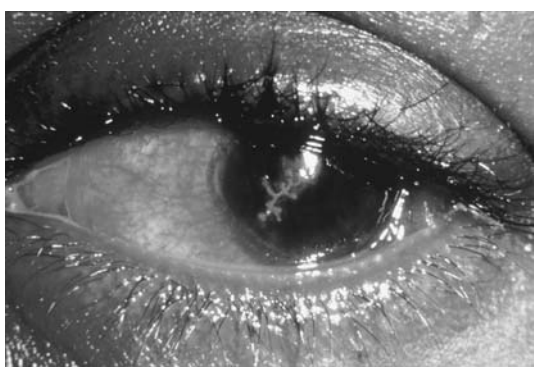


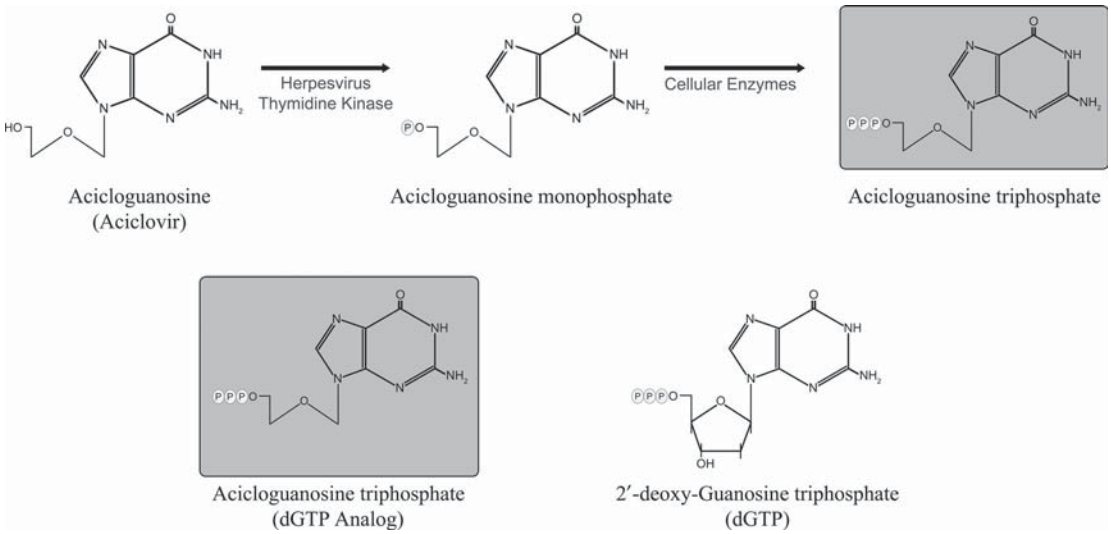
Figure 11.5. HSV-1 dendritic lesions on the cornea. HSV-1 can cause dendritic lesions on the cornea, which can be visualized by fluorescein staining. (For figure in color, please see color plate section.)

While the majority of HSV ocular infections are clinically inapparent, severe disease can occur in some individuals. Herpes stromal keratitis is the leading infectious cause of blindness in the United States and is the result of damage to the stromal layer of the cornea due to recurrent reactivations of HSV [27]. While it is not clear why some individuals are more susceptible to this disease than others, it is believed to have an autoimmune component in which an inappropriate immune response against stromal antigens is triggered by recurrent HSV reactivations [28,29]. Moreover, data suggests that inappropriate cytokine response to the frequently reactivating virus triggers a nonspecific cell-mediated response that damages the cornea [29]. In either event, antiviral therapies alone are insufficient to control this disease, and severe cases require multiple corneal transplants, with each ultimately failing after reactivating virus reseeds the new cornea.

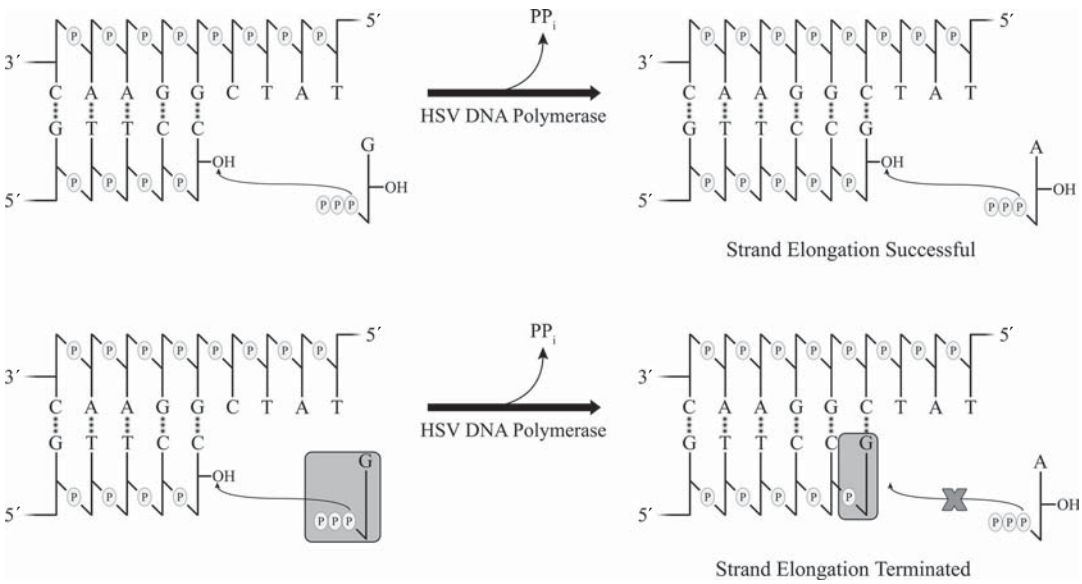
Treatments

Antivirals

Acicloguanosine (Aciclovir, formerly called Acyclovir) and its derivatives (e.g., ganciclovir, valiciclovir, and famciclovir) are effective antivirals with a high therapeutic index for the treatment of acute HSV infections (see also Chapter 19). Aciclovir, which is a nucleoside analog, is only efficiently phosphorylated to a monophosphate by the HSV thymidine kinase (tk) and not by cellular enzymes, conveying a high degree of specificity and relatively low toxicity. After the initial phosphorylation, aciclovir can be phosphorylated by cellular enzymes to acicloguanosine triphosphate, which acts as a chain terminator to inhibit viral DNA polymerase (Figure 11.6). Acicloguanosine triphosphate's preferential incorporation by the HSV DNA polymerase, as opposed to cellular polymerases, gives the drug additional selectivity against HSV-infected cells. Strains of HSV



A



B

Figure 11.6. Mechanism of aciclovir inhibition of HSV replication. (A) Acicloguanosine (aciclovir) is preferentially acted upon by the HSV thymidine kinase (tk) to yield acicloguanosine monophosphate. Once phosphorylated by the HSV tk, the monophosphate form can be further phosphorylated by cellular kinases to produce acicloguanosine triphosphate, a dGTP analog. (B) The acicloguanosine triphosphate can be used as a substrate by the HSV DNA polymerase, blocking replication by chain termination.

that are resistant to the acicloguanosines, while not widespread, do exist and have been shown to contain mutations in either the HSV tk or polymerase genes.

Aciclovir can be given topically, reducing the severity and duration of lesions, and it can also be given orally to reduce frequency and severity of recurrent genital herpes infections. It is important to note that because aciclovir only acts on replicating virus, it cannot prevent or cure latent infections.

Prospects for a vaccine

While initial attempts at subunit or killed HSV vaccines have not been fruitful, recent success of live-attenuated VZV, or chicken pox, vaccine has rekindled hopes that a similar vaccine for HSV can be developed [30]. Because of the later age of acquisition and relatively more severe clinical disease, HSV-2 is the main target of vaccine efforts. Because acquired immune response to the original infection is unable to completely control HSV infections, individuals who have severe recurrent disease would benefit most from a vaccine. These individuals mount a cellular and humoral immune response to the initial HSV infection, although it is insufficient to control subsequent reactivations. Therefore, a successful HSV vaccine would likely enhance the immune response in individuals susceptible to recurrent disease.

Designing an effective vaccine that will block the virus from entering neurons and from establishing latency is difficult because: (1) even replication-defective mutants of HSV can establish a latent infection since the HSV virion need only gain access to the nerve termini near the skin's surface to infect [31], and (2) an individual who has previously been infected with one strain of HSV can be superinfected with a second strain. Therefore, it is unlikely that a vaccine akin to the VZV vaccine, in which the immune response is primed to completely clear the infection, will work (see also Chapter 12); instead, HSV vaccine development will require a greater understanding of, and the ability to augment, an immune response that efficiently controls recurrent disease in most individuals.

Current research

Animal models

A challenge of HSV biology, including latency and reactivation, is that at present there is no cell culture system shown to sufficiently mimic HSV *in vivo* latency. Therefore, the numerous questions surrounding the regulation of HSV latency rely on the use of animal models.

Mouse model

Several models of latency and reactivation employ the mouse. Once the mouse is infected through the eye, footpad, or another route of epithelial inoculation, the virus replicates and can cause encephalitis, paralysis, and even death. Because HSV-2 infection in mice is severe, aciclovir is often administered to the infected animals to prevent complete mortality of the experimental group. After acute infection, a latent infection can be established in the sensory ganglia that enervates the site of initial infection [32]. Reactivation is most often induced by hyperthermia or explant of ganglia [33].

Spontaneous reactivation by the latent HSV at the site of initial inoculation in the mouse does not reproducibly occur, placing a limitation on the relevance of mouse models to human infection. However, mouse systems are more relevant than cell culture, are cost-efficient, and while reactivation occurs in an *in vitro* manner, the mouse provides useful insight into molecular events related to latency and reactivation.

Rabbit eye model

In human HSV-1 infection, latency occurs in the TG, and reactivation occurs at the site of primary infection, commonly the mucosal epithelia. This is similar to what is observed in the rabbit eye model: rabbits are infected through the corneas, latency occurs in the TG, and upon reactivation infectious virus can be recovered at the site of initial infection. In addition to spontaneous reactivation, it is possible to induce reactivation in latently infected rabbits.

Iontophoresis of epinephrine via a direct current to the eye has been shown to induce viral shedding from latently infected rabbits at high frequencies [34]. The use of the rabbit eye model for studying latency and reactivation is only relevant for HSV-1 and is strain dependent (reviewed in [35]).

Guinea pig model

HSV-2 latency and reactivation can be studied using female guinea pigs infected via the vaginal route. The primary infection can cause mortality, with viral latency occurring in the surviving animals. Spontaneous reactivation occurs as vesicles in the vaginal region from which infectious virus or viral DNA can be recovered, but reliable induced reactivation is not possible (reviewed in [35]).

Role(s) of the LAT in latency and reactivation

When HSV-1 becomes latent in a neuron, there is an overall shutdown of the genome, with the exception of the latency-associated transcript (LAT) region [36,37]. The primary LAT is approximately 8.3–8.5 kb in length [38,39,40] and can be spliced to yield 2.0- and 1.5-kb species in HSV-1 [41,42] and a 2.2-kb product in HSV-2 [43] (Figure 11.7). Stability of the LAT splice products – including a half-life of the HSV-1 2.0-kb intron of almost 24 hours in cell culture [44] – suggests importance to the virus, although no precise function has been ascribed to them. Furthermore, the 2.0-kb intron is dispensable for normal reactivation [45].

The exact role of the primary LAT is still unknown. Promoter deletion mutants not expressing LAT retain wild-type levels of establishment and maintenance of latency [46,47,48,49], arguing against a critical role for the LAT in those functions. The LAT has been implicated in neuronal survival and antiapoptosis [50,51,52,53,54,55,56], and a clear role for the LAT appears to be in reactivation from latency, since promoter deletion mutants that do not express LAT do not display efficient *in vivo* reactivation [57,58].

An emerging picture shows LAT playing a number of different functions during latency and reactivation, including preventing and facilitating reactivation. To date, there is no clear evidence that the LAT encodes a protein, and current hypotheses center on the role of the LAT as a functional RNA.

Transcription from the HSV-1 genome is regulated epigenetically

In contrast to viruses that only cause transient acute infections, DNA viruses that episomally persist in cells must maintain their genomes and regulate transcription for long periods of time. Like the DNA of

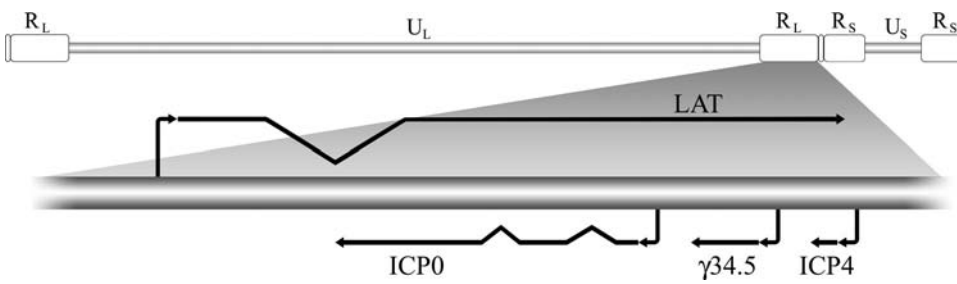


Figure 11.7. Map of the HSV genome and the LAT region. The organization of the HSV genome is shown at the top, with the unique regions of the genome (U_L and U_S) depicted by lines and the repeat segments (R_L and R_S) depicted by open boxes. The expanded region represents the entire long repeat (R_L) and a portion of the short repeat (R_S). Illustrated are the locations of the latency-associated transcript (LAT) and three lytic viral genes (ICP0, γ34.5 and ICP4) that are transcribed from the opposite strand and overlap with the LAT. The primary LAT RNA is an 8.3–8.5-kb transcript that is spliced to yield a stable intron of 2 kb.

cellular chromosomes, the latent genomes of many of these viruses, including HSV, associate with histones and assemble into a protein-DNA complex called chromatin. The simplest subunit consists of the nucleosome, composed of four different histone proteins. Specific modifications to the N-terminal tails of histones can be indications of transcriptional activity in cells. The HSV genome is not associated with histones in the capsid; however, nucleosomal structures can be detected during lytic infection in cell culture and during latent infection of the mouse CNS [59,60]. Further, during latent infection of murine DRG and TG, lytic genes appear to be associated with repressive histone modifications such as diMe-H3 K9 [61], whereas the LAT region is associated with transcriptionally permissive modifications such as diAc-H3 K9,14 and diMe-H3 K4 [62]. These findings implicate histone modifications in regulating latent patterns of transcription in an epigenetic manner, and unlike some herpesviruses, CpG methylation of the latent genome does not seem to occur in HSV [63,64].

Several recent studies using a LAT promoter-deletion mutant have implicated the LAT in regulating lytic gene expression during latency [61,65]. Moreover, analysis of histone modification profiles and LAT expression during explant-induced reactivation from mouse DRG suggests that within the first hour postexplant, the LAT region is remodeled to a less transcriptionally permissive state [66]. This remodeling is followed by a decrease in LAT expression, suggesting that a loss of LAT expression may be an early prerequisite for reactivation. By 3 to 4 hours postexplant, a nearby lytic gene (ICP0) is remodeled from a transcriptionally nonpermissive to a transcriptionally permissive state. While it is still unclear which viral gene(s) first respond(s) to stress stimuli to initiate the reactivation cascade, it is clear that the process is tightly regulated and that even during explant reactivation only a small number of cells harboring latent genomes productively reactivate (1–5%) [33]. Clearly, a critical component of latency and reactivation is control of individual neurons infected and the manner in which latency is established in those cells.

HSV latency is established in a subset of sensory neurons

While it has been shown that HSV establishes a latent infection within sensory ganglia, this represents a heterogeneous population of different cell types including pain-sensing, nociceptive and tactile-responsive cells; only about 10% of ganglionic cells are neurons [67]. In experimental ocular infections of mice, HSV-1 establishes latency preferentially within A5 neurons, a subtype of sensory neurons expressing Galβ1–4GlcNAc-R epitopes [68,69]. However, ocular infection with HSV-2 results in establishment of latency primarily in KH10 neurons, a completely different subpopulation of sensory neurons expressing Galα1–3Galß1–4NAc-R epitopes [15,68]. In addition to suggesting that HSV-1 and HSV-2 have adapted to subtle differences between sensory ganglia at the anatomic sites where they most often reside, this also suggests that the cell type provides a critical environment for the establishment of latency. Moreover, recent studies suggest that differences between the HSV-1 and HSV-2 LATs may play a role in directing the establishment of latency to these two distinct neuronal populations in the different ganglia [16], further implicating the LAT in regulatory pathways involved in establishment and maintenance of HSV latency.

Future directions

In addition to work on a novel vaccine for prevention of HSV infection and recurrent disease, strides are being made toward HSV vector development for delivering therapeutic peptides to the nervous system [70]. Clinical trials currently under way use HSV vectors as treatments for diabetic neuropathy and chronic pain [71]. Modified HSV is also being used as an oncolytic approach to treating certain tumors, including glioblastoma multiforme [72,73,74,75] (see Chapter 22). Successes in these areas are likely to bring new applications of HSV as a therapeutic vector (see also Chapter 23). Finally, while much is known about the replication strategies

of HSV during a natural infection, greater insight into viral immune system evasion and how HSV regulates gene expression during latency and reactivation will likely provide clues into subtleties of gene regulation within neurons. Such findings would provide new treatment strategies for HSV infections and improve the utility of HSV as a gene therapy vector as well as provide insight into still unknown aspects of cellular gene regulation.

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12

The pathogenesis of varicella-zoster virus neurotropism and infection

Leigh Zerboni and Ann M. Arvin

Introduction

Varicella-zoster virus (VZV) is a neurotropic human herpesvirus that causes varicella, which is commonly known as chicken pox, as the primary infection in susceptible individuals. In the healthy host, varicella is usually a mild, self-limiting febrile illness characterized by a generalized, pruritic vesicular rash (Figure 12.1A). Like other alphaherpesviruses, VZV gains access to sensory ganglia of the peripheral nervous system during primary infection and establishes lifelong persistence at these sites. VZV reactivation from latency causes herpes zoster, called "shingles," and is associated with a vesicular rash localized to one of the cutaneous dermatomes of the face, trunk, or extremities (Figure 12.1B). The dermatomal rash reflects the region of skin innervated by the cranial nerve or dorsal root ganglion where reactivation is occurring. VZV is highly contagious and is maintained in the human population by close contact with individuals who have varicella or herpes zoster. Both primary and recurrent VZV infections are more severe in immunocompromised patients because resolution requires an effective cell-mediated immune response. Antiviral drugs that inhibit VZV replication are effective in most high-risk patients with varicella or herpes zoster. VZV is the only human herpesvirus for which vaccines that prevent or modify the severity of primary and recurrent infections have been developed. These vaccines are made from the VZV/Oka strain, attenuated by passage *in vitro*.

While the clinical manifestations of varicella and herpes zoster are well-documented, knowledge about the mechanisms of VZV pathogenesis in the human host is limited because primary and recurrent infections are rarely fatal and VZV infection is highly species-specific for the human host. VZV infection of human skin, thymus/liver, and dorsal root ganglia (DRG) xenografts in the severe combined immunodeficiency (SCIDhu) mouse model of VZV pathogenesis has provided a novel system for identifying VZV gene products that mediate VZV tropisms (Figure 12.2). Infecting SCIDhu skin, T cell, and DRG xenografts with recombinant VZV mutant viruses that have targeted deletions or mutations of specific genes or their promoters enables the assessment of functions of VZV protein subdomains and viral gene promoter elements during VZV replication in differentiated human cells *in vivo*. The SCIDhu DRG model provides new opportunities to examine VZV neuropathobiology and interactions with human neurons and nonneuronal cells within the DRG tissue microenvironment.

Aspects of the basic virology of VZV

Taxonomic classification

VZV is classified as a member of the family *Herpesviridae* based on a shared genomic organization and virion architecture. Herpesviruses have likely arisen through cospeciation with their hosts in the kingdom *Animalia* for at least 200 million years, and

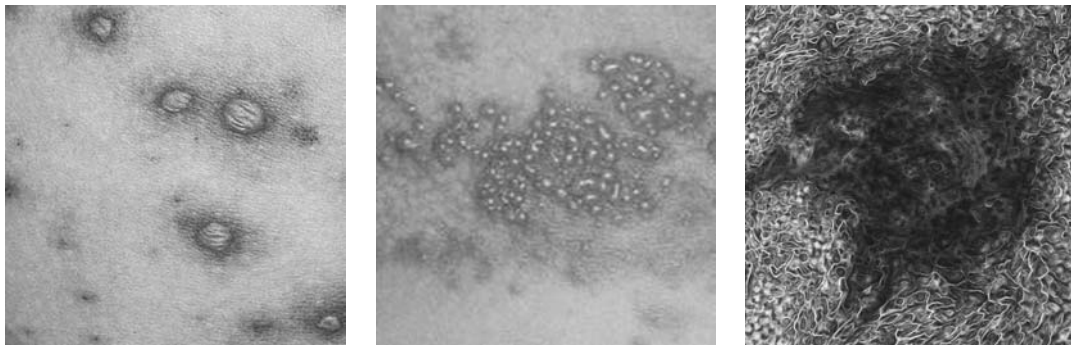


Figure 12.1. Varicella and herpes zoster lesions and VZV replication in cultured cells. (A) Photograph of a typical crop of chickenpox lesions. Varicella lesions erupt following a 10 to 21-day incubation period. Skin lesions usually develop first on the face, scalp, and trunk. Lesions begin as pruritic erythematous macules that enlarge as they fill with vesicular fluid containing infectious particles. The lesions crust after 24 to 48 hours. (B) Photograph of "shingles" (herpes zoster) caused by VZV reactivation from latency. VZV reactivation results in a vesicular rash corresponding to the cutaneous dermatome that is innervated by neurons in the involved cranial nerve or dorsal root ganglion. Cutaneous lesions form small clusters with the morphology of varicella vesicles and contain infectious virus. (C) Typical VZV multinucleated syncytia on cultured melanoma cells (3 days postinfection). VZV-specific proteins were stained with antihuman VZV polyclonal antibody and detected using Fast Red substrate. A methyl green nonspecific counter stain was applied. (For figure in color, please see color plate section.)

more than 130 herpesviruses have been identified to date [1,2]. All *Herpesviridae* share four biological properties, which are (1) a DNA genome with open reading frames (ORFs) encoding for a wide array of enzymes involved in DNA synthesis (e.g., viral helicase/primase), nucleic acid metabolism (e.g., viral dUTPase), and protein processing (serine/threonine protein kinase), (2) DNA synthesis and herpesviral capsid assembly restricted to the nucleus, (3) a lytic productive infection of host cells, and (4) maintenance of the viral genome within certain host cells in a nonlytic form, known as "latency" [2]. All herpesviruses are capable of re-emerging from latency to replicate and, in some cases, to cause recurrent disease in their natural hosts [2].

Herpesviruses are further divided into subfamilies based on their host cell tropisms and replication cycle. The subfamily *Alphaherpesviridae* includes three neurotropic human viruses: herpes simplex virus (HSV)-1, HSV-2, and VZV, as well as several other mammalian and avian herpesviruses. Establishment of latency in peripheral sensory nerve ganglia is a principal criterion of *Alphaherpesviridae* [2]. VZV, simian varicella virus (SVV), pseudorabies virus-1 (PRV-1), and equine herpesvirus-1 (EHV-1), are assigned to the genus *Varicellovirus* whereas HSV-1, HSV-2, and other nonhuman alphaherpesviruses comprise the genus *Simplexvirus* [3].

Genomic organization

As with all *Varicelloviruses*, VZV possesses a genomic organization in which either of two isomeric forms may be present in the virion by inversion of a unique long region and a unique short DNA region, which is flanked by repeat elements (Figure 12.3A) [4,5]. Several VZV isolates have been fully sequenced; sequence characteristics that have geographical associations have been identified, but most of these differences do not alter the gene product [6,7,8]. The VZV genome is the smallest of the human herpesviruses, consisting of approximately 125 kb and containing ORFs that are known or predicted to encode approximately 70 proteins [6]. The VZV

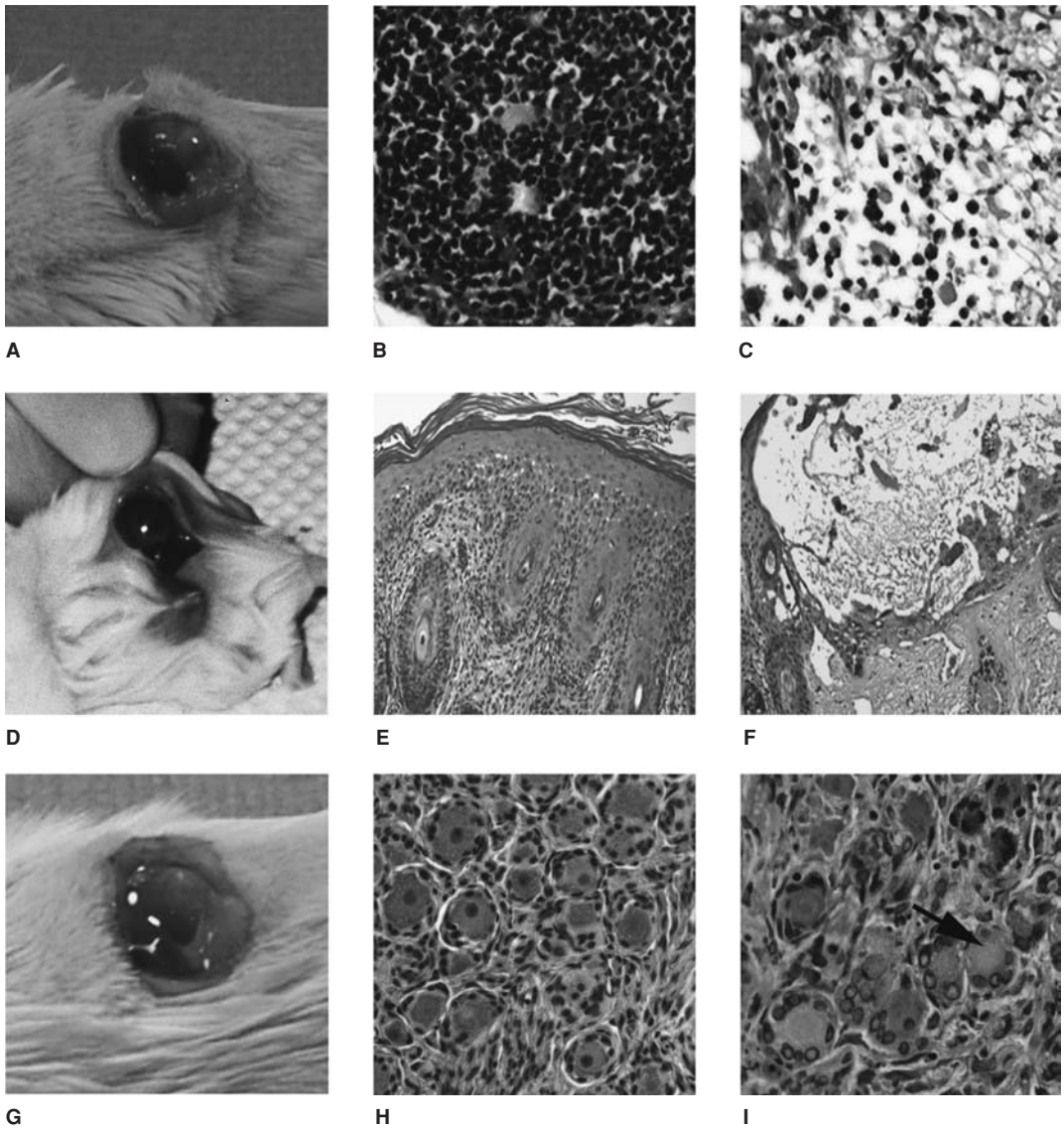


Figure 12.2. The SCIDhu mouse model of VZV pathogenesis. VZV infection of human skin, T cells (thy/liv), and dorsal root ganglia (DRG) xenografts in the severe combined immunodeficiency (SCIDhu) mouse model of VZV pathogenesis has provided a novel system for identifying VZV gene products that mediate VZV tropisms. (A) Photograph of a thy/liv xenograft, 9 months postxenotransplantation. (B) Uninfected thy/liv xenograft, with abundant hemotoxylin- and eosin- (H & E) stained lymphocytes. (C) VZV-infected thy/liv xenograft, 21 days postinfection, with severe depletion of lymphocytes. (D) Photograph of a skin xenograft, 5 weeks postxenotransplantation. (E) Uninfected skin xenograft, H & E stain. (F) VZV-infected skin xenograft, 21 days postinfection, with a large vesicular fluid-filled lesion. (G) DRG xenograft, 12 weeks postxenotransplantation. (H) Uninfected DRG xenograft. (I) VZV-infected DRG xenograft, 14 days postinfection, with significant cytopathic effect in sensory ganglionic cells (black arrow). Figure 12.2G-I from Zerboni *et al.*, 2005 [76]. (For figure in color, please see color plate section.)

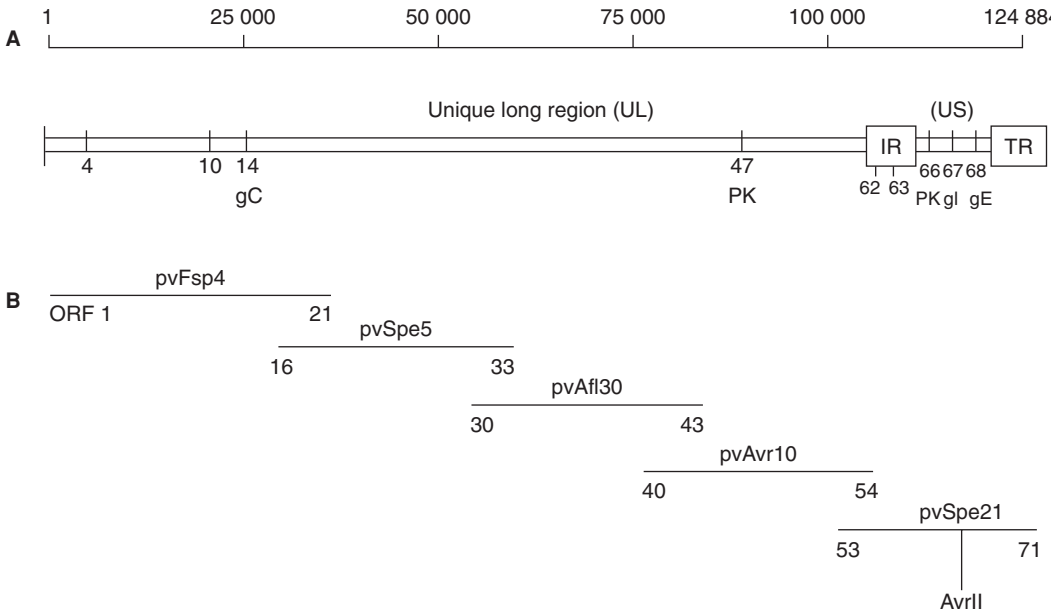


Figure 12.3. The VZV genome and VZV cosmids. (A) The VZV genome is 124 884 bp, which contains open reading frames (ORFs) that encode for approximately 70 proteins, 40 of which are highly conserved among herpesviruses. VZV possesses a genomic arrangement in which a unique long region (UL) and unique short region (US) are separated by repeat elements (IR = internal repeat, TR = terminal repeat). (B) VZV genomic cosmids, made from VZV DNA recovered from infected cells, contain overlapping fragments of the VZV genome and yield infectious recombinant virus when cotransfected into a susceptible cell line. Depicted are five cosmids that span the vaccine Oka genome and the ORFs contained within each fragment. Genetic elements or sequences of interest can be modified by PCR-directed mutagenesis, creating a mutated cosmid that is then cotransfected with the other intact cosmids to yield a recombinant VZV genome with a specific mutation in a coding or promoter region.

genome contains at least 40 ORFs that are core genes conserved among the neurotropic human alphaherpesviruses, but the level of sequence identity is typically limited and some essential genes of HSV-1 and HSV-2, such as the ORF-encoding glycoprotein D, are not present in VZV [6]. Table 12.1 delineates a subset of key viral ORFs, several of which are discussed in this chapter (Table 12.1).

Virion structure

Structural features of herpesviral particles are highly conserved and include an icosadeltahedral viral nucleocapsid containing a single copy of a linear toroidal double-stranded DNA core [2]. Surrounding the nucleocapsid is an amorphous assemblage of viral proteins, referred to as the virion tegument, that contains viral transcriptional regulator proteins [2,9]. The virion envelope is derived from host cellular membranes that have been modified to express viral glycoproteins [10]. The infectious VZ virion is approximately 180–200 nm in diameter and is pleomorphic in form and electron density (Figure 12.4A).

Viral replication

Compared to the neurotropic *Simplexviruses* and other alphaherpesviruses, which infect a spectrum of cell types *in vitro* and various species *in vivo*, VZV infection is highly host restricted. In the laboratory, VZV is routinely propagated in human diploid fibroblast cell lines, human melanoma cell lines,

Table 12.1. Function of select VZV genes

ORF	Function
Regulation of transcription	
IE4	Viral gene transactivator and tegument protein
IE62 (duplicated on ORF71)	Viral gene transactivator and tegument protein
IE61	Viral gene transactivator/transrepressor
IE63 (duplicated on ORF70)	Viral gene transactivator and tegument protein
ORF10	Viral gene transactivator and tegument protein
DNA synthesis	
ORF28	DNA polymerase (large subunit)
ORF29	Single-stranded DNA-binding protein
ORF51	Origin of replication-binding protein
Viral enzymes	
ORF47	Viral protein kinase
ORF66	Viral protein kinase
ORF36	Viral thymidine kinase
Nucleocapsid proteins	
ORF33.5	Capsid assembly
ORF40	Major capsid protein
Viral glycoproteins	
gB	Role in entry, fusion
gE-gI heterodimer	Role in entry, fusion
gH-gL heterodimer	Role in entry, fusion

monkey kidney (Vero) cells, and to a more limited extent in guinea pig embryo fibroblasts. In contrast to HSV, VZV replicates efficiently in human $CD3^+$ T cells [11,12]. This robust T cell tropism appears to be a critical factor in VZV pathogenesis [11,13,14]. VZV is also unique among other alphaherpesviruses in the failure to release infectious particles into the extracellular media of cultured cells. Electron microscopy studies have demonstrated that an abundance of VZV particles in cultured cells appear to be defective or are degraded intracellularly before egress can occur (Figure 12.4B) [10,15]. VZV spreads in cell culture from cell to cell, forming large multinucleated syncytia (Figure 12.1C).

The inability to produce high-titer infectious cell-free VZV preparations has precluded a precise analysis of the VZV replication cycle, but VZV replication appears to closely parallel replication of HSV-1, the prototypical alphaherpesvirus. Replication of HSV-1 is a highly coordinated process with a cascade of immediate-early, early, and late gene expression [16]. As with HSV-1, VZV entry into permissive host cells is presumed to begin with attachment of the viral envelope to the host cell surface by nonspecific electrostatic interaction with heparan sulfate glycosaminoglycans on cell surface proteoglycans, followed by fusion of viral and host cell membranes [17,18]. Heparan sulfate proteoglycans (HSPGs) are widely distributed on the cellular plasma membranes and basal laminae of mammalian cell types, including human sensory neurons and Schwann cells, and function in several key neuronal cell processes [19,20]. HSV-1 glycoprotein (g)B and gC interact with HSPGs, whereas only binding by VZV gB has been demonstrated [21].

Electrostatic attraction of the viral envelope via VZV gB and possibly by gC interactions with HSPGs may stabilize the virion on the plasmalemma, allowing additional coreceptors to bind and trigger cell-virus membrane fusion. Several cellular proteins have been identified which may function as co-receptors for HSV-1 entry into permissive cells, including nectin-1 (HVEM), an immunoglobulin-like adherens junction protein that mediates HSV entry into human sensory neurons through binding to HSV-1 glycoprotein D [22]. The technical difficulty of generating high-titer cell-free VZV has prevented analysis of VZV entry by these pathways. Four VZV glycoproteins, gB, gE, gH, and gI, contain mannose-6-phosphate (Mann-6P) residues, which may act as ligands for the cation-independent mannose-6-phosphate receptor (M6PR), as blocking of M6PR with heparan sulfate reduces VZV infection and M6PR-deficient cell lines resist infection by cell-free VZV [18,23,24]. VZV gE has been shown to colocalize with ZO-1, a tight junction protein of polarized epithelial cells [25], which suggests

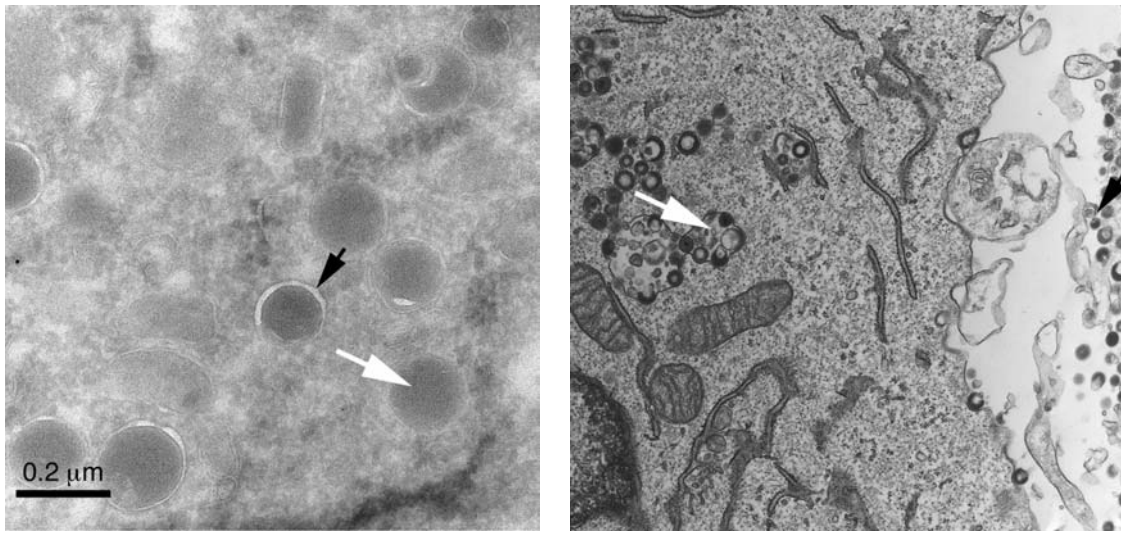


Figure 12.4. VZ virion morphology. (A) Cryoelectron micrograph of the ultrastructural features of a varicella particle. The infectious VZ virion is approximately 180 to 200 nm in diameter. Several cytoplasmic particles are shown. Nucleocapsids within enveloped particles are not visible due to the electron dense tegument (white arrow), but the viral envelope with glycoprotein spikes is well-preserved (black arrow). Photograph by Mike Reichelt. (B) Large secretory vesicles packed with pleomorphic particles are visible in this cultured human melanocyte. Most intracellular (white arrow) and extracellular (black arrow) particles are partially degraded in their morphology. This may account for the failure to release infectious virus into extracellular media of infected cell cultures. Photograph by Leigh Zerboni.

a role for gE in spread across intracellular junctions. Recently, Li *et al.* (2006) demonstrated gE binding to insulin-degrading enzyme, an intracellular thiol metalloendopeptidase localized to peroxisomes, and that blocking of IDE expression with siRNA reduces cell-cell spread of VZV [26].

Following virion entry, the VZV nucleocapsid and associated tegument proteins are transported to the nucleus where viral gene transcription is initiated [3]. Within 4 to 10 hours of infection, VZV immediate-early genes are transcribed and translated, and their protein products return to the nucleus to initiate early gene transcription. In turn, early gene protein products facilitate viral DNA replication and late gene transcription. Like HSV, VZV genome replication is presumed to follow a rolling-circle mechanism within the nucleus to generate genomic concatemers, which are subsequently cleaved by virally encoded nucleases into linear molecules [6,27]. Unit length linear genomes are packaged into capsids; capsid proteins encoded by ORFs 20,23,33,33.5,40, and 41 are translated from mRNAs in the cytoplasm and translocated to the nucleus for capsid assembly.

VZV egress from the infected cell is poorly understood. The observation that most VZV particles in cultured cells are contained within large vesicles and are degraded and pleomorphic in appearance has led to two models of viral egress. One model, based on observations of VZV-infected human fibroblasts, proposed that viral capsids bud through the inner nuclear membrane, acquiring a temporary envelope, which is lost upon fusion with the lumen of the rough endoplasmic reticulum (ER) [10]. Naked cytosolic capsids are directed to redistributed Golgi-derived vesicles, which have been modified to display viral envelope glycoproteins on their inner surface, and associated with viral tegument proteins on their outer surface. Capsids acquire a second

envelope by wrapping of the vesicle around the particle so that the inner membrane of the wrapping vesicle becomes the viral envelope while the outer membrane becomes a transport vesicle [10,28]. These Golgi-derived vesicles may coalesce with other virus-containing vesicles and fuse with cell membranes to release infectious virus, but most are diverted to late endosomes due to the presence of Mann-6P side chains on viral envelope glycoproteins. Most infectious virions are degraded within late endosomes, which accounts for the failure to release cell-free infectious virus [18,23]. The alternate model, based on observations in a human melanoma cell line, postulated that the initial envelope acquired by budding through the nuclear membrane is retained but modified by fusion with Golgi-derived secretory vesicles containing viral glycoproteins. Some vesicles are diverted to prelysosomes where the viral contents are degraded, but others release infectious virus at the cell surface [15]. These two models likely reflect cell type-specific differences in viral egress pathways.

Varicella envelope proteins are transmembrane glycoproteins, most of which are cotranslationally assembled in the ER and modified in the Golgi network prior to insertion into the plasma membrane and/or viral envelope. VZV gE and gB are the most abundantly produced viral glycoproteins in cultured cells. VZV encodes other known or predicted glycoproteins, including gC, gH, gI, gL [3]. VZV gE forms a noncovalently linked heterodimer with gI, which is required for efficient membrane expression of gE [29,30]. Similarly, gH, which has fusogenic properties, forms a complex with gL; gH function requires coexpression of gL [31,32]. Evidence that gB has fusogenic properties, and that gB, gH, and gL are targets for neutralizing antibodies, suggests that they play a role in viral entry [33,34,35]. gC is not required for VZV replication, and plaque-purified isolates have been generated that do not express gC fully, including the Oka vaccine strain [36].

Generation of VZV recombinants

Infectious VZV can be recovered after transfection of permissive cells with full-length genomic DNA, but in contrast to HSV, the highly cell-associated pattern of VZV replication prevents plaque purification of VZV mutants made by homologous recombination. VZV cosmids that contain overlapping fragments of the VZV genome yield infectious virus when cotransfected (Figure 12.3B). Cosmids have been made from VZV DNA recovered from cells infected with the vaccine Oka virus or the original clinical isolate, parent Oka [37,38,39,40]. The cosmid that carries a gene or sequence of interest can be modified by polymerase chain reaction (PCR)-directed mutagenesis, creating a mutated cosmid that is then cotransfected with the other intact cosmids. If the mutation is lethal, no infectious virus is recovered and rescue and complementation experiments can be done to confirm that the gene or motif is essential. Our experience using VZV mutants to analyze viral gene functions *in vitro* and *in vivo* is summarized in Table 12.2. VZV bacterial artificial chromosome (BAC) methods have also been developed recently that will facilitate studies of VZV molecular virology [41].

VZV epidemiology and transmission

VZV is a ubiquitous human pathogen with a worldwide geographic distribution. Annual epidemics arise most frequently during late winter and spring and are more prevalent in temperate climates (reviewed in reference 42; see also Chapter 17). Prior to vaccine licensure in the United States, the annual incidence of varicella was equivalent to the birth rate, with approximately 3.5 million cases per year. Varicella is highly contagious, with an attack rate among susceptible household contacts of 90% [42]. While aerosol spread of VZV can occur, most transmission is likely to be due to contact with fluid from cutaneous varicella lesions, which contain high titers of cell-free virus. Transient exposures, such as daycare or classroom settings, have transmission rates of 10–35%, indicating that aerosol transmission is not efficient. In temperate climates, most children acquire varicella before 10 years of age so that susceptibility rates are less than 5% in adults over 18 years of age

Table 12.2. The analysis of VZV gene and promoter functions by cosmid mutagenesis and evaluation for effects on replication *in vitro* and in the SCIDhu mouse model for VZV pathogenesis

		Cell culture *in vitro*		SCIDhu mice *in vivo*	
	Virus	Growth kinetics	Plaque size	Skin xenografts	T cell xenografts
Recombinant oka	+	+++	**Normal**	++	++
glycoproteins					
gE YAGL (internalization motif)	−				
gE AYRV (endocytosis motif)	+	++++	Normal	+	+
gE SSTT (phosphorylation motif)	+	++++	Normal	++	++
ΔgE P27-P187 (ectodomain deletion)	−				
ΔgEY51-P187 (ectodomain deletion)	+	+	Small	−	
ΔgE P27-Y51 (ectodomain deletion)	+	++	Small		ND
gE S49A (ectodomain mutant)	+	++	Normal	++	ND
gE S31A (ectodomain mutant)	+	+	Normal	+/−	ND
gE linker P187 (ectodomain mutant)	+	++	Normal	ND	ND
gE linker I146 (ectodomain mutant)	+	++	Normal	ND	ND
gE linker G90 (ectodomain mutant)	+	++	Normal	ND	ND
gE linker Y51 (ectodomain mutant)	+	++	Normal	++	ND
gE linker P27 (ectodomain mutant)	+	+	Small-Norm	+	ND
gE linker G16 (ectodomain mutant)	−				
ΔgI/gE (double deletion)	−				
ΔgI (complete deletion)	+	++	Small	−	−
ΔgI-N (N-terminus deletion)	+	++	Small	ND	ND
ΔgI-C (C-terminus deletion)	+	++	Small	ND	ND
ΔgI repair (gI repair at ectopic site)	+	++	Normal	++	++
ΔgK (complete deletion)	−				
rOka HSV gK (replace with HSV gK)	−				
ΔgK repair (gK repair at ectopic site)	+				
Regulatory genes					
ΔORF4 (complete deletion)	−				
ORF4 K4435 (KYFKC motif)	−				
ΔORF62 (complete deletion)	+	++	Normal	++	
ΔORF71 (complete deletion)	+	++	Normal	++	
ΔORF62/71 (double deletion)	−				
ΔORF62/70 rep62 (single copy repair)	+	+	Small	−	
Δ63/70 (double deletion)	−				
Δ63/70 rep63 (single copy repair)	+	++	Normal	++	++
Δ63 (single copy deletion)	+	++	Normal	++	++
ORF63 T171 (phosphorylation mutant)	+	+	Small	+	++
ORF63 S181 (phosphorylation mutant)	+	+	Small	+	++
ORF63 S185 (phosphorylation mutant)	+	+	Small	+	++
ORF63 phosphorylation mutants (N = 19)	−				
Δ64/69 (double deletion)	+	++	Large	++	++
Δ64 (single copy deletion)	+	++	Normal	++	++
Δ69 (single copy deletion)	+	++	Normal	ND	

Table 12.2. *(Continued)*

		Cell culture *in vitro*		SCIDhu mice *in vivo*	
	Virus	Growth kinetics	Plaque size	Skin xenografts	T cell xenografts
Tegument proteins					
ORF47-ΔC (C-terminal deletion)	+	++	Normal	+	−
ORF47P-S1 (kinase motif mutant)	+	++	Normal	++	ND
ORF47P-S2 (kinase motif mutant)	+	++	Normal	++	ND
ORF47D-N (kinase motif mutant)	+	++	Normal	+	−
ORF66S (stop codon mutant)	+	+			+
ORF66 S48A (kinase domain mutant)	+				
ORF66 S331A (kinase domain mutant)	+				
ORF66 G102A (kinase domain mutant)	+	++		+	+/−
ORF66 S250P (kinase domain mutant)	+	++		++	++
ΔORF10 (deletion mutant)	+	++	Normal	+	++
ORF10 P28A (acidic domain mutant)	+	++	Normal	++	
ORF10 P28S (acidic domain mutant)	+	++	Normal	++	
Promoter mutants					
gI-AP1 (trans factor binding mutant)	+	++	Normal	++	++
gI-SP1 (trans factor binding mutant)	+	++	Normal	+/−	+
gI-USF (trans factor binding mutant)	+	++	Normal	++	++
gI-SP1/USF	+	++	Normal	−	+/−
gI-29RE-1 domain	+	++	Small-Norm	+	++
gI-29RE-2 domain	+	++	Normal	++	++
gI-29RE-3 domain	+	++	Normal	+/−	+/−
gI-29RE-4 domain	−				
ORF10-Δpro	+	++		+	
ORF10-Δpro +39/+63	+	++		++	
ORF10-pro ΔUSF	+	++		+	
Uncategorized mutants					
ΔORF35	+	+	Small	+	+
ΔORF65	+	++	Normal	++	++

[42]. For reasons that are not understood, varicella is less common and many adults remain susceptible to primary VZV infection in tropical regions [43].

Von Bokay suggested the epidemiological link between varicella and herpes zoster in 1892, based on the observation that children developed varicella following exposure to an adult with zoster lesions [44]. In 1925, Kundratitz demonstrated that inoculating children with fluid from herpes zoster lesions caused varicella [45]. Subsequently, Garland and Hope-Simpson proposed that herpes zoster was caused by reactivation of latent VZV occurring many years after varicella [46,47].

The incidence of herpes zoster in the United States is 11.12 cases per 1000 person-years, in subjects 60 years of age or older [48]. Herpes zoster occurs only in persons who have had primary VZV infection, but mild cases of varicella often escape diagnosis. Host factors that disrupt T-cell-mediated immune functions predispose to the reactivation of latent VZV and progression to the clinical signs of herpes zoster. Immunosenescence is associated with a

decline in VZV-specific T cell responses and explains the increasing incidence of herpes zoster with age [48,49]. The risk of herpes zoster is also increased in patients with cellular immunodeficiencies caused by cancer and cancer therapies, HIV infection, or immunosuppression after solid organ transplantation or for rheumatoid or other chronic diseases. VZV reactivation is rare among children, with an incidence of 0.74 per 1000 person-years. An inadequate host response to primary VZV infection acquired during the first year of life or *in utero* is a risk factor for herpes zoster in childhood.

Several VZV isolates have been sequenced, and subclassifications have been suggested that link particular isolates by their geographic origin [8,50]. However, antigenic variation appears to be minimal, based on the highly predictable protection conferred by previous VZV infection. One VZV strain, VZV-MSP, has been identified that has a gE mutation and an altered growth phenotype but is not known to have increased pathogenic potential in the natural host [51].

Characteristics of primary VZV infection, latency, and VZV reactivation

Varicella

The incubation period of primary VZV infection is well-defined because the exposure is easy to identify; the range is 10 to 21 days, with most cases occurring at about 2 weeks [52]. In contrast to HSV, household surveillance studies prove that VZV infection of a susceptible host is almost always symptomatic, even though many cases in young children are mild enough to be overlooked. Early symptoms, often appearing 24 to 48 hours before the rash, include fever, fatigue, and headache and continue for a few days after the onset of the typical vesicular rash of varicella or "chicken pox." Most initial skin lesions develop on the face, scalp, or trunk. The first stage of each lesion is an erythematous macule, which enlarges to a fluid-filled vesicle and then begins to crust after 24 to 48 hours (Figure 12.1A, Figure 12.5). VZV can be detected in peripheral blood mononuclear cells during this acute phase; the cells that harbor VZV DNA, detected by *in situ* hybridization, have the morphology of lymphocytes [53]. In addition to skin, lesion formation may occur on mucous membranes, producing painful ulcers. Varicella lesions are intensely pruritic and contain high titers of infectious virus. New lesions appear for 1 to 7 days, resulting in a total of about 100 to 300 lesions but ranging from 2 or 3 to more than 1500. Older children are likely to have more lesions than younger children, and the varicella rash in healthy adolescents and adults is typically quite extensive. Skin damage from chronic conditions, such as eczema, or acute damage (e.g., sunburn), increases the severity of varicella. Later "crops" of cutaneous lesions appear on the extremities but may not progress to the vesicular stage. Deep dermal lesions leading to skin scars are not common except where the first lesions appeared, often along the hairline or eyebrows.

Varicella is usually uncomplicated, though, if laboratory tests are done, most cases are associated with low lymphocyte and neutrophil counts in the acute phase, and liver function tests are often mildly abnormal. Most serious complications of varicella are caused by superinfection with *Staphylococcus aureus or Streptococcus pyogenes* (group A betahemolytic streptococcus) [3]. VZV can infect the lungs, causing severe or fatal viral pneumonia in healthy adults, but varicella pneumonia is quite rare in children. Adults are also susceptible to thrombocytopenia and hemorrhagic complications of primary VZV infection. The neurologic complications of primary VZV infection include meningoencephalitis and cerebellar ataxia [54,55,56]. In contrast to HSV encephalitis, which is associated with extensive necrosis of brain parenchyma, VZV encephalitis usually resolves within a few days. The etiology of VZV encephalitis and of cerebellar ataxia may be immune-mediated, rather than resulting from lytic infection of cells in the central nervous system. Other complications of primary VZV infection, such as transverse myelitis, optic neuritis, arthritis, nephritis, and mycocarditis, occur but are rare.

The resolution of primary VZV infection requires the induction of VZV-specific T cells, although the

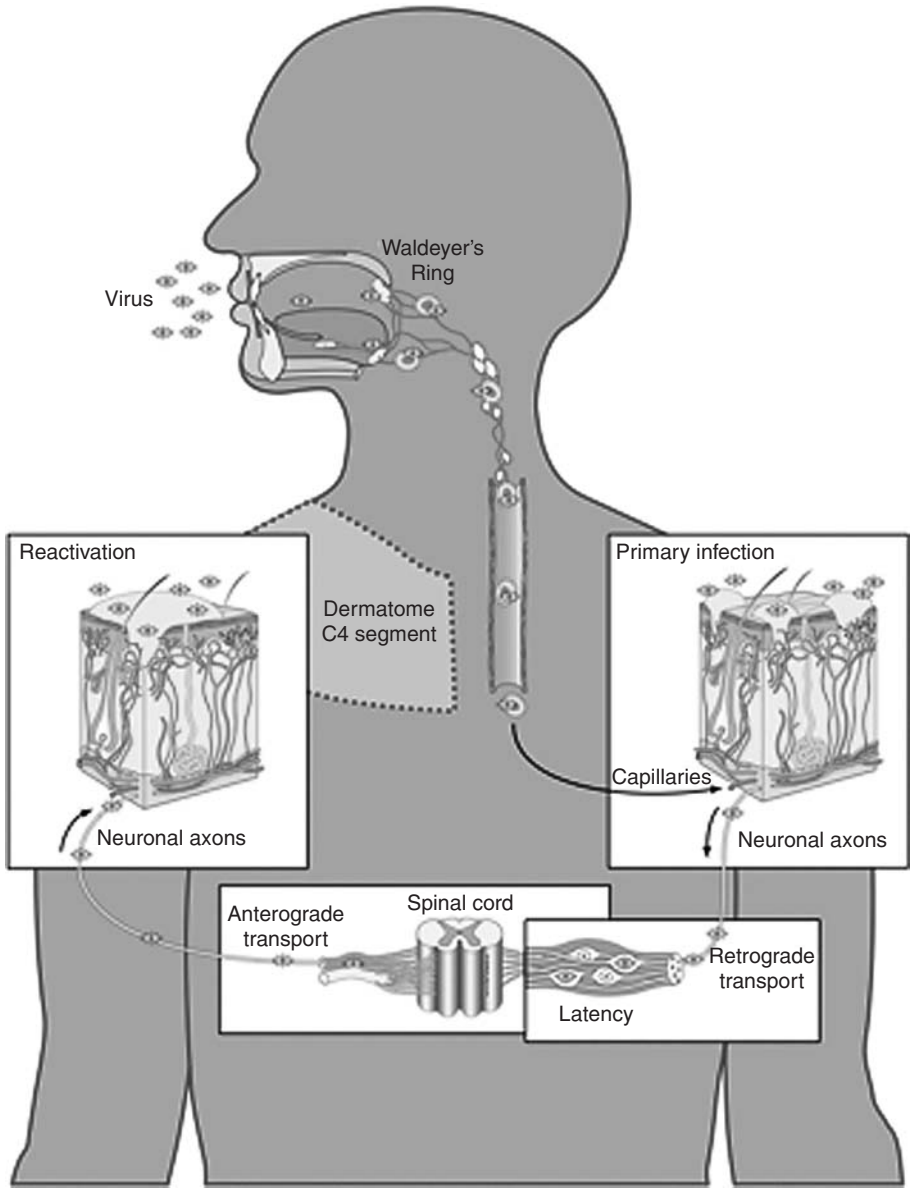


Figure 12.5. Model of the course of VZV infection in the human host. VZV infection is acquired by inoculation of mucosal epithelial cells via the respiratory route. Infection of T cells in Waldeyer's ring amplifies the virus and allows transport to the skin via a cell-associated viremia. Infection of skin produces the vesicular rash associated with chicken pox. During skin infection, VZ virions gain access to the sensory nerve cell body by retrograde axonal transport from fine nerve endings to establish a lifelong latent infection within the sensory ganglia. Clinical reactivation of latent VZV results in herpes zoster, during which VZ particles gain access to skin via anterograde axonal transport. (For figure in color, please see color plate section.)

infection also elicits a robust humoral immune response. The cessation of new lesion formation is associated with lymphocytosis and appearance of activated T cells that recognize VZV antigens. As a result, patients who have underlying diseases, such as leukemia or other cancers, congenital T cell immunodeficiencies, or HIV/AIDS, or who are receiving medical treatments that interfere with cellular immunity, such as drugs given to prevent the rejection of organ transplants, are at risk of progressive primary VZV infection [3]. Under these circumstances, and in newborn infants, varicella can lead to VZV pneumonia, hepatitis, coagulopathy, encephalitis, and other life-threatening complications. These patients require antiviral therapy with acyclovir or related drugs to compensate for their impaired cell-mediated immunity and terminate viral replication [3]. Rarely does maternal varicella in pregnancy result in transplacental transmission of the virus and severe damage to the fetus – recognized as congenital varicella syndrome [3].

VZV latency

Primary VZV infection appears to result in persistence of the virus in sensory ganglia invariably, even if varicella is mild. However, little is known about the initial infection of sensory ganglia because primary VZV infection is seldom fatal. VZV is presumed to reach ganglia via retrograde transport by nerve axons from skin lesions, but VZV viremia also allows for possible hematogenous spread [3,57]. VZV is found in ganglia from patients who died during the varicella incubation period, and zosteriform rashes may occur simultaneously with the usual varicella rash. These observations indicate that VZV can enter sensory ganglia before skin lesions appear. VZV, like HSV-1 and -2, establishes lifelong infection of sensory ganglia. Investigations of VZV latency in sensory ganglia obtained at autopsy many decades after primary VZV infection have yielded differing estimates of VZV genome copies, ranging from 6.0–9000/10^5 ganglia cells, and estimates of infected cell frequencies have varied from 0.10–30% of neurons and 0–30% of nonneuronal cells [3]. However, improved techniques reported more recently that use dissociated ganglia cells or laser capture microscopy and PCR indicate that VZV persists in 1.5–4.1% of neurons [58,59,60]; while some satellite cells harbor VZV genomes, these studies disproved earlier evidence that VZV persists primarily or exclusively in satellite cells [61]. VZV latency in sensory ganglia differs from HSV in that VZV has no latency-associated transcripts (LATs) [16,62]. In contrast, several lytic VZV genes appear to be transcribed. Of these, ORF63 is reported most consistently, ORFs 62 and 29 (ssDNA-binding protein) are common, and ORF4 (IE regulatory), ORF21 (nucleocapsid), and ORF66 (viral kinase) transcripts have been detected [63,64,65,66]; ORFs 10, 28, 36, 40, 61, and ORFs encoding glycoproteins are not usually found. ORFs 4, 21, 29, 62, and 63 mRNA are also detected in ganglia after paraspinal injection of rodents [67]. In addition to gene transcription, expression of VZV proteins in the cytoplasm of neurons has been described in autopsy specimens from subjects without evidence of herpes zoster at the time of death. IE63-expressing cells were reported in 4/21 [68], 3/3 [69], and 10/10 adult ganglia [70] but not infant/fetal ganglia controls. Two of these reports also describe IE62, IE4, ORF21, and ORF29 proteins in some specimens, and ORF66 protein has also been reported [63,69,70]. Because investigation is limited to autopsy studies, it is difficult to exclude the effects of postmortem changes on expression of viral as well as host cell proteins [71], and some estimates of the frequencies of protein-expressing cells are higher than data about the numbers of cells that contain VZV DNA. One hypothesis is that IE proteins are made but are sequestered in the neuronal cell cytoplasm, thereby blocking their transactivating activity [69]. However, VZV gene transcripts are detected, suggesting that some IE62 reaches the nucleus but does not transactivate VZV glycoproteins and other genes. Current information about VZV latency remains limited and descriptive and, as is true of HSV, the mechanisms by which VZV genomes persist in neurons and, apparently, to some extent in satellite cells within sensory ganglia are not understood.

Herpes zoster

When VZV reactivates from latency, it is likely that reactivation remains either localized to the involved neuron and is abortive or progresses to the production of VZ virions that traffic from the cell body along neuronal axons to the skin. Delivery of viral particles to the epidermal cells can be expected to result in some replication. However, whether cutaneous lesions appear at the skin surface and how extensive the rash becomes probably depends on host immune factors. If reactivation progresses to clinical symptoms, VZV causes a vesicular rash in a distribution corresponding to the cutaneous dermatome that is innervated by neurons in the involved cranial nerve or dorsal root ganglion (Figure 12.1B; Figure 12.5) [3]. The rash is usually preceded by intense pain of unknown etiology, not diagnosed until the rash develops. In the first phase, small clusters of cutaneous lesions, with the morphology of varicella vesicles, appear within the affected dermatome and contain infectious virus. Because of the involvement of sensory neurons, acute herpes zoster is accompanied by neuropathic pain and marked hypersensitivity of the skin. When cerebral spinal fluid is obtained, most patients have increased protein and lymphocytes; VZV may be recovered from the specimen. Extension of the rash within the dermatome usually continues for several days; in the worst cases, the complete dermatome is involved. Resolution of the rash is associated with a robust VZV-specific T cell response; full cutaneous healing often requires several weeks, and hypersensitivity can persist for several months. VZV reactivation that fails to progress to cutaneous lesions is called "zoster sine herpete" and may be associated with prolonged pain and paresis. Postherpetic neuralgia (PHN) is the most common complication of herpes zoster. PHN causes serious morbidity in elderly and immunocompromised patients. Encephalitis is a rare complication; cerebral angiitis, characterized by vasculitis, thrombosis, and cerebral infarcts can occur with herpes zoster involving the ophthalmic or other cranial nerves in elderly individuals and has a high mortality rate [57]. The motor weakness or facial palsies that complicate some cases of herpes zoster are usually transient but may last for many months. Because of their cellular immune deficiencies, immunocompromised patients typically have more severe dermatomal involvement and require antiviral treatment because they are at risk for VZV dissemination to the lungs, liver, and central nervous system. The appearance of scattered cutaneous lesions, resembling a varicella rash, is evidence of viremia and dissemination. A syndrome of atypical, nonlocalized herpes zoster occurs in some immunocompromised patients who have viremia due to VZV reactivation without evidence of dermatomal disease and has a high mortality rate. Patients with HIV/AIDS may develop a chronic cutaneous form of herpes zoster that responds poorly to acyclovir and other antiviral drugs. Rarely, immunocompromised patients may develop a multifocal leukoencephalopathy due to VZV reactivation that was asymptomatic or occurred months before the central nervous system involvement was diagnosed.

Investigations of VZV pathogenesis and immunobiology in the SCIDhu mouse model

Clinical studies have defined some elements of VZV pathogenesis, such as the detection of infectious virus in skin lesions and VZV DNA in sensory ganglion cells. However, since VZV is a human-restricted pathogen, no small animal model mimics the life cycle of VZV in the natural host. As defined clinically, VZV pathogenesis has three stages (Figure 12.5). The first stage is a 10 to 21-day incubation period, presumed to be initiated by inoculation of respiratory mucosal epithelial cells. During this stage, the virus must spread from the site of inoculation to multiple skin sites of replication. The second stage is acute disease, during which scattered discrete cutaneous vesicular lesions appear; lesion biopsies show infected cells extending from the dermis to the epidermal skin layers, with cytopathic changes in infected cells and later, a lymphocytic inflammatory response. The third stage is latency in the sensory nerve ganglia, which may be associated with

subsequent reactivation that progresses to herpes zoster.

The development of the SCIDhu mouse model has made it possible to investigate some of these events in VZV pathogenesis (reviewed in reference 72). VZV is not infectious for mice, rats, or rabbits, and infection in guinea pigs is very restricted or abortive. The capacity to engraft human tissue xenografts in mice with the severe combined immunodeficiency (SCID) mutation has enabled studies of VZV interactions within differentiated human target cells in an intact tissue microenvironment *in vivo*. VZV replication is limited to the human tissue xenografts because the host restriction of VZV prevents infection of the mice, despite their immunodeficient condition. Using SCIDhu mice, it allowed the analysis of VZV tropism for human T cells within thymus/liver xenografts, dermal and epidermal cells in skin xenografts, and sensory neurons and satellite cells in DRG xenografts (Figure 12.2).

VZV T cell tropism

SCID mice with human thymus/liver (thy/liv) tissue xenografts were established to examine VZV lymphotropism [12]. Thy/liv implants are constructed by coimplantation of fetal thymus and liver tissue, which differentiate *in vivo* and contain mature $CD4^+$ and $CD8^+$ T cells and immature $CD4^+/CD8^+$ T cell subpopulations within thymic epithelial stroma (Figure 12.2A-B). When these xenografts were inoculated with VZV-infected fibroblasts, VZV infected T cells, replicating efficiently in all subpopulations, which demonstrated that VZV is a lymphotropic herpesvirus (Figure 12.2C) [12]. Infectious VZV was released from T cells infected *in vivo*, and VZV-infected T cells did not exhibit fusion with other T cells despite their close proximity.

VZV lymphotropism mediates spread to skin tissues

The capacity of VZV to infect and replicate in lymphocytes provides a vehicle for viral spread within the human host that is unique among human alphaherpesviruses. In the absence of opportunities to investigate VZV pathogenesis in small animal models, one hypothesis was that primary VZV infection resembled mousepox pathogenesis [73]. According to this model, inoculation via the respiratory route is followed by infection of peripheral blood mononuclear cells in regional lymphoid tissue, which are released to cause a primary viremia that carries the virus to reticuloendothelial organs. Viral amplification within reticuloendothelial organs leads to a second viremic phase that delivers the virus to the skin. Based on our observation that VZV exhibited tropism for T cells in thymus/liver xenografts, we proposed the more detailed hypothesis that VZV might initiate infection by transfer from respiratory epithelial cells into tonsil T cells (Figure 12.5) [11,13,14]. Respiratory epithelial cells line the tonsillar crypts, and T cells migrate across these surfaces into and out of the tonsils that form Waldeyer's ring in the posterior pharynx. We found that tonsil T cells were highly permissive for VZV infection *in vitro* and, interestingly, memory $CD4^+$ T cells that express the skin homing markers, cutaneous leukocyte antigen (CLA), or chemokine receptor (CCR) 4, were especially permissive (Figure 12.5) [14]. Thus, VZV may gain access to T cells shortly after initial replication in mucosal epithelial cells, not unlike the model of Epstein-Barr virus pathogenesis, in which EBV infects B cells in the tonsils.

Based on this hypothesis, we investigated whether VZV-infected tonsil $CD4^+$ T cells could transfer VZV to skin in SCIDhu mice with full thickness human dermal xenografts. Direct inoculation of these skin xenografts causes epidermal changes that are indistinguishable from biopsies of natural varicella lesions, except for the absence of the lymphocytic inflammatory response (Figure 12.2F) [14,74]. When SCIDhu mice with skin xenografts were inoculated with VZV-infected tonsil T cells via the tail vein, human T cells were detected in skin at locations surrounding hair follicles within 24 hours, and progressive infection of the skin xenografts followed [11,13,14]. Intravenous inoculation of VZV-infected fibroblasts did not result in VZV transfer to skin xenografts. These experiments suggest that VZV

tropism for tonsil T cells provides a direct mechanism for cell-associated spread of VZV to skin during primary VZV infection. Moreover, VZV has evolved to exploit the normal process of programmed immune surveillance in skin through preferential infection of T cells that express skin homing markers.

Pathogenesis of VZV skin infection

Although VZV-infected T cells homed to human skin xenografts within 24 hours after introduction into the circulation, viral replication within human skin xenografts required 10 to 21 days to form the cutaneous lesions extending through the skin surface that are characteristic of "chickenpox" [13,14]. Whereas VZV replication in human fibroblasts *in vitro* is a rapid lytic process, the tempo of VZV replication in differentiated human dermal and epidermal cells *in vivo* is much slower. Analysis of the pathologic changes in VZV-infected skin xenografts shows gradual cell-cell spread and formation of polykaryocytes by fusion of VZV-infected skin cells.

Because SCID mice lack any adaptive immune response mechanisms, these observations suggested that innate antiviral responses might modulate the pathogenic process. Interferon-alpha (IFN-α), a known inhibitor of herpesviral replication, is not produced during VZV infection of fibroblasts *in vitro*. However, immunohistochemistry analysis of VZV-infected skin xenografts revealed extensive expression of IFN-α in uninfected epidermal cells surrounding foci of VZV-infected cells [13,14]. IFN-α was not detected in the cells that expressed VZV proteins [13,14]. Uninfected cells adjacent to skin lesions also exhibited phosphorylation and nuclear translocation of Stat1 protein, which is an indicator of activation of the global IFN pathway [13,14]. The biological relevance of IFN upregulation was demonstrated by giving antibody that blocked the IFN-a/b receptor to mice with VZV-infected skin xenografts; lesions were much larger and yields of infectious VZV from the xenografts were significantly higher in treated compared to mock-treated mice (Figure 12.6). Activation of the NF-kB pathway was also demonstrated in epidermal cells surrounding VZV-infected cells in skin xenografts [75]. Given these barriers to the progression of VZV infection in skin, it is not likely that delivery of VZV to skin by a late, secondary viremia at the end of the incubation period could produce skin lesions, as suggested by the mousepox model. Instead, the data are more consistent with early transport of the virus to skin by infected tonsil T cells, with the potential for further amplification as uninfected T cells migrate through sites where skin lesions are developing, become infected, and re-enter the circulation to distribute virus to more skin sites (Figure 12.6). This process would also account for the several "crops" of VZV lesions that appear during varicella. It is possible that some amplification also occurs in reticuloendothelial cells, but this phase is not necessary to explain the events in VZV pathogenesis according to this model.

VZV neurotropism

Most recently, we have developed a model of VZV neurotropism by establishing DRG xenografts in SCID mice [76]. These DRG xenografts contain the cell bodies of afferent spinal nerves, which continue to express human neural cell markers and maintain the expected cellular morphology, as well as supportive cells, within the normal dense connective tissue matrix (Figure 12.2G-H). VZV tropism for sensory neurons was assessed by direct inoculation of DRG xenografts using low passage clinical isolates, including the parental vaccine strain, Oka (Figure 12.2I). Our results demonstrated that the initial phase of VZV infection in DRG xenografts is characterized by productive replication of VZV. High viral genome copy numbers (7.1×10^7–8.0×10^8 copies/10^5 cells) were associated with recovery of infectious virus at 14 days after infection. VZ virions were detected in neurons, and VZ proteins were detected in neurons, as well as encapsulating satellite cells. Within 8 weeks of infection, VZV genome copy numbers decrease 100-fold and infectious virus is no longer recovered, indicating the transition from productive to persistent infection. Differential regulation of VZV gene expression characterizes VZV persistence in DRG, with continued transcription of

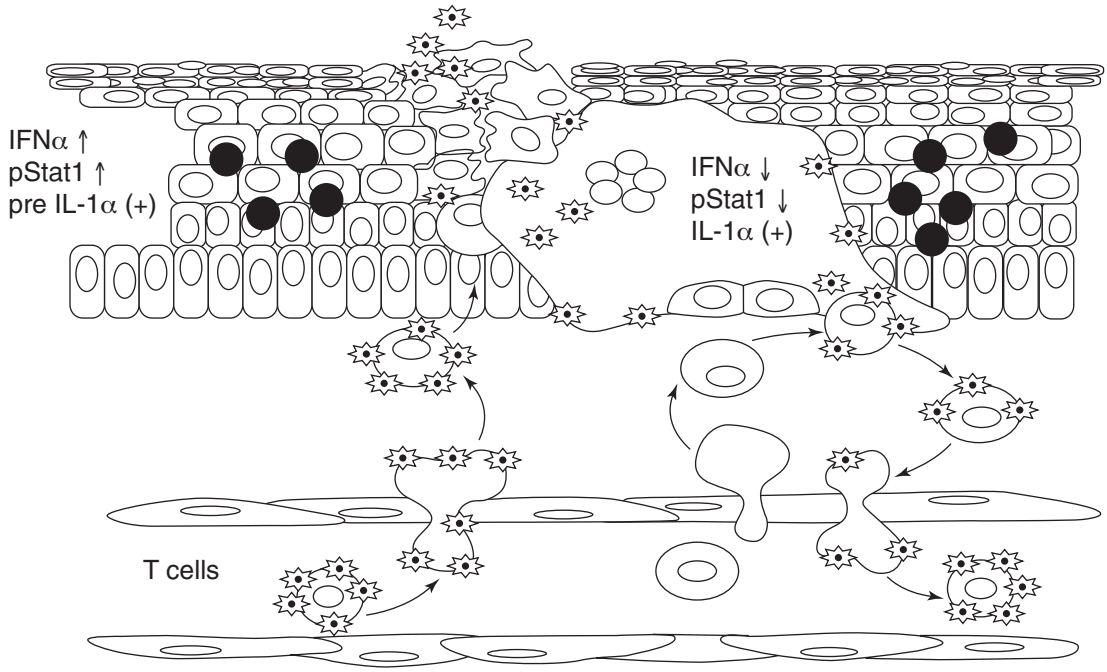
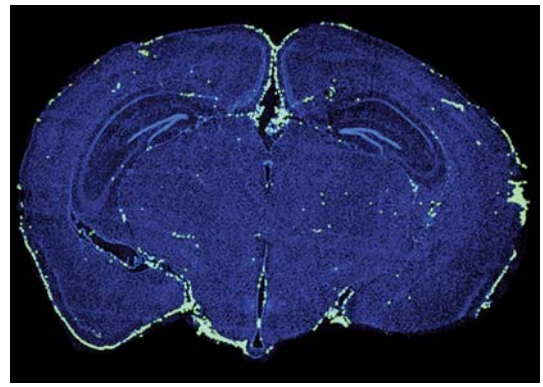


Figure 12.6. A model for pathogenesis of VZV skin infection. Transfer of virus from T cells initiates VZV replication in deep dermal skin tissues during primary VZV infection. During the course of the 10 to 21-day incubation period, innate IFN responses in epidermal cells slow the progression of the VZV lesion. IFN-α-stimulated signaling of neighboring cells attenuates cell-cell spread. Secondary crops of lesions occur due to infection of migrating T cells. A similar innate immune response of epidermal cells is expected to modulate the progression of skin infection during herpes zoster. Figure adapted from Ku et al., 2004 [14].

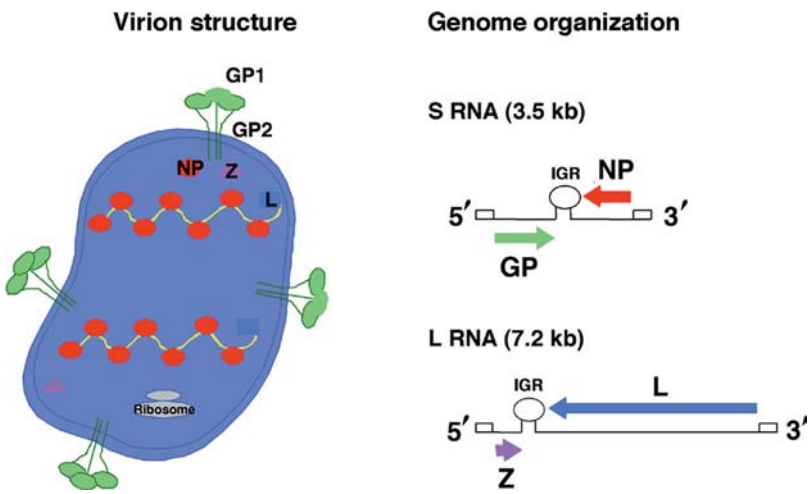
ORF63 but not of ORF31, which is a late gene-encoding glycoprotein B, by quantitative reverse transcriptase (RT)-PCR for mRNA [76]. A similar block of glycoprotein synthesis was observed during VZV infection of human neural stem cells in the brain in a nonobese diabetic SCID mouse model [77]. Thus, the course of VZV infection of human sensory neurons appears to be a biphasic process in which productive replication precedes a phase in which the VZV genome persists in neurons, absent infectious virus production but characterized by sustained ORF63 transcription, whereas late glycoprotein genes are not transcribed. The SCID DRG model of VZV neuropathogenesis recapitulates acute and persistent VZV infection of sensory neurons during infection in the natural host. The transition to persistence occurs without any requirement for immune control by VZV-specific adaptive immune mechanisms. This significant observation implies that VZV has evolved self-regulatory measures, which temper acute replication in sensory ganglia so that sites for VZV latency remain intact.

Experiments in the SCIDhu DRG model also indicate that VZV T cell tropism may facilitate neurotropism [76]. When VZV-infected T cells were given intravenously to SCIDhu mice with DRG xenografts, the virus was transferred to the human neural tissue and infection progressed as it does after direct inoculation. While it is presumed that VZV is likely to reach neuronal tissues via retrograde transport along nerve axons from sites of replication in skin, these observations suggest that VZV lymphotropism may provide a second mechanism by which the virus reaches DRG sites of persistence [76].

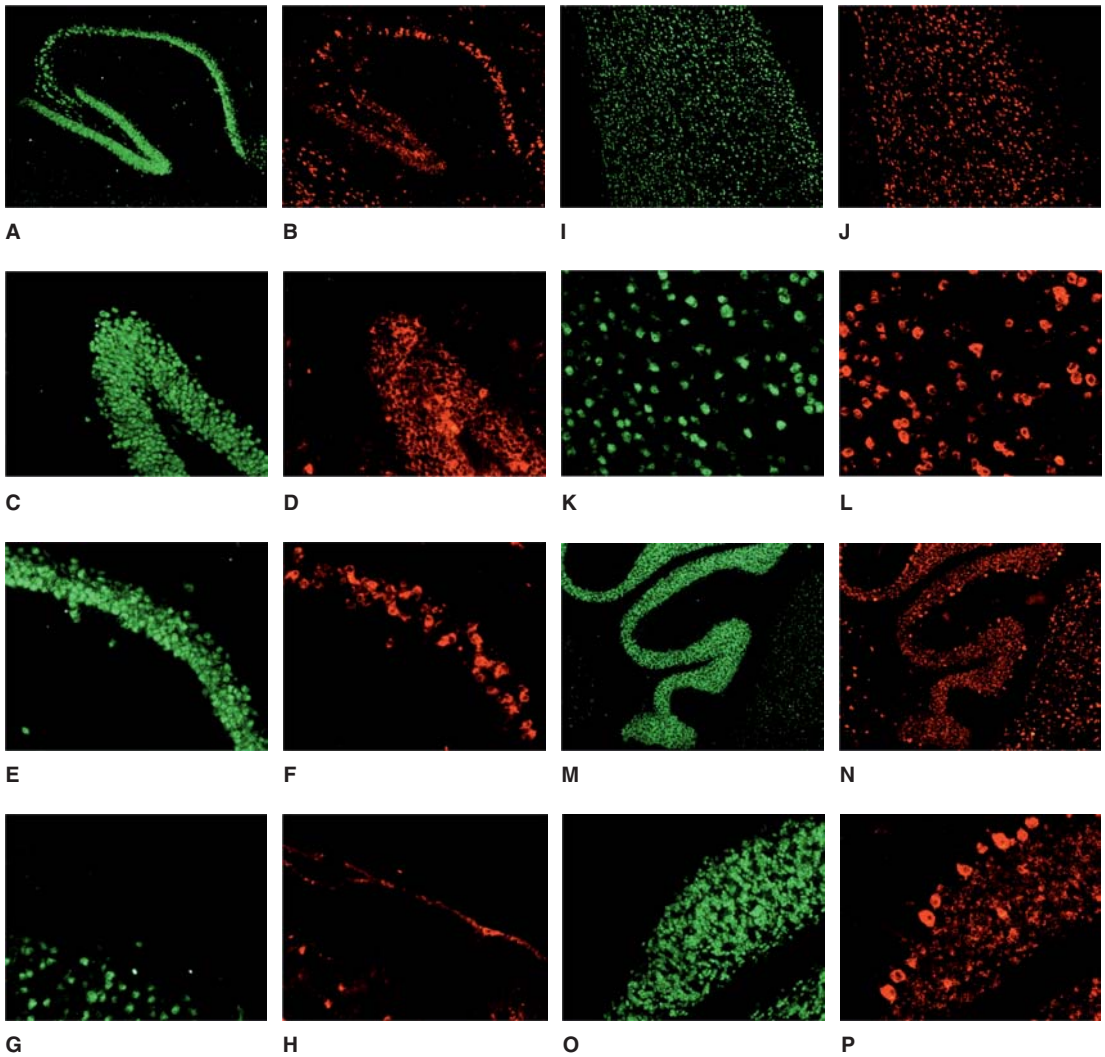
Color plate 3.1. Rabies virus infection in human postmitotic neurons (NT2-N). Human neurons, infected for 24 hours with rabies virus (strain CVS), were stained with FITC-labeled antibodies directed against nucleocapsid (green). Viral nucleocapsids accumulate in inclusion bodies near the cell's nucleus. Bars represent 10 μm.



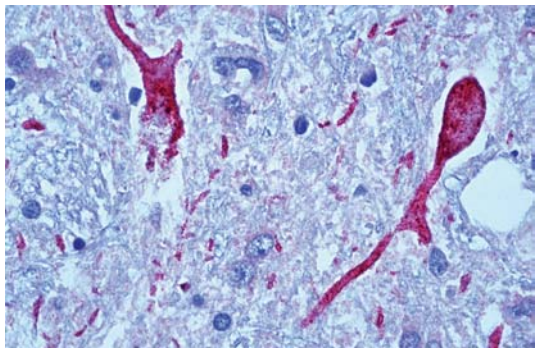
Color plate 5.2. Virus distribution in the brain of mice suffering from acute LCM. Adult mice were inoculated i.c. with LCMV and sacrificed on day 5 of diseases. Viral antigen was detected in whole brain sections by immunofluorescence staining using hyperimmune guinea pig serum to LCMV and a FITC-labeled secondary antibody (green). Cell nuclei in brain tissue were counterstained with DAPI (blue). Image courtesy of Dr. Dorian B. McGavern.



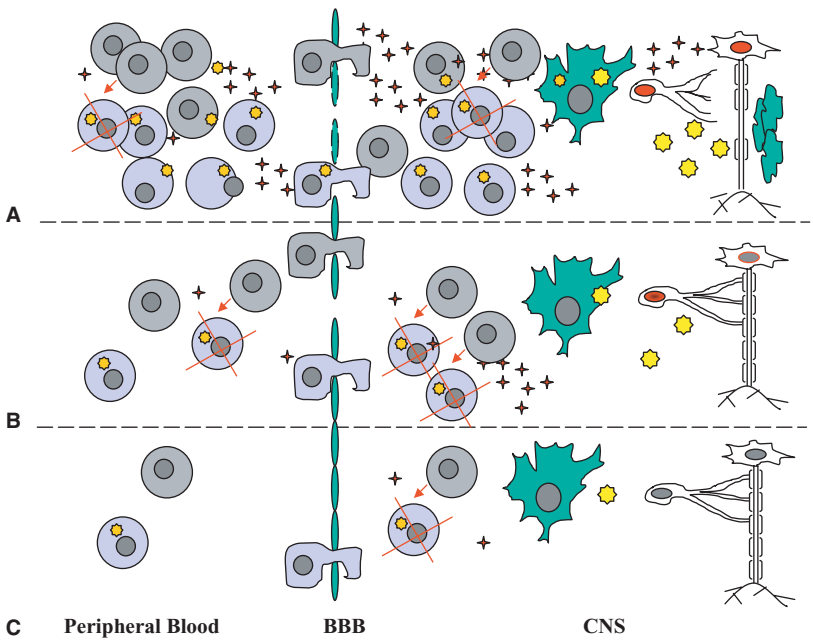
Color plate 5.1. Schematic of arenavirus particles and their genome organization. For details, see text.



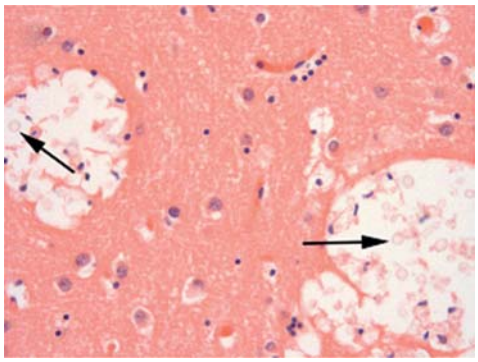
Color plate 5.3. Virus distribution in the brain of LCMV-Pi mice. Viral antigen was detected in whole brain sections of 6-month-old LCMV-Pi mice by immunofluorescence staining using hyperimmune guinea pig serum to LCMV and a Rhodamine Red-X-labeled secondary antibody (red). Neurons were labeled with an antibody to NeuN and a FITC-labeled secondary antibody (green). Hippocampus (a, b), dentate gyrus (c, d), and C1 region (e, f). Meninges (g, h). Cerebral cortex (i – l) and cerebellum (m – p). Reprinted with permission from [135].



Color plate 7.5. Photomicrograph of immunohistochemical staining of brain tissue from a fatal West Nile encephalitis case, showing West Nile antigen-positive neurons and neuronal processes in the brain stem and anterior horn cells (in red). (From W.-J. Shieh and S. Zaki, CDC, 1999.)



Color plate 8.5. Hypothesis of CNS damage in HTLV-1 infection. A. The pathogenic mechanism of HAM/TSP is unknown, but the current hypothesis suggests that inappropriate infiltration of T cells ($CD8^+$: gray cells, $CD4^+$: lilac cells, Tax protein: orange star) into the CNS, across a damaged blood-brain barrier (BBB), produces an excess of proinflammatory cytokines (red stars) that result in glial activation (green cells), production of reactive oxygen species (yellow stars), and neuronal damage (white cells). **B.** At an early stage of HAM/TSP development, or in an AC with a high proviral load, we propose that T cell infiltration and neuronal damage occur, but at a lower level such that single neurological symptoms and clinical signs may be apparent. **C.** In ACs with a low HTLV-1 proviral load, T cell infiltration into the CNS occurs at a low level where HTLV-1 activity and inflammation are controlled efficiently and no neuronal damage results.



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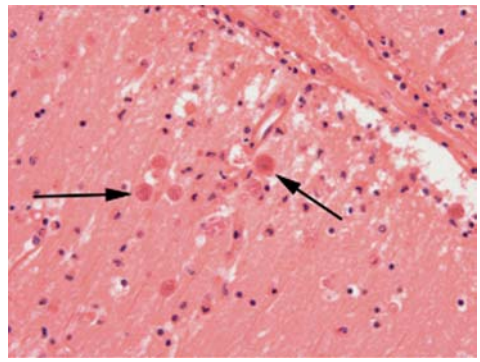

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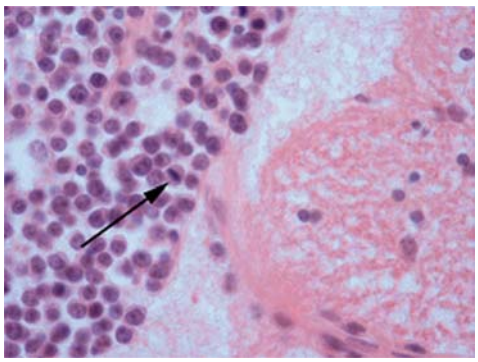

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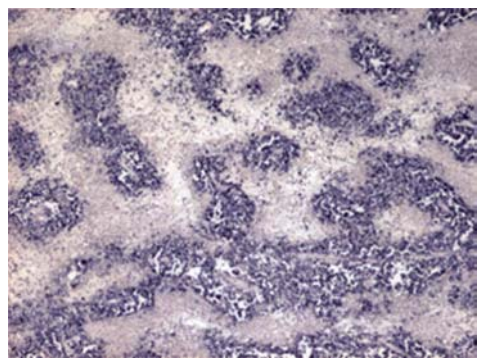

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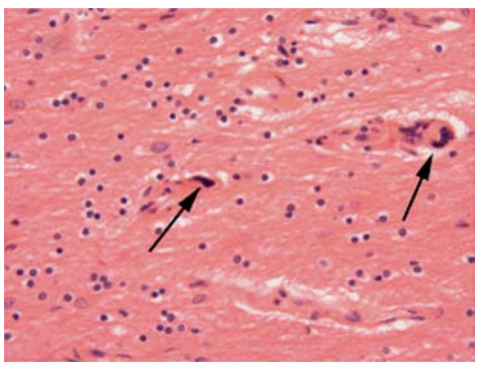

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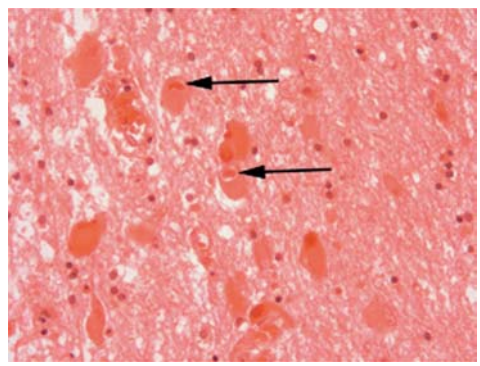

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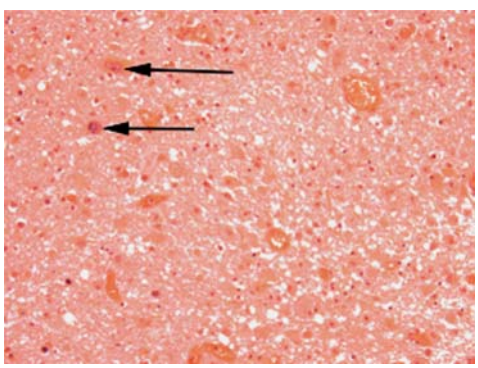
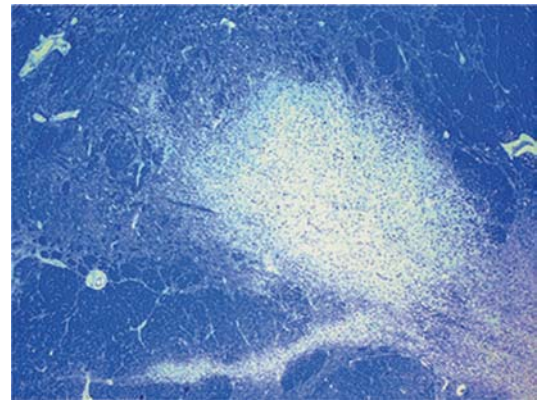


G

Color plate 9.8. Neuropathological findings in HIV-infected subjects (A) Cryptococcus (×200). The arrows indicate two of many cryptococcus organisms in cystic cavities within the basal ganglia. (B) Toxoplasma (×200). Showing many cysts containing organisms in the cerebellar cortex. (C) Primary central nervous system lymphoma (PCNSL) (×400). Showing malignant B lymphocytes abutting on white matter in the basal ganglia. A mitotic cell is arrowed. (D) Primary central nervous system lymphoma (PCNSL) (×100). Showing *in situ* hybridization for Epstein-Barr virus. (E) HIVE (×200). Showing perivascular giant cells in the white matter. (F) CMV (×200) showing enlarged cells with intranuclear viral inclusions. (G) PML (×100) showing necrotizing demyelination of the white matter with enlarged bizarre glial cells.

A



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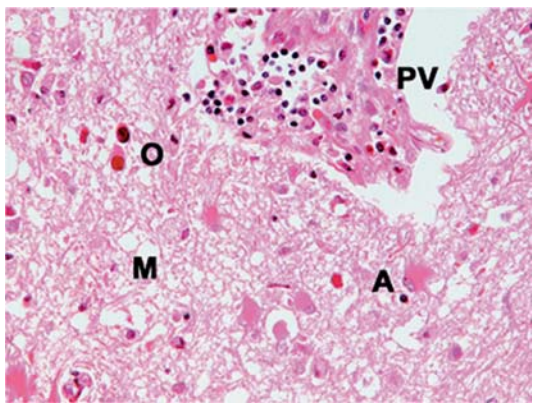

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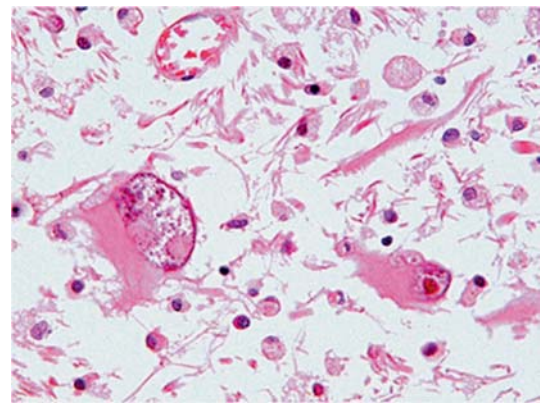

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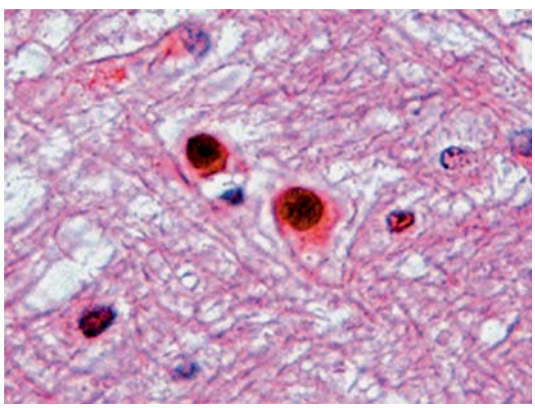

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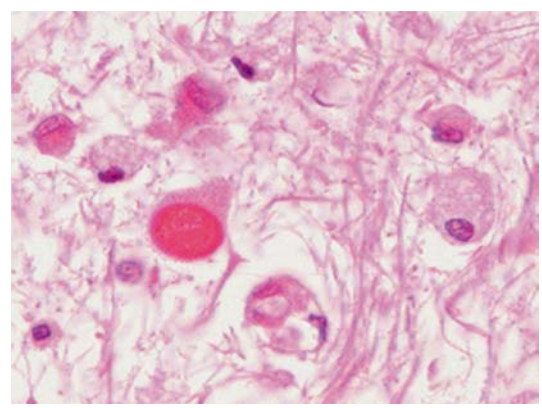
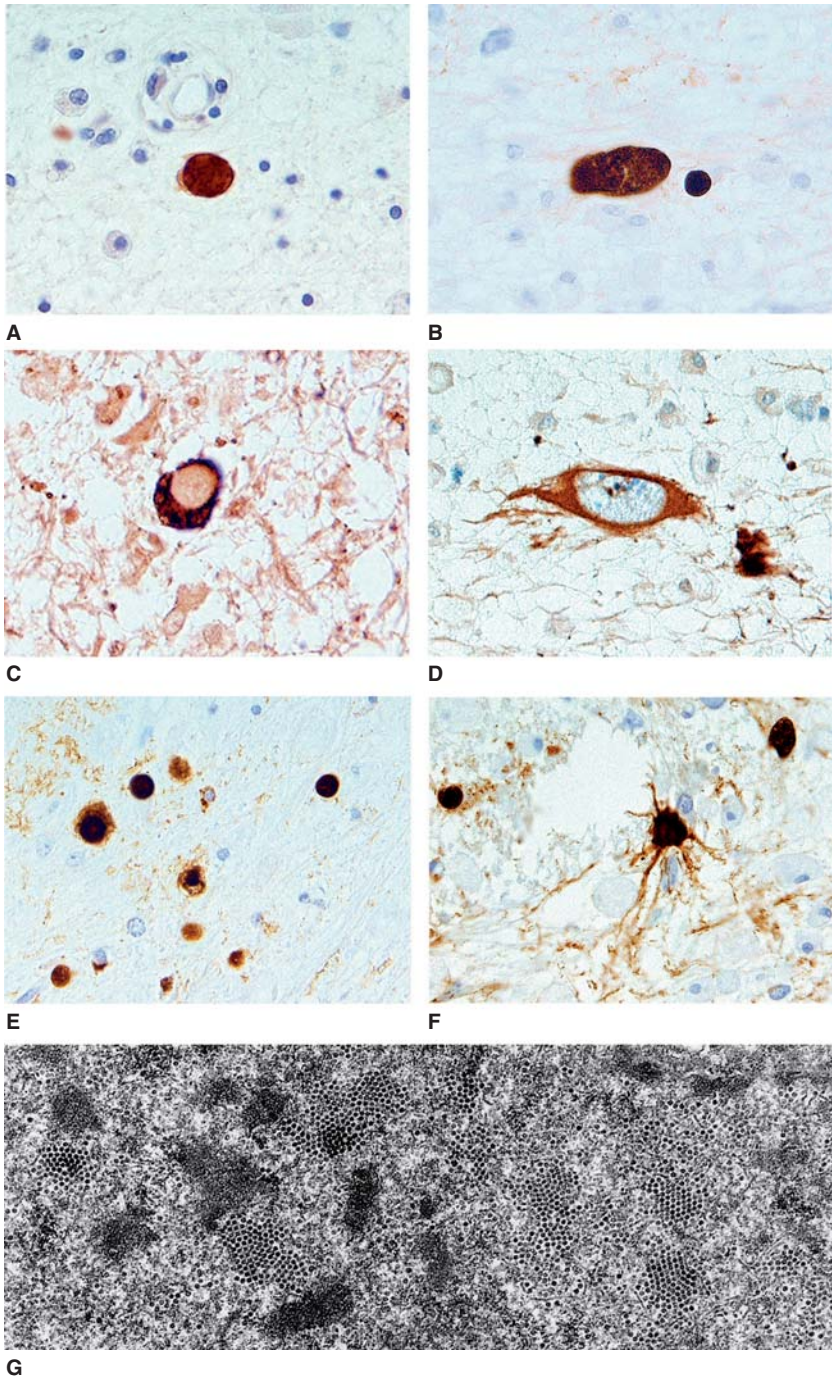
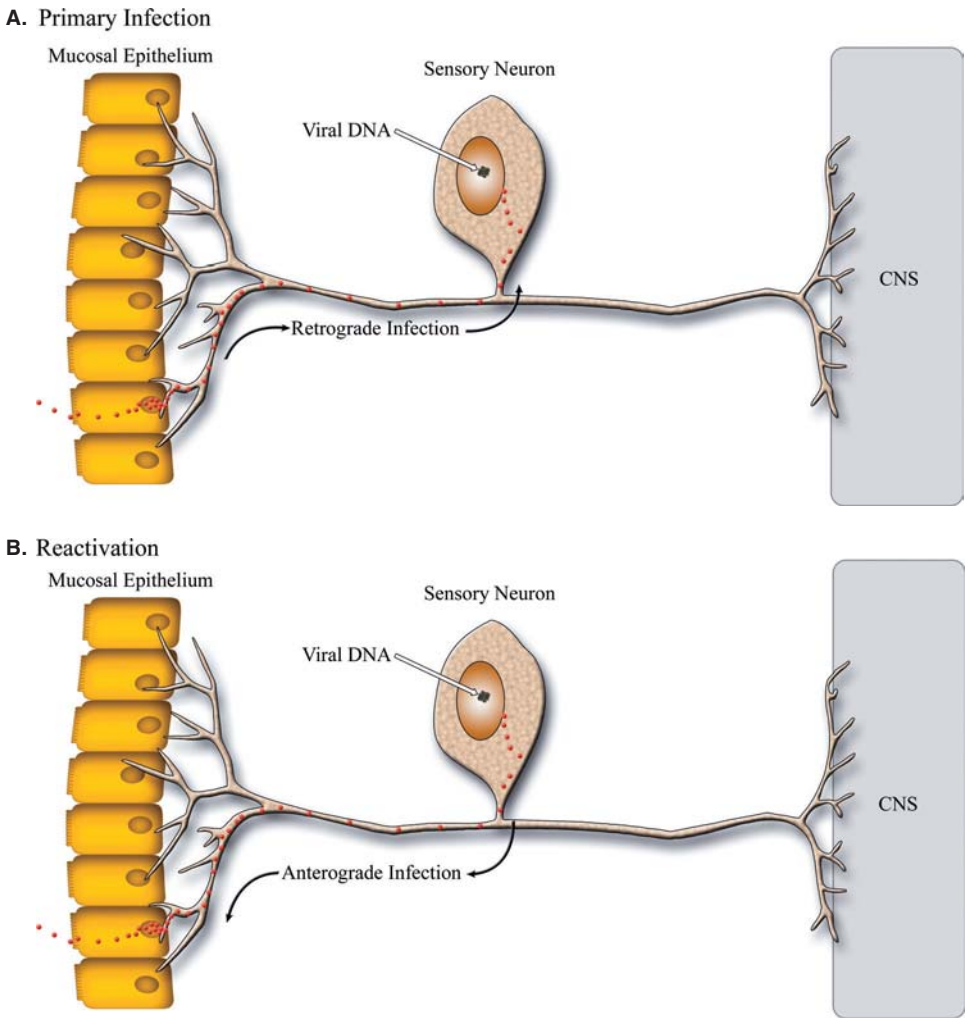


F

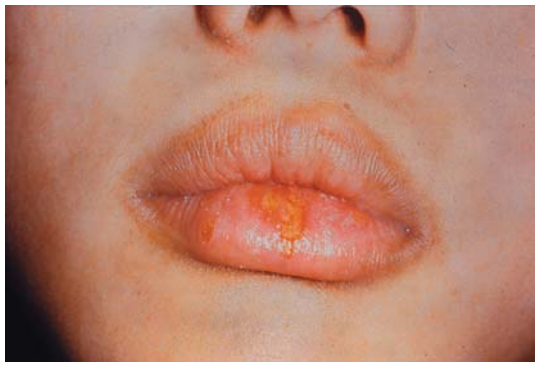
Color plate 10.6. Histological characteristics of progressive multifocal leukoencephalopathy. Montage of the frontal lobe of a patient with PML demonstrating multiple confluent plaques of demyelination in the subcortical white matter (Panel A). The center of a demyelinated plaque is shown in a low magnification view (Panel B, Luxol Fast Blue, original magnification ×20). The most prominent features of PML are shown in this view of a demyelinated plaque, oligodendrocytes harboring intranuclear inclusion bodies (O), bizarre astrocytes (A), and foamy macrophages (M), adjacent to a blood vessel infiltrated by perivascular cuffs of lymphocytes (PV) (Panel C, original magnification ×200). Bizarre astrocytes (Panel D) and enlarged oligodendrocytes with intranuclear inclusion bodies in plaque at early (Panel E) and late stages (Panel F) of demyelination are shown (H–E, original magnification ×1000).



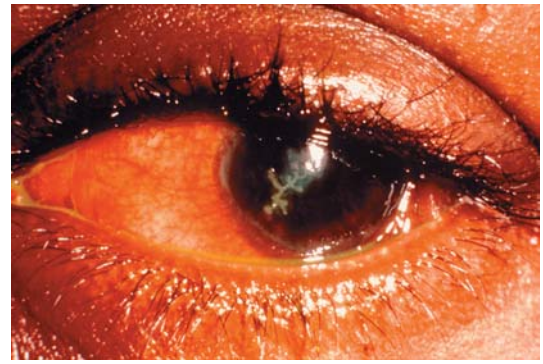
Color plate 10.7. Detection of JCV proteins in PML. Immunohistochemistry with a specific T-antigen antibody shows its location in the nuclei of enlarged oligodendrocytes (Panel A) and bizarre astrocytes (Panel B). In contrast, the late accessory product agnoprotein is detected in the cytoplasm of oligodendrocytes (Panel C) and bizarre astrocytes (Panel D). The JCV capsid protein VP-1 is robustly expressed in the nuclei and cytoplasm of both phenotypes of cells, oligodendrocytes harboring inclusion bodies (Panel E), and bizarre astrocytes (Panel F), indicating active viral replication. Panels A to D original magnification 1000×, E, and F 400×. Electron microscopy of the inclusion bodies shows the characteristic icosahedral viral particles (Panel G).



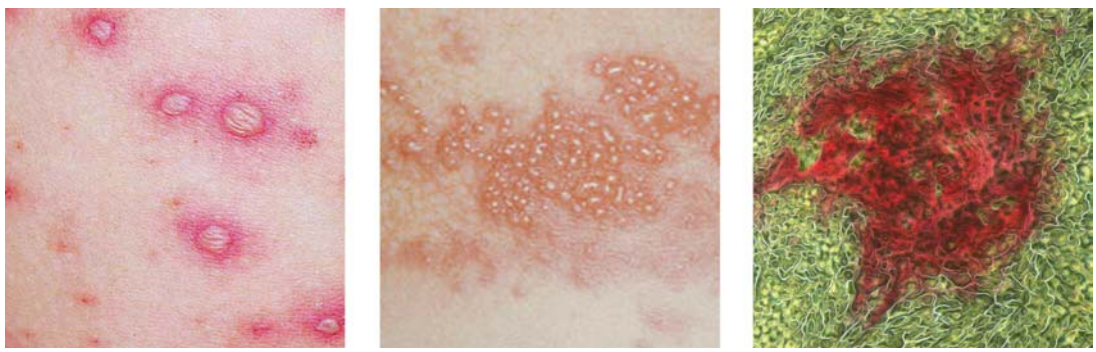
Color plate 11.3. Diagram of the HSV lytic/latent infection cycle. (A) During the typical course of the initial (primary) infection, HSV infects the mucosal epithelium and gains access to sensory nerve termini. After entry into the termini, the capsid is transported by retrograde fast axonal transport to the neuron's cell body, where the viral DNA enters the nucleus. Here, the virus can become latent. (B) Periodically, the latent genome can reactivate. When this occurs, progeny virions are transported in an anterograde manner to the site of the primary infection, where the virus can reinfect the mucosal cells and shed virus.



Color plate 11.4. Recurrent herpes simplex labialis. Common recurrent lesion (cold sore) on the lip produced by HSV-1 reactivation.

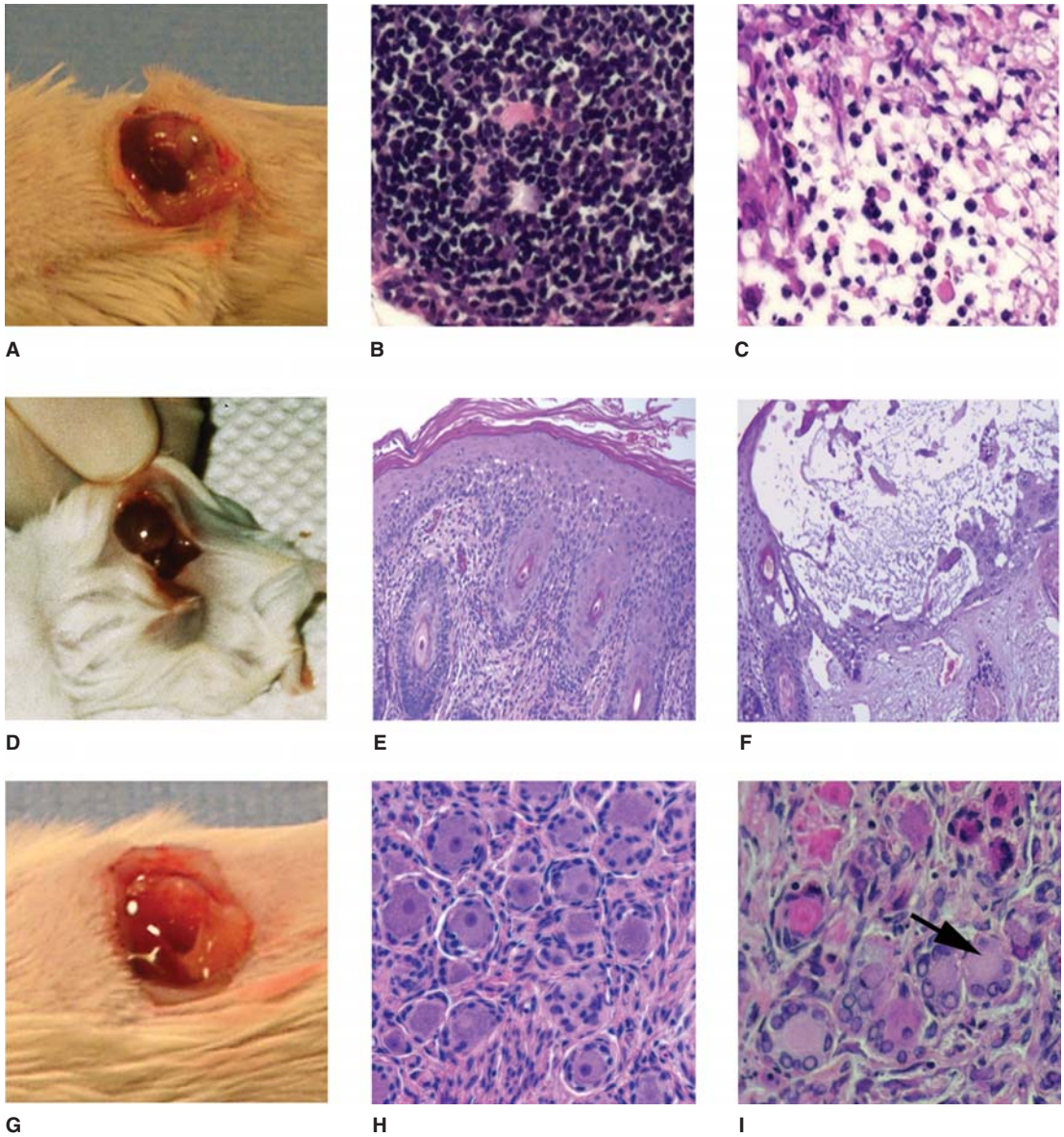


Color plate 11.5. HSV-1 dendritic lesions on the cornea. HSV-1 can cause dendritic lesions on the cornea, which can be visualized by fluorescein staining.

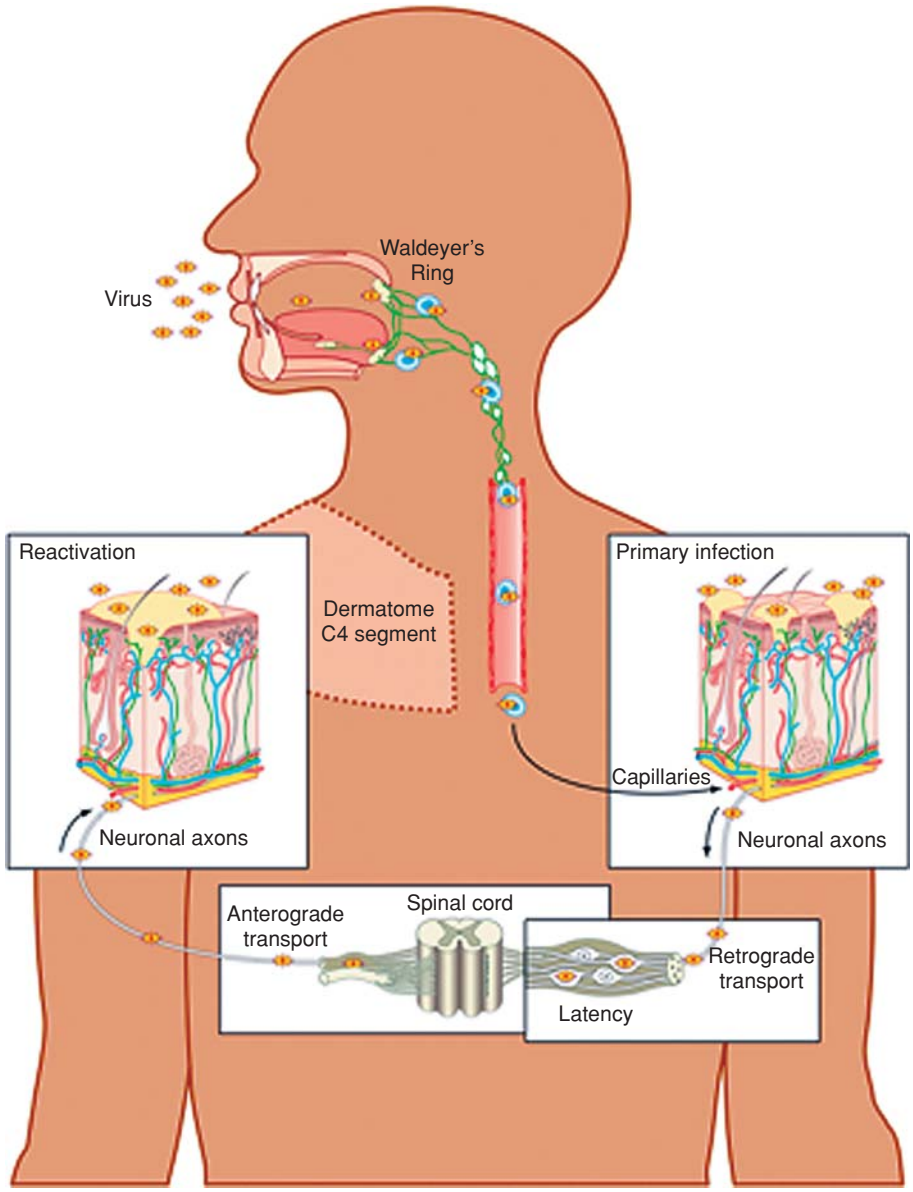


A B C

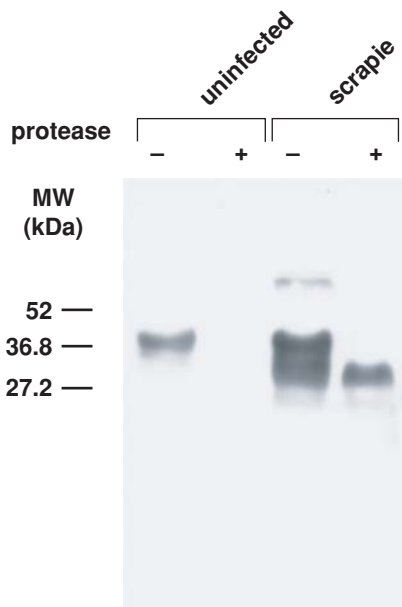
Color plate 12.1. Varicella and herpes zoster lesions and VZV replication in cultured cells. (A) Photograph of a typical crop of chickenpox lesions. Varicella lesions erupt following a 10–21-day incubation period. Skin lesions usually develop first on the face, scalp, and trunk. Lesions begin as pruritic erythematous macules that enlarge as they fill with vesicular fluid containing infectious particles. The lesions crust after 24–48 hours. (B) Photograph of "shingles" (herpes zoster) caused by VZV reactivation from latency. VZV reactivation results in a vesicular rash corresponding to the cutaneous dermatome that is innervated by neurons in the involved cranial nerve or dorsal root ganglion. Cutaneous lesions form small clusters with the morphology of varicella vesicles and contain infectious virus. (C) Typical VZV multinucleated syncytia on cultured melanoma cells (3 days postinfection). VZV-specific proteins were stained with antihuman VZV polyclonal antibody and detected using Fast Red substrate. A methyl green nonspecific counter stain was applied.



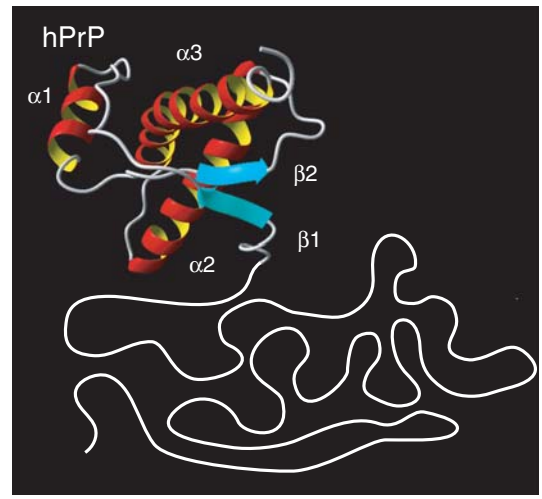
Color plate 12.2. The SCIDhu mouse model of VZV pathogenesis. VZV infection of human skin, T cells (thy/liv), and dorsal root ganglia (DRG) xenografts in the severe combined immunodeficiency (SCIDhu) mouse model of VZV pathogenesis has provided a novel system for identifying VZV gene products that mediate VZV tropisms. (A) Photograph of a thy/liv xenograft, 9 months postxenotransplantation. (B) Uninfected thy/liv xenograft, with abundant hemotoxylin- and eosin- (H & E) stained lymphocytes. (C) VZV-infected thy/liv xenograft, 21 days postinfection, with severe depletion of lymphocytes. (D) Photograph of a skin xenograft, 5 weeks postxenotransplantation. (E) Uninfected skin xenograft, H & E stain. (F) VZV-infected skin xenograft, 21 days postinfection, with a large vesicular fluid-filled lesion. (G) DRG xenograft, 12 weeks postxenotransplantation. (H) Uninfected DRG xenograft. (I) VZV-infected DRG xenograft, 14 days postinfection, with significant cytopathic effect in sensory ganglionic cells (black arrow). Figure G-I from Zerboni *et al.*, 2005 [76].



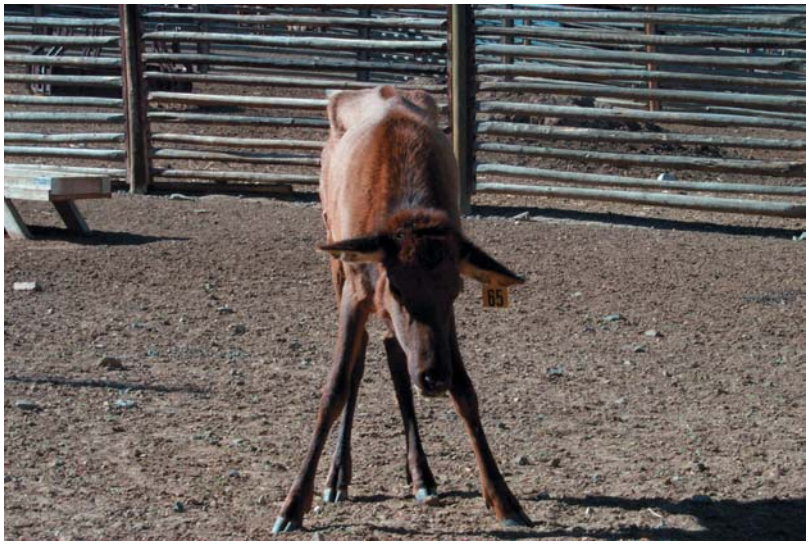
Color plate 12.5. Model of the course of VZV infection in the human host. VZV infection is acquired by inoculation of mucosal epithelial cells via the respiratory route. Infection of T cells in Waldeyer's ring amplifies the virus and allows transport to the skin via a cell-associated viremia. Infection of skin produces the vesicular rash associated with chicken pox. During skin infection, VZ virions gain access to the sensory nerve cell body by retrograde axonal transport from fine nerve endings to establish a lifelong latent infection within the sensory ganglia. Clinical reactivation of latent VZV results in herpes zoster, during which VZ particles gain access to skin via anterograde axonal transport.



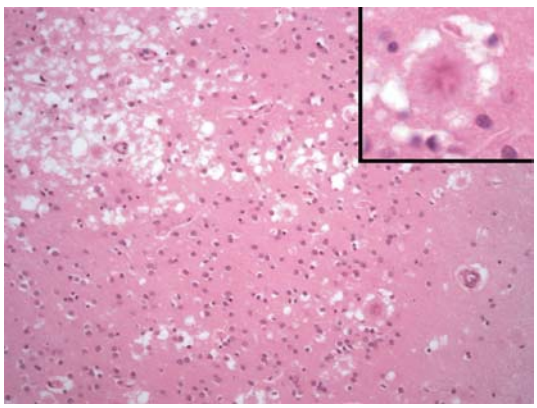
Color plate 13.1. Western blot of mouse brain homogenates probed with anti-PrP primary antibody. Brain homogenates taken from uninfected and scrapie-infected CD-1 mice were either treated (+) with proteinase K or untreated (–), as indicated.



Color plate 13.2. Three-dimensional structure of α-helical human PrP, determined by nuclear magnetic resonance (NMR) spectroscopy. The α-helices are shown in red and yellow, and the first and second β-strands are shown in green and blue, respectively. The N-terminus, which is able to bind copper ions, is unstructured. Courtesy of Dr. Roland Riek (Salk Institute, La Jolla, CA).



Color plate 13.3. Elk in late stage of chronic wasting disease (CWD). The animal shown displays broad gait, emaciation, lowered head position, and drooped ears. Courtesy of the Wyoming Game and Fish Department.



Color plate 13.4. Neuropathology of variant Creutzfeldt-Jakob disease (vCJD). Hematoxylin and eosin stain shows severe vacuolation and florid plaques. Inset shows close up magnification of a florid plaque, consisting of a ring of vacuoles arranged like a halo around a central plaque. Courtesy of Dr. Brent Harris (Dartmouth-Hitchcock Medical Center, Lebanon, NH).

Investigations of the contributions of VZV proteins and promoter elements to VZV pathogenesis and host cell tropism in the SCIDhu mouse model

The analysis of viable recombinant VZV mutants in SCIDhu models reveals niche-specific VZV genes and promoter elements that are necessary for, or that modulate, VZV infection of differentiated human cells within their unique microenvironments (reviewed in reference [78]). Using these models, we have demonstrated that VZV genes that are dispensable in cultured cells are often required in such environments, and their functions differ between T cells and skin *in vivo*. VZV gene promoters, like those of all herpesviruses, have elements that are recognized by ubiquitous host cell regulatory proteins. Combining the tools of making VZV mutants by cosmid mutagenesis and evaluating these mutants in the SCIDhu xenografts makes it possible to determine if particular gene products, motifs within VZV proteins, or promoter elements affect virulence *in vivo*. This approach has been used to characterize the role of viral glycoproteins, viral kinases, and regulatory proteins and promoter elements in skin and T cells. Major findings are summarized in this section. The availability of the DRG model will make it possible to begin to assess molecular mechanisms of VZV neurotropism using similar methods [79].

Role of the VZV glycoproteins, gE and gI, in VZV skin and T cell tropism

Unlike other alphaherpesviruses, VZV gE (ORF68) and gI (ORF67) are the only glycoproteins encoded in the U_S region. Functions of gE and gI in cell-cell spread and envelopment are closely linked through the formation of gE/gI heterodimers [80].

Glycoprotein E

VZV gE is essential for replication [81]; it is a major envelope protein and the most abundant glycoprotein in VZV-infected cells. VZV gE has similarities to gE homologs in regions important for interactions with gI but it also has a large, nonconserved N-terminal ectodomain [82]. Mutational analysis of this unique 188 amino acid region showed that it mediates functions that are essential even if other regions involved in gE/gI interactions or C-terminal functions are not disrupted. Whereas a complete deletion, excluding the 27 amino acid leader sequence, was lethal, partial deletions removing residues P27-Y51 and Y51-P187 allowed replication, albeit with decreased cell-cell spread. However, the Y51-P187 domain was required for normal cytoplasmic envelopment of virions and was essential for skin infection *in vivo*. Another mutant, in which the gE N-terminus was modified by a linker insertion at Y51, exhibited less gE incorporation into virions but had no effect on virus yields, syncytia formation, or gE trafficking *in vitro*, or on skin xenografts *in vivo*, suggesting that limited amounts of gE in the virion envelope do not affect the characteristic VZV polykaryocyte formation when cell surface expression of gE expression is intact. These experiments also showed that the serine at position 31 was important for VZV virulence in skin even though this mutation did not alter gE intracellular trafficking, gI interactions, or virion assembly or egress *in vitro* or *in vivo*. This residue may mediate an as-yet-undefined interaction with other viral and cellular proteins during VZV infection of epidermal cells *in vivo*. Thus, the unique region of the VZV gE ectodomain, which is absent from HSV and other alphaherpesviruses, has essential or important subdomains that function in the critical tropism of VZV for skin.

The small gE C-terminus (62 amino acids) has functional motifs, including YAGL (aa 582–585) required for gE endocytosis [83], AYRV (aa 568–571) that mediates gE trafficking to the trans-Golgi network [83], and a phosphorylated acid cluster, SSTT (aa 588–601) [84]. Mutagenesis of the gE C-terminus using VZV cosmids demonstrated that this region is essential for VZV replication, reflecting a requirement for the YAGL endocytosis motif and indicating that in contrast to other alphaherpesviruses, VZV gE must undergo endocytosis during viral replication [85]. The trans-Golgi network (TGN) trafficking

domain was dispensable *in vitro*, but disrupting TGN trafficking reduced virulence *in vivo*, with a more pronounced effect on replication in skin compared to T cell xenografts. The SSTT phosphorylation motif was not required *in vitro* or *in vivo*. Taken together, these analyses of functional domains in the N- and C-terminal regions demonstrate the multifunctional role of this protein in VZV replication and pathogenesis and help to explain why VZV gE is essential while this protein is dispensable in related viruses. Further, functional motifs in gE that are dispensable *in vitro* were shown to be essential in skin and T cells *in vivo*.

Glycoprotein I

While ORF67 is dispensable for replication, VZV gI is necessary for normal syncytia formation and growth in cultured cells through functions that are mediated both by its N-terminal and C-terminal regions [39]. Deletion of ORF67 or mutations that eliminate N- or C-terminal residues have dramatic effects on the intracellular trafficking of gE, cause an aberrant punctate expression of gE on cell surface membranes, and disrupt virion envelopment in the TGN [39]. These experiments indicate that other fusogenic proteins, including gB and the gH:gL complex, cannot compensate for gI functions in mediating cell-cell spread. Importantly, these gI functions are critical for the pathogenesis of VZV infection in SCIDhu skin and T cell xenografts *in vivo* [86]. Since polykaryocyte formation is the hallmark of skin infection, these experiments suggest that gI and gE interactions are necessary for VZV cell-cell spread in differentiated epidermal cells in their tissue microenvironment. Because VZV infection of T cells *in vivo* does not induce cell fusion, gI functions needed for virion assembly and egress appear to be essential for T cell tropism.

Role of the VZV serine/threonine kinases, ORF47 and ORF66 proteins, in skin and T cell tropism

VZV encodes two serine/threonine kinases, including ORF47, which is highly conserved in all herpesviruses, and ORF66, which is found only in the alphaherpesviruses. Both proteins are present in the VZ virion tegument.

ORF47 protein

ORF47 protein, which is related to HSV U_L13 and the cellular CKII kinases, is dispensable *in vitro*, as shown by insertion of an initial stop codon in ORF47 [87]. However, ORF47 expression was essential for the pathogenesis of VZV infection in skin and T cell xenografts [88], demonstrating that ORF47 functions were not complemented by related cellular kinases present in host cells or by the ORF66 protein *in vivo*. To investigate whether this requirement for ORF47 was related to its kinase activity or to other functions, VZV recombinants were made that had a ORF47 C-terminal truncation or targeted mutations in two putative kinase motifs, DYS and PPE, present in the ORF47 C-terminus [89]. Removing the C-terminus and disrupting the DYS motif in this region eliminated ORF47 kinase activity, but ORF47 homodimer formation and ORF47 protein binding to IE62 protein persisted. Further, the elimination of ORF47 kinase activity through the C-terminal deletion or the DYS mutation caused a significant reduction of VZV infection in skin xenografts. However, in contrast to blocking ORF47 expression, which was lethal, low levels of replication occurred despite eliminating ORF47 kinase function, as long as binding to IE62 protein, which is also a tegument protein, remained intact.

The ORF47 kinase defective mutants retained the capacity to create VZV polykaryocytes in skin. ORF47 kinase directs the phosphorylation of gE, which is necessary for its trafficking to cellular membranes and accumulation in the TGN for virion envelopment [90]. Cell-cell fusion was observed in skin infected with the ORF47 kinase mutants even though gE membrane expression was quite limited, suggesting that other fusogenic proteins allow some polykaryocyte formation when gE trafficking is disrupted by blocking gE phosphorylation by ORF47 protein. Nevertheless, VZ virion production was severely diminished in skin xenografts in the absence of ORF47

kinase activity. Subsequent electron microscopic (EM) analysis of cultured cells showed a similar disruption of virion formation when ORF47 expression or kinase activity were blocked, even though plaque numbers and plaque morphology were normal in standard VZV titration assays. Whereas these mutants retained limited infectivity in skin, eliminating ORF47 kinase activity was lethal in human T cell xenografts *in vivo* [91]. This observation supports the concept that VZV T cell tropism requires assembly and release of VZ virions because infected T cells do not fuse, whereas infection can occur in skin despite very restricted virion assembly as long as some cell fusion occurs. Thus, functional motifs in VZV proteins are differentially required depending on the target cell *in vivo*.

ORF66 protein

The VZV protein encoded by ORF66, which is related to HSV-1 U_S3, is dispensable *in vitro*, as shown by introducing an ORF stop codon mutation into the genome using cosmids derived from vaccine Oka [92] or parent Oka [93]. ORF66 protein was further analyzed by creating point mutations in conserved protein kinase subdomains revealed by bioinformatics comparison to the sequences of known cellular kinases [94]. Two ORF66 mutants were generated including a G102A substitution and an S250P substitution. Mutating G102 was shown to block the kinase activity of ORF66 protein and resulted in retention of IE62 in the nuclei at late times in cultured cells, whereas the S250P substitution had no effect. Aberrant IE62 localization *in vitro* is associated with disruption of ORF66 kinase activity [95].

Evaluation of the ORF66 stop codon mutants in SCIDhu skin xenografts showed no further attenuation of the vaccine Oka-derived mutants and only a slight decrease in replication of the parent Oka virus in which ORF66 expression was blocked [88,93]. However, replication of both ORF66 stop codon mutants was reduced substantially in T cell xenografts *in vivo*. Very few intact VZ virions were detected in T cells infected with the parent Oka ORF66 mutant in contrast to robust virion production associated with parent Oka infection. Targeted elimination of ORF66 kinase activity by the G102A mutation, leaving ORF66 expression intact, resulted in the same pattern of slightly less replication in skin and a significant impairment of growth in T cell xenografts *in vivo*. However, this interference with ORF66 kinase function did not cause IE62 nuclear retention in VZV-infected T cells. These observations suggested that decreased virion production in T cells was due to ORF66 protein functions other than IE62 phosphorylation. Experiments with the ORF66 stop codon and targeted ORF66 kinase-defective mutant showed that these mutants had lost the capacity to block apoptosis that was observed in T cells infected with intact VZV [93,94]. Thus, the requirement for ORF66 kinase in VZ virion production can be explained by the need to block T cell apoptosis long enough for virus assembly to occur. During VZV pathogenesis, prolonging T cell survival would be expected to be critical for transfer of VZV to skin by infected T cells. Of interest, blocking ORF66 expression and inhibiting its kinase activity also reduced the capacity of the virus to interfere with upregulation of IFN signaling following exposure to IFN-g [93]. ORF66 protein is also important for downregulating MHC class I expression on VZV-infected cells [96]. Thus, in addition to enhancing infection by blocking apoptosis, ORF66 protein also has immune evasion functions that are likely to support T cell tropism during VZV pathogenesis.

Role of VZV tegument proteins in VZV skin and T cell tropism

While some progress has been made in determining the proteins that comprise the VZV tegument, information about the VZ virion structure remains limited. In addition to the viral kinase proteins ORF47 and ORF66, the regulatory proteins IE4, IE62, and IE63 are components of the tegument, along with ORF10 protein. Of these, IE62, IE63, and ORF10 have been examined using the strategy of cosmid mutagenesis and evaluation of resulting VZV mutants in the SCIDhu skin and T cell models of VZV pathogenesis.

Deletion of the ORF encoding IE4 was incompatible with recovery of infectious virus from cosmid transfection, indicating that IE4 is essential for virus replication [97].

IE62 protein

IE62, which is the major transactivator of VZV gene expression, is encoded by the duplicated genes, ORF62/71, located in the repeat regions that flank the U_S segment of the VZV genome (Figure 12.3). One copy of this duplicated gene is required; expression from a nonnative site was sufficient for VZV replication in cultured cells, but plaque size and infectious virus yields were diminished and both IE62 and gE protein expression were decreased by ectopic expression of this critical regulatory protein [98]. IE62, a 1310 aa phosphoprotein, has a nuclear localization signal, an N-terminal activation domain, and a DNA-binding domain [3]. IE62 is phosphorylated by ORF47 and ORF66 proteins and cellular kinases. IE62 motifs that affect interactions with the IE4 protein and a putative site that mediates ORF9 protein binding were not essential for VZV replication *in vitro* [98,99]. The deficiencies associated with IE62 expression from an ectopic site blocked replication of this IE62 mutant in skin xenografts *in vivo*. Experiments in which one copy of ORF62 or ORF71 was deleted and the other copy was intact demonstrated that VZV replication was associated with restoring the deleted copy to yield complete genomes both in cultured cells and in skin xenografts *in vivo* [98].

IE63 protein

IE63 is a small 278 aa protein, predominantly localized to the nucleus of infected cells through a nuclear localization signal, but it is also found in the cytoplasm at later stages of infection [9]. Like IE62, IE63 is also expressed from duplicated genes, ORF63/70. The ORF63/70 sequence resembles the HSV $U_S1.5$, which encodes a smaller protein expressed colinearly with ICP22 [100]. Deleting both ORF63 and ORF70 from VZV cosmids appears to be lethal for VZV replication unless cells are also transfected with a plasmid expressing IE62 [100,101]. The presence of either ORF63 or ORF70 at the native site allows normal VZV replication in fibroblasts and T cells infected *in vitro* and in SCIDhu skin xenografts *in vivo*. When functional domains of IE63 were characterized by transient expression methods, S165, S173, and S185 were phosphorylated by cellular kinases, and mutations altering two putative nuclear localization signal (NLS) sequences were associated with a change in IE63 localization from predominantly nuclear to a cytoplasmic/nuclear pattern [100]. IE62 and IE63 form heterodimers; the N-terminal domain required for IE63 binding to IE62 was mapped to aa 55–67, with R59/L60 being critical residues. VZV mutants were generated with changes in ORF63, expressed from the ectopic *Avr*II site, which resulted in alanine substitutions of T171, S181, or S185. The consequences of these mutations were a small plaque phenotype, reduced viral titers, and decreased ORF47 protein and gE synthesis *in vitro*, suggesting that IE63 must be phosphorylated at these sites to enhance IE62-induced expression of these gene products [102]. These mutations in serine/threonine residues in IE63 caused a significant reduction in virulence in skin but not in T cell xenografts. Thus, IE63 functional domains appear to influence VZV tropism for skin and T cells differentially during VZV pathogenesis *in vivo*.

ORF10

VZV ORF10 is a regulatory protein located in the virion tegument, which has the capacity to enhance IE62-mediated transactivation of VZV genes; it has an acidic activation domain and a motif that is predicted to bind to human cellular factor 1 (HCF-1). In contrast to VP16, which is its homolog, deleting ORF10 is compatible with replication from vaccine Oka and parent Oka cosmids [103,104]. As expected because the complete gene was dispensable, targeted mutations in the acidic activation domain and HCF-1 site did not alter VZV replication, IE gene transcription, or virion assembly *in vitro*. ORF10 expression was completely dispensable for VZV T cell tropism *in vivo*, as assessed by infectious

virus yields from SCIDhu T cell xenografts. In contrast, ORF10 protein was identified as a virulence determinant in SCIDhu skin xenografts because its deletion resulted in diminished VZV replication and smaller skin lesions. Electron microscopy analysis demonstrated that epidermal cells infected with the VZV mutant lacking ORF10 had reduced numbers of DNA-containing nucleocapsids and complete virions, as well as many aggregates of intracytoplasmic viral particles not observed in skin infected with parent Oka. However, point mutations in the activation or putative HCF-1 domains did not have any impact on the pathogenesis of VZV infection in skin. In contrast to ORF66 protein, which is necessary for VZ virion formation in T cells and not in skin, VZV ORF10 protein is not required in T cells but is needed for efficient VZ virion assembly in skin and is a determinant of VZV virulence in epidermal and dermal cells *in vivo*.

Effects of cellular transactivating proteins on the expression of VZV gene products and their contributions to VZV pathogenesis

Sequence analysis of the promoters of VZV genes reveals the presence of consensus binding sites for cellular proteins that function in the transactivation of host cell genes. These cellular proteins and their putative binding sites in VZV gene promoters have been mapped and examined for their role in enhancing transactivation by IE62 and other VZV regulatory proteins using reporter construct methods. We have made targeted mutations in consensus sites in the promoters for gI and ORF10 protein to examine the hypothesis that these cellular proteins might contribute to skin and T cell tropism during VZV pathogenesis.

gI promoter

The gI promoter contains sequences that bind the cellular proteins specificity factor 1 (Sp1) and upstream stimulatory factor (USF), and an open reading frame 29 (ORF29)-responsive element (29RE), necessary for enhancing IE62-induced transcription by the single-strand ORF29 DNA-binding protein [105,106]. VZV recombinants in which binding sites for cellular transactivators within the gI promoter were disrupted were used to assess whether Sp1, USF, or both of these cellular transactivating factors influenced VZV infection of differentiated human cells *in vivo* [106]. VZV mutants with two base pair substitutions in Sp1 or USF sites showed normal replication *in vitro*. However, disrupting the Sp1 site reduced VZV virulence significantly in skin and T cells *in vivo*. Whereas mutating only the USF site had no effect, disrupting both Sp1 and USF promoter sites together reduced plaque sizes and viral titers in cultured cells. This dual SP1/USF promoter mutant was incompatible with any replication in skin, although some infectious virus was produced in T cell xenografts. Since the effect of the combined Sp1/USF mutation was a reduction in gI expression, these experiments indicate that less gI is required for productive VZV infection of T cells compared to the levels needed for cell–cell spread in skin. These observations suggest that particular cellular transactivators can contribute to VZV virulence in skin and that their activity may vary depending on the target cell type (e.g., epidermal cells versus T cells *in vivo*).

ORF10 promoter

As discussed above, VZV ORF10 is required for normal VZV infection of skin but not for T cell tropism in the SCIDhu skin and T cell xenografts [103]. We examined ORF10 transcription and identified the minimal ORF10 promoter in luciferase construct experiments [103]. The ORF10 promoter has a consensus binding site for USF. USF bound specifically to its consensus site within the ORF10 promoter and was required for IE62 transactivation while other mutations, altering putative TATA boxes or the Oct-1 binding site, did not affect reporter transcription. When this USF site was mutated to create a VZV mutant virus, production of ORF10 protein was blocked. Both the deletion of the ORF10 promoter and the targeted mutation of the USF binding site

were associated with a reduction in virulence in skin comparable to the effects of removing the ORF10 gene. These experiments provide a second example, in addition to mutagenesis of the gI promoter, indicating that the virulence of VZV in skin depends upon functions of cellular transactivators in concert with IE62 protein.

Taken together, these experiments show that VZV gene products, their functional domains, and cell transactivators that modulate viral gene expression have effects on VZV virulence that are highly specific in different cell types that must be infected during VZV pathogenesis.

Antiviral drugs and VZV vaccines

Antiviral therapy

VZV ORF36 encodes deoxypyrimidine kinase, the viral thymidine kinase that is the target for acyclovir [3]. Phosphorylation of acyclovir by the ORF36 gene product induces conversion to an active form, which competes with the normal substrate for the viral DNA polymerase and, once incorporated into synthesized DNA, acts as a chain terminator. VZV is susceptible to inhibition by acyclovir and related nucleoside analogs, such as valacyclovir and famciclovir [107,108] (see also Chapter 19). These drugs are licensed and used widely for varicella and herpes zoster in healthy and immunocompromised patients (see also Chapter 12). Their clinical efficacy requires administration shortly after the onset of clinical symptoms. Antiviral resistance can emerge in patients who are treated for prolonged periods; resistance results from emergence of thymidine kinase-defective mutants. Alternative drugs such as foscarnet are used in this clinical circumstance. However, in most cases, antiviral therapy fails because the host response remains deficient rather than because VZV becomes resistant to nucleoside analogs. Antiviral drugs can also be given as prophylaxis to reduce the incidence of herpes zoster in high-risk patients, but prolonged use is not advised because of the emergence of resistance.

VZV vaccines

The attenuation of the parent Oka strain of VZV, which was recovered from a skin lesion of a child with varicella, was achieved by tissue culture passage in human and guinea pig embryo fibroblasts [109]. The virus was passaged first in human cells and then in guinea pig embryo cells at low temperature and again in human diploid fibroblasts for large-scale vaccine preparation. VZV vaccines have been licensed to prevent varicella and to reduce zoster morbidity in the elderly [48,110]. The vaccine was licensed in Japan in the mid-1970s and in the United States in 1995. Varicella immunization is recommended as a routine childhood vaccine, given at 12 to 18 months, with a second dose now recommended 3 months later; susceptible older children and adults are also vaccinated with a two-dose regimen [111]. Two doses of Oka-based vaccines elicit primary VZV immunity in more than 90% of vaccine recipients. Routine varicella vaccination has dramatically reduced the incidence of varicella and associated complications and deaths. Breakthrough cases of varicella occur in immunized individuals, but pre-existing immunity can be expected to reduce the extent of illness in most individuals. Most recently, a more potent vaccine made from the vaccine Oka strain has been licensed for immunization of healthy older adults in order to reduce the risk of herpes zoster. This vaccine boosts VZV immunity and ameliorates the age-related decline in VZV immunity that predisposes this age group to herpes zoster [48]. The live attenuated VZV vaccines for varicella and herpes zoster are not considered safe for most immunocompromised patients. However, an inactivated preparation of the varicella vaccine appeared to reduce the risk of herpes zoster in bone marrow transplant recipients given a dose before the transplant and three doses after, when compared to patients randomized to receive no vaccine [112].

The molecular basis of the attenuation of the Oka vaccine strain is unknown, but it was demonstrated in extensive clinical studies performed in susceptible children and adults before licensure. While vOka-derived vaccines are very effective, vOka

retains some capacity to cause varicella in immunocompromised patients and to establish latency and reactivate in high-risk and healthy individuals [110,113,114]. Molecular analyses show that vOka is a mixture of intact and altered VZV genomes, some of which exhibit skin tropism and allow vOka transmission [115,116]. Further investigations using the SCIDhu mouse model for VZV pathogenesis demonstrated that vaccine Oka strain has a reduced capacity to replicate in skin compared to parental Oka strain, whereas infectivity for T cells and DRG is not impaired compared to parental Oka strain [28,74,79]. When parent Oka, a second low passage isolate, vaccine Oka, and a standard, high-passage laboratory strain were compared in skin xenografts, only the low passage viruses had full virulence in skin [74]. In the absence of any adaptive immunity, these observations indicate that the vaccine Oka virus has genetic changes that reduce virulence rather than that the cutaneous administration of vaccine Oka is responsible for the attenuation phenotype. When the laboratory strain that had been passed >100 times in cultured human cells was tested, no VZV replication was detected in skin, indicating that tissue culture passage can result in the accumulation of mutations that eliminate VZV virulence in skin.

The availability of paired sets of recombinant cosmids from parental (pOka) and vaccine (vOka) Oka made it possible to generate chimeric recombinants in which blocks of VZV genes were derived from the parent virus or the vaccine [117]. The evaluation of the virulence of these chimeric viruses in SCIDhu skin xenografts demonstrated that various combinations of VZV genome fragments were associated with an attenuation phenotype. These experiments indicated that the attenuation of the vaccine was not the result of a single mutation but probably reflects minor mutations in several VZV genes that cumulatively reduce the capacity to replicate in skin.

Conclusion

Considering the fact that the VZV genome was sequenced only 20 years ago and tools for making VZV mutants and evaluating their consequences in human tissue xenografts were developed only about 10 years ago, substantial progress has been made in understanding the pathogenesis of VZV infection. Although many questions remain unresolved, further progress can be expected given the techniques that are now available. Approaches to treatment and prevention of VZV-related disease have also been introduced for widespread use. Further improvements in the clinical management of VZV infection should emerge in parallel with better insights into VZV molecular virology and pathogenesis.

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13

Transmissible spongiform encephalopathies

Surachai Supattapone and Judy R. Rees

Introduction

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are a unique group of slowly progressive and invariably fatal infections of the central nervous system, which can occur in infectious, sporadic, and inherited forms. Some examples of TSEs include kuru and Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and elk, transmissible mink encephalopathy (TME) in mink, and scrapie in sheep. The infectious agent of TSEs is an unconventional, proteinaceous entity, which has been termed a "prion." Unlike conventional infectious agents such as viruses or bacteria, infectious prions are remarkably resistant to most physical or chemical inactivation methods and can form spontaneously in hosts unexposed to exogenous infection. The "protein only" hypothesis postulates that prions lack informational nucleic acids and are composed exclusively of an infectious protein termed PrP^{Sc}. Experimental evidence indicates that PrP^{Sc} molecules form through the misfolding of a host-encoded glycoprotein termed PrP^{C} in an autocatalytic process. However, the molecular mechanism of prion formation and precise composition of infectious prions remain unknown. Currently, much research is focused on developing effective methods for presymptomatic diagnosis and therapy for TSEs, and recent advances toward these goals have been achieved.

The infectious agent

The precise nature of the TSE infectious agent has been the subject of great controversy for decades. Early studies showed that the scrapie agent is unusually resistant to various forms of physical and chemical inactivation that normally destroy conventional viruses and bacteria, such as boiling or exposure to alcohol [23]. In 1966, Tikvah Alper reported that scrapie infectivity was not destroyed by large doses of ultraviolet light capable of damaging nucleic acids [1]. These unexpected results led several investigators to hypothesize that the scrapie agent may not contain any nucleic acids and therefore might represent a completely novel form of infectious agent, distinct from viruses, bacteria, and eukaryotic pathogens. This hypothesis was controversial because it was not immediately apparent how an infectious agent lacking nucleic acids could replicate, although some theoretical models were proposed [53].

In 1982, Stanley Prusiner and his colleagues used a biochemical approach to purify the scrapie agent [85]. These studies identified a protein with three glycoforms, which copurified with infectivity. The infectivity of the purified preparation was not altered by enzymatic or chemical treatments that degrade nucleic acids but was destroyed by protein denaturing agents such as guanidine and urea. These observations led Prusiner to coin the term "prion," to indicate "proteinaceous infectious agent." Further studies unexpectedly revealed that the prion

protein (PrP) is also expressed in the brains of normal, uninfected animals [3] and that this normal cellular protein of unknown function (PrP^C) exhibits different properties than the apparently infectious molecules purified from scrapie-infected animals (PrP^{Sc}). Biophysical studies revealed that both PrP^C and PrP^{Sc} contain a glycophosphatidylinositol (GPI) anchor, two N-linked carbohydrate chains, and an intramolecular disulfide bond [46,69,107,117]. PrP^C and PrP^{Sc} appear to have identical molecular weight as determined by mass spectroscopy, but Fourier transform infrared (FTIR) and circular dichroism (CD) spectroscopic studies revealed that PrP^{Sc} contains substantially more β-sheet secondary structure [79,93]. Thus, PrP^C and PrP^{Sc} molecules appear to have different conformations, which for many years were operationally distinguished by the relative detergent-insolubility and protease-resistance of PrP^{Sc} molecules (Figure 13.1). More recently, immunoconformational assays have been used to detect a subset of disease-associated PrP^{Sc} molecules that are protease-sensitive [94].

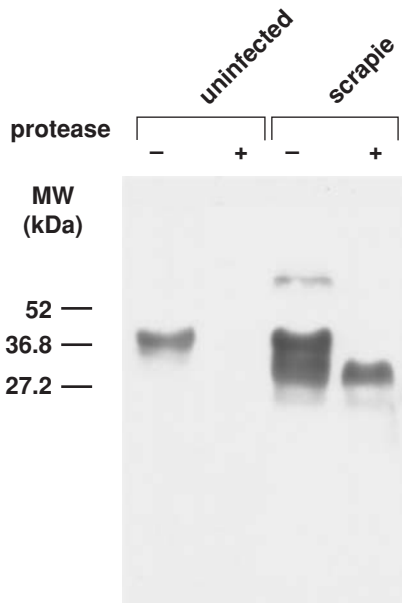

Figure 13.1. Western blot of mouse brain homogenates probed with anti-PrP primary antibody. Brain homogenates taken from uninfected and scrapie-infected CD-1 mice were either treated (+) with proteinase K or untreated (−), as indicated. (For figure in color, please see color plate section.)

According to the "protein-only" hypothesis, the TSE agent is comprised of only PrP^{Sc} molecules, and these molecules are produced from PrP^C molecules by a process of induced conformational change [86]. Consistent with this hypothesis, $Prnp^{0/0}$ mice lacking PrP^C molecules are not susceptible to TSE infection [26]. Furthermore, analytical studies suggest that purified infectious prions are not likely to contain informational nucleic acids, >22 nucleotides in length [95]. The "protein-only" hypothesis also provides a reasonable explanation for the etiology of sporadic and inherited forms of prion disease, which do not appear to be caused by exposure to exogenous sources of infection [81]. However, an important challenge to the "protein-only" hypothesis is raised by the existence of distinct TSE "strains." Strains are defined as isolates of infectious prions with distinctive clinical and neuropathological features that can arise when prions are transmitted, usually inefficiently, between different animal species, and that can then be faithfully maintained upon serial passage within the same animal species [25,27]. Several studies have shown that prion strains are associated with PrP^{Sc} molecules that possess distinctive biochemical characteristics [8,40,82,94,113]. However, the molecular basis of strain variation remains unknown.

Some investigators have proposed that the TSE agent is either a conventional virus or a "virino," composed of replicating informational nucleic acids protected by a proteinaceous coat [64]. However, recent studies have shown that infectious prions can be propagated *in vitro*, in the absence of replicating nucleic acids [31]. Synthetic prions have also been produced in transgenic mice by inoculation of refolded, recombinant PrP amyloid fibrils [66]. Taken together, these results indicate that nucleic acid replication is not required for the amplification of TSE infectivity. However, it remains possible that infectious prions contain a second component bound to PrP, which may influence or even dictate strain properties.

Pathogenesis

Thus far, PrP^C is the only specific host factor that has been directly shown to be required for susceptibility to TSEs. Moreover, the amino acid sequence of host PrP^C molecules also influences disease susceptibility. For instance, species-dependent differences in PrP^C sequence are responsible for transmission barriers between animal species, and specific PrP^C polymorphisms influence the likelihood of acquiring both the infectious and sporadic forms of prion disease [38,97,98]. The three-dimensional molecular structure of PrP^C has been determined by nuclear magnetic resonance (NMR) spectroscopy and contains three α-helices and two antiparallel strands of β-sheet [60,90] (Figure 13.2). The secondary structure of PrP^C contains 42% α-helix and 3% β-sheet, whereas PrP^{Sc} contains 30% α-helix and 43% β-sheet [79,93]. However, the precise nature of the dynamic transition from PrP^C to PrP^{Sc} during the pathogenic process remains uncertain, because the three-dimensional structure of PrP^{Sc} has not yet been elucidated.

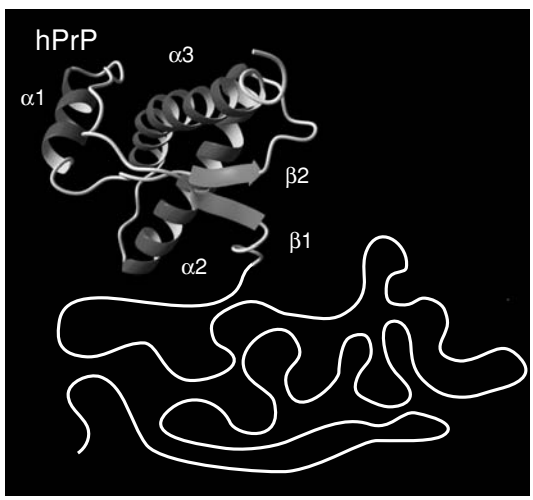

Figure 13.2. Three-dimensional structure of α-helical human PrP, determined by nuclear magnetic resonance (NMR) spectroscopy. The α-helices are shown as spirals and the first and second β-strands are shown by arrows, respectively. The N-terminus, which is able to bind copper ions, is unstructured. Courtesy of Dr. Roland Riek (Salk Institute, La Jolla, CA). (For figure in color, please see color plate section.)

The molecular mechanism of PrP^{Sc} formation is also undetermined. Two different models have been proposed: template assistance and nucleation-polymerization. In the template assistance model, it is hypothesized that the rate-limiting step of prion formation is the conversion of PrP^C oligomers into an intermediate state prior to interaction with infectious PrP^{Sc} oligomers [2,36]. In contrast, the rate-limiting step in the nucleation-polymerization model is the formation of a PrP^{Sc} nucleus, and subsequent addition of PrP^C oligomers to the elongating ends of PrP^{Sc} polymers would proceed quickly [59]. Radiation inactivation studies suggest that infectious prions have a molecular size of 55 kDa, corresponding to approximately 2 PrP^{Sc} monomers [4], while recent size fractionation studies have shown that the most infectious scrapie particles are 300–600 kDa in size, corresponding to approximately 14–28 PrP^{Sc} monomers [103].

Studies using model systems have suggested that host-encoded factors other than PrP^C may be required to propagate prions *in vitro* and *in vivo* [43,68,71,92,108,114]. Furthermore, the restricted range of neuronal and nonneuronal cell types that are susceptible to infection by prions also suggests the existence of prion propagation cofactors [13,45,88]. Although no specific cofactors have been identified to date, several studies have shown that various polyanionic compounds, such as host-encoded RNA and proteoglycan molecules, appear to stimulate prion-seeded conversion of PrP^C into PrP^{Sc} molecules *in vitro* [5,43,44,100,120].

Expression of PrP^C is required not only to replicate infectious prions but also for PrP^{Sc}-induced neuronal damage. This was first demonstrated by inoculating $Prnp^{0/0}$ mice harboring grafts of PrP^C-expressing brain tissue with infectious prions; despite the diffusion of PrP^{Sc} molecules out of the graft, the surrounding $Prnp^{0/0}$ neurons remained healthy [15]. Similar results were obtained *in vitro* using neurons derived from $Prnp^{0/0}$ mice [16].

Currently, the effects of PrP^C post-translational modifications on TSE pathogenesis are not

completely understood. However, recent work has shown that transgenic mice expressing only PrP^C molecules lacking a GPI anchor could replicate infectious prions without causing disease [35]. Notably, the anchorless PrP^C molecules expressed in these transgenic mice were also poorly glycosylated. Thus, it appears that whereas the GPI anchor may not be required to generate infectious prions, membrane attachment of PrP^C is necessary for the expression of PrP^{Sc}-induced neuronal damage. It is possible that PrP^C membrane attachment is required for disease expression because prion infection activates a pathogenic signaling cascade initiated by altered PrP^C trafficking. Circumstantial support for this possibility is provided by studies showing that intracerebral injection of anti-PrP^C cross-linking antibodies into mice causes neurodegeneration [105]. Alternatively, cellular attachment of PrP^C may be required because the process of prion-mediated neurodegeneration may not be cell-autonomous. Although white blood cells do not accumulate during the course of TSE infection, astrocytes and microglial cells within the brain proliferate and become reactive, and coculture studies have shown that microglia are required for neuronal death caused by an amyloidogenic PrP peptide [16].

Some TSE epidemics, such as kuru, BSE, and TME were predominantly transmitted by oral consumption of infected tissues. Thus, the TSE infectious agent appears to be able to penetrate the gastrointestinal epithelium and eventually invade the central nervous system. Some of the steps of the neuroinvasive pathway have been identified, while others remain unknown. Elegant histopathological and genetic reconstitution studies have shown that prions most likely enter the body through lymphoid cells of the gut and progress into the central nervous system (CNS) through follicular dendritic cells of the spleen, which appear to be a critical reservoir [17,70,75]. The precise pathways responsible for transferring prion infectivity from gut lymphoid cells to the spleen, and from the spleen into the CNS, have not been confirmed, but they may involve autonomic nerve fibers [6,52]. Progression of prion infection within the CNS requires close contact between adjacent neurons and may be facilitated by the release of exosomes containing PrP^{Sc} molecules [47].

History and epidemiology

The earliest record of TSE dates from the eighteenth century, when scrapie was described to be causing a fatal infectious disease among flocks of sheep and goats in Europe [19]. It was recognized even at this early date that scrapie could be transmitted horizontally among sheep grouped together in a flock, and therefore many farmers believed that the disease was likely to be contagious. However, it was difficult to obtain scientific evidence that scrapie was infectious because many attempts to transmit scrapie experimentally failed. The reason for these experimental failures remained unclear until the 1930s, when two independent events concurrently proved the infectious nature of the scrapie agent. In France, Cuillé and Chelle considered the observation that scrapie usually took years to spread when a diseased sheep was introduced into a healthy flock, and they decided to observe their experimental animals for prolonged periods after inoculation. These patient investigators were rewarded with the first successful transmissions of scrapie following incubation periods of 1–2 years [42]. At the same time in England, other workers found that vaccinating sheep against louping ill, an unrelated disease, accidentally caused an iatrogenic epidemic of scrapie. Apparently, the crude louping ill vaccine was contaminated with tissues derived from scrapie-infected animals.

Scrapie has been documented in many countries worldwide among sheep and goats. Two notable exceptions include Australia and New Zealand, which have never had a documented case of scrapie. In 1978, in an effort to eradicate scrapie from endemic areas of Iceland, farmers killed all the indigenous Icelandic sheep and goats and imported scrapie-free animals from New Zealand 3 years later. However, scrapie re-emerged within several of the imported flocks, demonstrating the persistence of the infectious agent in an unidentified environmental reservoir, possibly hay mites [28]. A more

successful strategy to combat scrapie was selective breeding, which produced inbred animal stocks that were apparently resistant to the disease. Subsequent genetic testing of selectively bred Suffolk sheep showed that scrapie susceptibility was associated with a polymorphism of the gene-encoding PrP, specifically homozygosity for glutamine at codon 171 [118]. In 1961, Chandler successfully transmitted scrapie to rodents, greatly facilitating the pace of TSE research [34]. There is no epidemiologic evidence that exposure to scrapie has caused disease in humans.

Scrapie was the first in a series of TSE epidemics documented worldwide in various mammalian species, including humans. In the 1950s, the disease kuru was spread by the practice of ritualistic cannibalism among the Fore people of Papua New Guinea. Epidemiologic investigation of the kuru epidemic, which killed over 2000 people, revealed that infectivity appeared to be most concentrated in tissues of the CNS, because people who consumed these tissues were far more likely to contract kuru than people who consumed other tissues, such as muscle [51]. These observations were confirmed in 1966 by experimental transmission of kuru to chimpanzees by intracerebral inoculation of infected brain homogenates [50]. Notably, the longest kuru incubation times appear to exceed 50 years, since cannibalism ceased in the 1950s and the last case was diagnosed in 2004 [41]. Notably, all the patients with prolonged kuru incubation times were heterozygous at the polymorphic codon 129 of the gene-encoding PrP.

A different TSE epidemic was first noticed among deer and elk species in North America in the 1960s. This disease, CWD, is notable for being the only TSE thus far identified in free-ranging wildlife. Horizontal transmission is highly efficient, and between 1–15% of wild deer surveyed in an endemic region of Colorado and Wyoming were infected with CWD [74]. Originally, CWD was clustered near commercial elk farms in the Midwestern United States, but cases have also been identified in Canada and South Korea. The CWD epidemic continues to grow in North America, but thus far no cases have been found among noncervid animals. The susceptibility of humans to CWD is uncertain, but both *in vitro* and transgenic mouse studies suggest that humans may have a relatively low risk of acquiring CWD [24,65,89,110]. Like scrapie, CWD is spread horizontally and through environmental reservoirs. Experiments with confined deer have shown that horizontal transmission can occur as a result of exposure to saliva [72] and that CWD infectivity can persist in soil [63,73].

In the 1980s, a large epidemic of bovine spongiform encephalopathy (BSE) originated in Great Britain. Over 180 000 cases of BSE have been documented in this epidemic, which was transmitted predominantly by the practice of reprocessing cattle carcasses into meat-and-bone meal (MBM) [104]. The incidence of BSE in Great Britain has declined steadily since 1992 as a result of a ruminant feed ban that prohibited farmers from feeding MBM to cattle or other ruminant species. However, during this same period, new cases of BSE have been documented in cattle from other countries of Europe, Asia, and North America [104]. Moreover, consumption of MBM and BSE-infected beef products by other animal species has caused a number of related TSE epidemics, such as feline spongiform encephalopathy, exotic ungulate encephalopathy, and most notably the human disease known as variant Creutzfeldt-Jakob disease (vCJD). To date, more than 160 cases of vCJD have been confirmed in the United Kingdom, and 28 cases have been confirmed in other countries [14,37]. The full scale of the vCJD epidemic cannot yet be predicted accurately because the incubation time and susceptibility factors for the disease are unknown. Thus far, all but one of the British patients have been homozygous for methionine at the polymorphic PrP codon 129, whereas the prevalence of M129 homozygosity in the Northern European population is 38% [14,37,76]. Some of the patients who developed vCJD were vegetarians, and the precise route(s) by which vCJD patients are exposed to BSE remains unknown. In addition, it is likely that both vertical transmission and horizontal transmission of vCJD by blood transfusion have occurred [18,67,80]. The causal link between BSE in cattle and vCJD in humans was unexpected

because previous observations that scrapie did not transmit easily to nonungulate species led some experts to predict the existence of "species barriers" between evolutionarily divergent species. Later it became clear that BSE could infect a wider variety of animal species than scrapie, providing an important lesson that prions derived from different TSEs can have distinct infectious properties, and that the species susceptibility of specific TSE isolates can only be determined empirically. Efforts to monitor and control the spread of BSE in livestock are complicated by two additional factors: (1) Atypical strains of BSE have been detected in infected cattle in Europe and Japan, and the infectious properties of these alternative strains have not yet been determined [11,29,121]. (2) The most common BSE strain can be transmitted to sheep by oral challenge, and therefore cross-species infection with BSE must be considered alongside scrapie in the differential diagnosis of rapidly progressive neurological disease in this species [48].

Another prion disease caused by commercial feeding practices is transmissible mink encephalopathy (TME). Between 1947–1985, several outbreaks have been reported in various mink farms in the United States, Canada, Finland, Russia, and Germany. In the most recent outbreak, captive mink in Stetsonville, Wisconsin, were apparently fed the carcasses of local "downer" cattle whose cause of death was unknown, and approximately 60% of the 7300 exposed animals developed TME [101]. An interesting and important observation was made during the course of studies characterizing TME. Two different TME strains could be distinguished clinically and pathologically, which were termed "hyper" and "drowsy" to describe the opposite behavioral phenotypes [10]. Biochemical studies revealed that the PrP^{Sc} molecules associated with these two strains could be distinguished easily; "drowsy" PrP^{Sc} molecules are more easily denatured and exhibit a greater electrophoretic mobility shift upon proteinase K digestion than PrP^{Sc} molecules associated with the "hyper" agent [8,9].

Several outbreaks of human prion disease have been caused inadvertently by medical procedures [22]. For instance, 114 patients from 16 different countries who received contaminated dura mater grafts during neurosurgery developed iatrogenic CJD. An additional 139 pediatric patients from seven different countries developed prion disease after receiving injections of pooled cadaveric pituitary extracts, which were presumably contaminated with CJD prions. Iatrogenic CJD has also been caused by the use of contaminated electroencephalographic (EEG) stereotactic electrodes, neurosurgical instruments, and corneal transplants. Among the various causes of iatrogenic CJD, intracranial procedures using contaminated instruments and corneal transplants produced disease most rapidly, with a median incubation time of approximately 18 months.

Unlike conventional infectious diseases, which require exposure to an exogenous source of infectious agent, prion disease can also occur in sporadic or inherited forms. All known inherited forms of prion disease are associated with germline mutations of the PrP coding sequence, and remarkably, some of these forms can be transmitted to normal primates by intracerebral inoculation [20,111]. The sporadic form of human prion disease occurs worldwide with an incidence of 1 per million people per year. Epidemiologic studies have not shown association between sporadic CJD and any environmental exposures, and the major risk factors appear to be advanced age [81] and homozygosity at the polymorphic PrP codons 129 and 219 [39,62,78]. Sporadic CJD is experimentally transmissible, but unlike scrapie and CWD, it does not appear to be contagious (i.e., spread by contact [49]).

Clinical features and pathology

All TSEs have long, asymptomatic incubation periods followed by rapidly progressive neurological deterioration leading to death. Some people who harbor pathogenic PrP mutations do not develop disease within their lifetime, and it is possible that there are subclinical carriers of infectious prion disease who have not been identified [56,57,58,87,115]. The initial symptoms and pattern of neurological deficits vary between different TSEs and are closely

Figure 13.3. Elk in late stage of chronic wasting disease (CWD). The animal shown displays broad gait, emaciation, lowered head position, and drooped ears. Courtesy of the Wyoming Game and Fish Department. (For figure in color, please see color plate section.)

correlated with the regional distribution of pathology in the central nervous system. For instance, kuru patients generally present with ataxia related to lesions in the cerebellum, and cognitive decline appears only during the terminal stages of the disease. In contrast, patients who acquire iatrogenic CJD following direct exposure of the cerebral cortex to contaminated material present with dementia. Patients with vCJD often present with psychiatric symptoms, and the rate of disease progression in this disease appears to be faster than that of other human TSEs. Specific clinical signs are also useful for diagnosing TSE infection in animals. Scrapie is so-named because infected sheep often scrape off their wool against hard surfaces. Cows that are normally docile become highly agitated when infected with BSE, a phenomenon that led to the disease being known popularly as "mad cow disease." CWD-infected deer are characterized by weight loss, subtle behavioral changes, lowered head position, and drooped ears (Figure 13.3).

The central neuropathological changes observed in all TSEs are intraneuronal spongiform degeneration, reactive gliosis, and PrP^{Sc} deposition (Figure 13.4). The regional distribution of pathology (lesion profile) varies considerably between different TSEs and between different prion strains. For instance, most cases of BSE and vCJD have an identical stereotypic lesion profile, whereas the lesion profile of scrapie in sheep is too variable to characterize [61]. Specific microscopic changes are associated with certain TSE infections. For instance, florid plaques consisting of eosinophilic deposits arranged like a halo around a central vacuole are a characteristic feature of BSE, vCJD, and kuru neuropathology.

Prevention

Prions are extremely stable and resistant to most chemical and physical inactivation methods. Thus, persistent environmental contamination is a major concern when formulating strategies to prevent the spread of TSEs. The most effective disinfection protocols appear to be the ones that strongly denature proteins. Unfortunately, most of these protocols are also corrosive. Currently, the most widely used

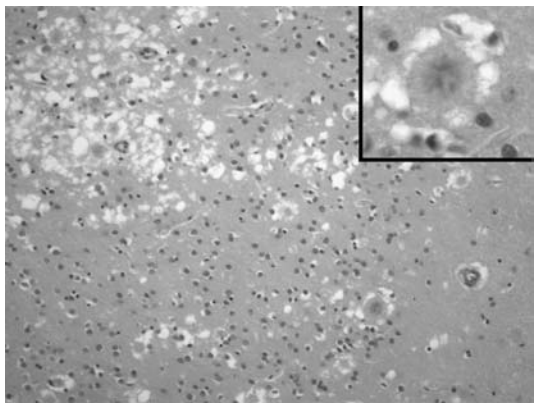

Figure 13.4. Neuropathology of variant Creutzfeldt-Jakob disease (vCJD). Hematoxylin and eosin stain shows severe vacuolation and florid plaques. Inset shows close up magnification of a florid plaque, consisting of a ring of vacuoles arranged like a halo around a central plaque. Courtesy of Dr. Brent Harris (Dartmouth-Hitchcock Medical Center, Lebanon, NH). (For figure in color, please see color plate section.)

disinfection methods include: (1) incineration; (2) contact with >20 000 p.p.m. sodium hypochlorite; (3) contact with 1 N NaOH plus autoclaving at 134°C [112]. Less corrosive methods that have been developed more recently include: (1) a mildly acidic solution of sodium dodecyl sulfate (SDS) [83]; (2) proteases derived from thermophilic bacteria [116]; and (3) ultra-high pressure inactivation [21].

The majority of documented iatrogenic CJD cases have resulted from direct exposure to infected CNS-associated tissue, such as pituitary extracts and dura mater grafts. Screening and/or avoiding such procedures is now certainly advisable. Epidemiologic analysis and *in vitro* studies suggest that prions can also be spread by blood transfusion [18,32,67,80]. New methods such as the protein misfolding cyclic amplification (PMCA) technique may soon allow blood supplies to be screened for prions prior to transfusion [32].

Several strategies are effective for controlling TSEs in livestock species. The front-line strategy in most instances involves mass eradication of TSE-infected flocks or herds, coupled with a documentation system that allows the rapid identification of animals that have moved from one farm to another. Another important measure that helped to control the BSE epidemic was the ruminant feed ban, which prevented potentially infected carcasses from being fed to other animals. Genetic strategies to limit the incidence of livestock TSE include the selective breeding of animals with protective PrP alleles containing dominant negative polymorphisms, such as arginine at codon 171 in sheep, and potentially the generation of PrP knockout livestock species.

Diagnosis and treatment

The diagnosis of TSE infection is usually confirmed by neuropathological studies and biochemical detection of PrP^{Sc} molecules. Due to the long incubation time of TSE infection, animal bioassays are not feasible methods for routine diagnosis and have been used mainly for public health surveillance and research purposes. In several animal species, it is also important to determine the prion strain type responsible for the disease. Several surrogate strain-typing procedures have been developed; the most commonly used method involves comparing the glycoform pattern of PrP^{Sc} molecules on Western blot. For instance, this method can be used to distinguish vCJD from sporadic CJD [40]. The identification of new cases of BSE or vCJD can cause large public health and economic impacts, therefore the diagnosis of these specific diseases must follow rigorous clinical guidelines [119] and be confirmed by neuropathological examination at autopsy. In all cases of confirmed TSE, sequencing of the PrP gene is advisable to screen for potential pathogenic mutations and to gather genetic data that can eventually be used to predict the effects of PrP polymorphisms on disease expression.

Currently, there is no clinically useful treatment for TSE infection. A large number of compounds have been shown to inhibit prion propagation in cultured cells, and some of these compounds have also been able to prolong the incubation time in experimentally infected animals when administered very soon after prion inoculation [30]. Unfortunately,

these compounds are ineffective when administered to symptomatic animals. Many of the inhibitory compounds, such as oligonucleotides, sulfonated dyes, sulfated glycans, and cyclic tetrapyrroles, contain planar conjugated rings and multiple negative charges, and therefore these structurally related compounds may all bind to a common target site on PrP [33]. Anti-PrP antibodies are also effective inhibitors of PrP^{Sc} formation *in vitro* and *in vivo* [7,55,84,102]. However, antibody-based therapies for CNS diseases may cause adverse autoimmune responses [12].

Recent developments

There have been many exciting developments in TSE research in recent years. Perhaps the most important of these has been the invention of PMCA, a method for amplifying and propagating PrP^{Sc} molecules *in vitro*, which is conceptually analogous to the polymerase chain reaction (PCR) method for amplifying nucleic acids [96]. PMCA appears to be the most sensitive diagnostic method available to detect infectious prions from a wide range of animal species and can be performed in less than a week [91,106]. Uniquely, PMCA can be used to detect prions in the blood of presymptomatic animals and thus offers a promising method to screen blood prior to transfusion [32,109]. The PMCA technique has also facilitated biochemical studies on the mechanism of prion formation. Such studies have shown that prion formation *in vitro* requires an accessory polyanion and is potently inhibited by copper ions [43,77].

There have also been recent advances toward understanding the composition of infectious prions. Legname *et al.* reported that injection of refolded, purified, recombinant PrP molecules into transgenic mice causes scrapie, which can subsequently be transmitted upon serial passage to wild-type mice [66]. Castilla *et al.* reported that infectious prions could be produced *in vitro* by serially propagating brain homogenates subjected to PMCA [31]. Silveira *et al.* used field flow fractionation to show that the minimum size of infectious prions is approximately 150 to 250 kDa, roughly equivalent to 6 PrP^{Sc} monomers [103]. These landmark studies eliminate the possibility that the infectious agent of scrapie is a conventional virus and confirm that misfolded PrP molecules are essential components of infectious prions. However, more work is required to determine whether *bona fide* infectious prions contain only PrP molecules or whether other, unidentified ligands may also be required components.

Important progress has also been made recently in understanding the mechanisms by which prion diseases are spread horizontally. Aguzzi and colleagues found that chronic inflammation increases the production of PrP^{Sc} molecules in the kidney, resulting in the excretion of infectious prions into the urine [54,99]. Other studies have shown that CWD can be transmitted horizontally either through contaminated soil [63,73], or by oral exposure to infected saliva [72]. It will be critical to understand the mechanisms by which TSEs are naturally transmitted in order to devise strategies to limit the spread of these incurable diseases.

Future directions

Despite significant advances in our understanding of TSEs over the past 50 years, most of the fundamental questions concerning these unorthodox infectious diseases remain unanswered. At a biochemical level, the precise composition of the infectious agent, the chemical basis of prion strain variation, and the mechanism of prion formation remain unknown. The development of PMCA has provided new opportunities to study some of these questions *in vitro*. However, the degree to which *in vitro* prion formation mimics the process of prion formation *in vivo* is still to be established, and the characteristics of *in vitro*-generated prions are yet to be fully described. To complement such biochemical studies, it would also be desirable to develop a cell culture or animal TSE model system amenable to genetic screening.

The cellular response to prion infection is another important area for future investigation. Cell culture

and animal studies have shown that PrP^{Sc} molecules can be cleared surprisingly quickly, but the mechanism by which cells accomplish this remains unknown. It is also unknown how prion infection causes the pathogenic cascade leading to glial proliferation and neuronal death. Interestingly, neurons must express membrane-attached PrP^{C} molecules to be susceptible to PrP^{Sc}-induced damage [15,35], suggesting that perhaps a specific, membrane-localized signaling system may be involved in the pathogenic process.

Several questions also remain unanswered about how infectious prions traffic through the body to reach the brain and spinal cord. In particular, the pathways by which prions (1) traverse the intestinal epithelium, (2) travel between the intestine to the spleen, and (3) invade the CNS remain important areas for future investigation.

Finally, advances in the clinical management of TSEs are urgently needed. Currently, there are no vaccines or effective therapies to combat these diseases in humans or livestock species. In addition, further work is required to develop and validate rapid methods for early noninvasive diagnosis and strain typing. Several of these practical goals will be facilitated by the development of PrP^{Sc}-specific antibodies that recognize clinically relevant prion strains, and by novel methods to deliver compounds to the sites of prion formation within the CNS.

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SECTION III

Introduction: immunity, diagnosis, vector, and beneficial uses of neurotropic viruses

Carol Shoshkes Reiss

In this section, Chapters 14–23 cover a wider range of topics dealing with the viruses that cause disease in the central nervous system (CNS). The first three chapters in the final section concern aspects of the immune response. The next three are about detection and treatment of infections and about the insects and animals that transmit the viruses to people. The final chapters focus on neurotropic viruses for gene transfers and for elimination of a brain tumor.

General principles of innate immunity, with special attention to the specific problems of infections in the brain, are covered in three chapters. The first describes important and often overlooked pathways that regulate innate immune responses and control virus infections (Chapter 14). The importance of lipid mediators and alternative pathways of inflammation, as well as the ways drugs may influence them, are described.

Then Chapter 15 highlights the Toll-like receptor pathway and other intracellular inflammatory pathways that are critical in the elicitation of the initial type I interferon response. This response is both antiviral and bridges innate and adaptive immunity.

The influence of the neuroendocrine networks on immune responses to viral infections is detailed in the following chapter (Chapter 16). It has long been recognized that the fight-or-flight response and stress have profound influences on both acute and persistent immune responses during infections.

Public health personnel rely heavily on the data associated with epidemiology as well as clinical signs to direct their diagnostic analyses when presented with a sick patient. The basic information about seasonality, geography, and other factors are key to dissecting the puzzle of diagnosis (Chapter 17). It is clear from this book that new, emerging, and re-emerging infections are not fantasy but are the reality that we have lived through over the last decade. A new set of diagnostic tools has been devised to identify novel pathogenic agents (Chapter 18), and this is related in the chapter on surveillance (Chapter 17) and discovery.

Once diagnosed, how a case is managed and treated is essential for the recovery of the patient. The current state of the art is well-described in the chapter on treatment of patients (Chapter 19). Very few antiviral drugs are available, and the blood-brain barrier constitutes a problem for delivery of antiviral therapy.

While many virus infections are shared among people by aerosols or by contact with fomites in the environment, others, like West Nile virus and Japanese encephalitis virus (Chapters 6 and 7), are transmitted by animal or insect hosts. The nature of these vectors and the relationship they have with the viruses are detailed in Chapter 20 of this book.

Between late 1998 and early 1999, new infections in domestic pigs and man have been characterized in Malaysia, Australia, and other areas of Southeast Asia. These diseases are caused by newly characterized paramyxoviruses, Hendra and Nipah, which are transmitted by fruit bats (Chapter 21). Like rabies, another bat-borne infection, these viruses

also have effective immune evasive proteins. Other bat-transmitted viruses include SARS.

Viruses that are neurotropic can be used for potentially beneficial purposes. Glioblastoma is an aggressive and fatal tumor. Attacking it with viruses that can destroy dividing cancer cells but spare normal brain tissue is a goal of a new area of therapeutics, which is the focus of Chapter 22. Delivery of genes to correct inborn errors of metabolism is becoming a reality using viral vectors. These can also be applied to deficiencies in the CNS (Chapter 23). Like the elimination of tumors, it is essential to be able to deliver viruses that do not subsequently cause disease.

14

Innate immunity in viral encephalitis

Carol Shoshkes Reiss

Introduction

Viruses enter the brain by many routes. Rabies virus enters via a bite from a rabid bat or animal, replicates locally, crosses the neuromuscular synapse, and travels retrograde to the central nervous system (CNS). Mosquitoes infected with West Nile virus (WNV) sting a bird or mammal; WNV replicates locally and then travels hematogenously, infecting the brain endothelium. Human immunodeficiency virus (HIV), whether the virus entered by injection or semen, enters lymph nodes, replicates, and then is carried to the brain by infected monocytes that traverse the microvascular endothelium and enter the perivascular space, ultimately transmitting HIV to microglia. Other viruses, such as reovirus, replicate in peripheral tissues, circulate as free infectious virions, and can infect the vascular endothelium of the CNS. Viruses can be inhaled and replicate in the olfactory neuroepithelium and spread caudally across the cribriforme plate along the olfactory nerve. Herpes simplex virus (HSV) can infect the eye (keratitis) or the oral or vaginal mucosa, enter the local nerve, and then be transmitted by retrograde passage to a ganglion and sometimes to the CNS, causing encephalitis. Once within the brain, viruses replicate in a variety of cell types and induce local innate immune responses.

Every cell type (endothelial cells, ependymal cells, perivascular macrophages and pericytes, astrocytes, microglia, oligodendrocyes, Schwann cells, and neurons) in the CNS can be infected by different viruses. Viral infections of the CNS challenge the host with a different set of problems than do peripheral viral infections. Among the complications are (1) cells that rarely if ever express class I major histocompatibility complex (MHC) molecules and are thus not suitable targets for either $CD4^+$ or $CD8^+$ MHC-restricted cytolytic T cells, (2) an enclosed volume that is constrained from swelling during inflammation, as well as poorly developed lymphatic drainage, and (3) the immunologic privilege of the CNS, which leads to extremely limited immune surveillance for pathogens [1]. Due to these conditions, the role of innate immunity, both from CNS-resident cells and their products and from circulating inflammatory cells and molecules that traverse the blood-brain barrier (BBB), are essential to "buy time," inhibiting viral replication and dissemination, until the host can marshal an antigen-specific cellular and humoral immune response.

Innate responses are evolutionarily ancient and are found in the most primitive organisms. These are highly conserved and are not pathogen-specific but are in response to classes of foreign molecules. Infections can be perceived both extracellularly and intracellularly by pathogen-associated molecular patterns (PAMPs) and their host cell ligands, pathogen recognition receptors (PRRs), among them, toll-like receptors (TLRs). The innate immune response to infection ranges from release of soluble preformed mediators or production of cytokines, chemokines, interferons, lipid mediators (prostaglandins, leukotrienes, cannabinoids, epoxides, etc.), complement cascade components, neurotransmitters, nucleotides, molecules that ultimately regulate

transcription factors (high mobility group, steroids), and more.

Many of these factors act on CNS-resident cells via surface receptors that trigger signal transduction cascades, activating many intracellular pathways; some of these pathways regulate ion channels, which in turn alter cellular metabolism without new gene expression. Transcription and translation of a myriad of genes result from receptor signaling. Some of these gene products are beneficial to clearing or restricting the infection, while others are anti-inflammatory. These factors may directly or indirectly regulate microvascular permeability, thus transiently opening the BBB to circulating molecules. In bornaviral disease, the host's inflammatory response results in pathology.

In addition, innate immunity activates and results in cellular migration of both CNS-resident astrocytes and microglia as well as recruitment across the BBB of circulating neutrophils, natural killer, monocytes, macrophages, dendritic cells, and ultimately T and sometimes B lymphocytes to the site of infection. These responding cells contribute their own secreted effector molecules as well as some interactions requiring intimate cell-cell contact for release of factors to transient tight junctions.

The immune responses are critical for host survival from the infection. Consequently, successful pathogens, especially those which persist, have developed a wide variety of evasive approaches to limit the inhibition of replication [2]. Many of these pathways are highlighted in individual chapters which precede this one. These evasive measures range from neutralizing host-secreted molecules (cytokines and chemokines) with soluble receptors, encoding anti-inflammatory proteins in their genome, preventing signal transduction, blocking inhibition of protein synthesis, preventing apoptosis, and blockading of the nuclear pore complex.

In this chapter, I will attempt to cover the breadth of the innate immune response to viral infection, but I will also devote more space to generally under-considered aspects than to the well-known components.

Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)

Viruses trigger host responses by engaging several different families of receptors, both outside and inside cells, which recognize generic patterns and not specific sequences (e.g., a peptide of viral surface protein). One of the best known families of these pattern recognition receptors is TLRs. Signaling through TLR2 may lead to neuroinflammation [3], however, for measles virus, TLR4 signaling suppressed the innate immune response [4]. Further discussion of TLRs in viral infections is found in Chapter 15 of this volume.

Other important intracellular members of this class have a common domain called caspase recruitment domain (CARD), helicase, and NACHT, are RIG-I (retinoic acid inducible gene-I), MDA5 (melanoma differentiation associated gene 5), Caterpillar, NOD, NALP, NAIP and CIITA [5,6,7,8,9,10, 11,12]. Nod2, an intracellular PRR, is closely associated with Crohn's disease and recognizes bacterial determinant(s) [13].

RIG-I binds 5′ uncapped single-stranded RNA, an essential intermediate in RNA virus replication [14,15]; RIG-I has been shown critical for responses to influenza, paramyxoviruses, HCV, and Japanese encephalitis [14,16]. MDA5, in contrast, is required for picornavirus responses [16]. Like TLRs, RIG-I activation leads to activation of a protein variously known as Cardif/IPS-1/MAVS/VISA [9,10, 17,18,19,20] upstream of IRF3 and NF-κB activation, which transduce the signals with nuclear translocation, leading to the production of interferon (IFN)-β and all the downstream IFN-stimulated genes (ISGs).

While these pathways have been well-documented in many cell types, they may not always "work" in the CNS. For instance, while vesicular stomatitis virus (VSV) replication is extremely sensitive to the antiviral effect of pretreatment of neurons with IFN-β, VSV infection fails to elicit IFN-β production in neurons [21]; these cells do not express TLR-7 [22]. Neuronal cell lines constitutively express RIG-I,

and both VSV infection and IFN-β treatment can upregulate RIG-I mRNA expression [23]; however, this does not appear to play a crucial role in controlling the infection. VSV also evades cell autonomous responses through one of many actions of the viral M protein, which essentially prevents mRNA export from the nucleus [24,25]. Although VSV infection elicits IFN-β production by plasmacytoid dendritic cells in peripheral lymphoid compartments [26,27, 28,29,30,31,32,33], no IFN-β is made in the CNS during the first week of VSV encephalitis [21]. In contrast, both Theiler's encephalomyelitis virus (TMEV) and LaCrosse virus elicited neurons to produce Type I IFN responses [34].

An intracellular PAMP protein family which suppresses HIV and retrovirus replication are the apolipoprotein B mRNA editing enzyme catalytic polypeptide 3 (APOBEC3) proteins [35,36]. APOBEC3 molecules are packaged with HIV virions and work in the secondarily infected cell to block replication but are targeted by Vif in some viral variants for proteasomal destruction.

The receptor for advanced glycation endproducts (RAGE), an activating receptor, is expressed on many cells including vascular smooth muscle cells, endothelial cells, monocytes, and microglia [37,38, 39,40]. It was originally recognized as a contributor to the inflammation seen in diabetes, and binds, as its name suggests, proteins which have been post-translationally modified with glucose.

Other ligands for the receptor are S100 family proteins, HMGB1, and insoluble complexes of Aβ peptide, which are released during tissue damage in arthritis, atherosclerosis, aging, neurodegeneration, pulmonary diseases, sepsis, and ischemia [41, 42,43,44,45,46,47,48,49]. This has led to the classification of RAGE as a damage-associated molecular pattern (DAMP) receptor like TLRs are PAMP receptors [50]. Thus, direct or indirect compromise of neurons and parenchymal cells during viral encephalitis leads to the release of HMGB1 [51] or S100, which can activate microglia, perivascular macrophages and pericytes, as well as the microvascular endothelial cells [38,52]. Release of S100 during VSV encephalitis did not contribute to the production of IFN-α by splenic plasmacytoid dendritic cells, since infusion of soluble RAGE did not suppress the response [21]. Thus, the BBB will be disturbed, cells within the CNS will secrete cytokines, chemokines, and other inflammatory mediators. This could be among the first of the sequential waves of innate immunity in response to the viral infection.

Interferon-induced antiviral responses

The initial report of a factor made by cells which inhibited viral replication was made approximately 50 years ago [53]. There are two types of IFN. Type I is more diverse, produced by virtually all cells and includes IFN-α, IFN-β, IFN-λ, and IFN-τ. Type has only one member, IFN-γ. IFN-γ is made principally by $CD4^+$, Th1, $CD8^+$, and NK cells (reviewed in [54, 55,56,57,58,59]).

IFNs, which may inhibit viral replication by pathways described below, may also lead to neurodegeneration and demyelination through the activation of microglial production of neurotoxins [60,61,62]. Therefore, the beneficial and pathologic effects of IFNs may depend on the quantity and duration of expression.

Once IFNs have been induced and secreted, these cytokine bind ubiquitously expressed receptors, which are able to induce a signal transduction cascade starting with Jaks and signal transducers and activators of transcription (STATs) and lead to nuclear translocation of phosphorylated STAT complexes that result in gene induction in virtually all cells [63,64,65,66,67,68]. As with most other signal transduction cascades, there are regulatory phosphatases that dampen the IFN-mediated induction; these include suppressors of cytokine signaling (SOCS) and protein inhibitor of activated STAT1 (PIAS) family members [69,70,71,72,73,74,75]. While the Jak-STAT pathway is predominant, secondary signal transduction pathways are also important for IFN's activity [64,68]. Although most of the consequences of IFN binding and signaling are

transcriptional, not all of the inductive effects of IFNs require new mRNA production; I will discuss that below.

Some IFN-stimulated genes (ISG) are critical for costimulation, antigen processing, and presentation, some for antiviral effects, others contribute to regulation of angiogenesis, cellular apoptosis, or stasis [76], and other physiological processes. Hundreds of IFN-regulated genes have been identified using microarray analysis [77,78,79,80,81,82]. Traditionally these were studied in isolation, and many antiviral pathways have been well-characterized including Mx, PKR, RNAseL, OAS, IDO. I will focus on a few of the more important antiviral pathways controlled by IFNs.

Inactivation of GTP

The first antiviral IFN-stimulated pathway studied in detail initially for myxovirus infections, Mx, was discovered in 1978 by Lindenmann and colleagues; they observed that some mice were spontaneously resistant to influenza virus replication and later showed that Mx had GTPase activity [83,84]. Other GTPases including very large inducible GTPase-1 and TGTP/Mg21/IRG-47 are induced by IFNs [85,86]. Guanylate-binding proteins are also ISGs. These include GBP-1, a dynamin superfamily member with GTPase activity [87,88,89].

Inhibition of protein synthesis

Probably the best known pathway is the dsRNA-dependent protein kinase (PKR) pathway in which dsRNA produced during viral infection activates the kinase PKR, which phosphorylates and inactivates the translation factor eIF2α, inhibiting the production of new proteins in infected cells. This pathway is important in many neurotropic viral infections including sindbis, WNV, VSV, TMEV, and HSV-1 [82,90,91,92,93,94,95,96,97,98]. The inactivation of eIF2α can also be accomplished by two other cellular pathways: stress in the endoplasmic reticulum (PERK) and GCN2, and viral infection can contribute to activating them [99,100]. Other viral stress-induced proteins, including VSIG, P56, and homologs, can suppress protein synthesis by complexing with other transcription factors including eIF-3c [101,102]. Viperin/cig5/vig is an interferon-stimulated gene (ISG) and also induced during infection by cytomegalovirus (CMV), JC virus, or VSV, and suppresses synthesis of some viral proteins [103,104, 105,106,107]. Inhibition of viral protein synthesis is an effective host response to infection.

Degradation of RNA

The substrate of 2′,5′-oligoadenylate synthase (OAS)-dependent RNAseL is dsRNA. This pathway is important in the resistance to HSV-1, flaviviruses, LCMV, and VSV infections of the CNS [108,109, 110,111,112,113,114,115,116,117]. RNAseL may contribute to the apoptosis of infected cells [118].

Another antiviral protein that recognizes and sequesters viral mRNAs is zinc finger antiviral protein [119]. A potent host antiviral response is sequestering or degradation of viral RNA. Zinc has been shown to contribute not only to this antiviral protein but to other proteases including MMPs and metallothionein necessary for diapedesis [120] and inhibition of polyprotein processing for many picornaviruses [121,122,123]. Zn^{2+} also significantly potentiates the antiviral action of type I IFNs [124].

Altered amino acid metabolism

Indoleamine 2,3-dioxygenase (IDO), a catabolic enzyme for tryptophan, is induced by IFNs. IDO may have regulatory effects on T cell activity [125,126,127] and may contribute to alterations in serotonin metabolism and thus mood [128]. However, IDO has also been shown both to enhance astrocyte viability [129] and contribute to the formation of toxic quinolinic acid [130]. IDO has antiviral activity against vaccinia virus, HTLV-1, measles, and HSV-1 [131,132,133,134,135,136]. However, it may be antagonistic to containment of HIV-1 [137]. IFN-induced alterations in amino acid metabolism can suppress infection.

Chemokines

IFNs induce expression of chemokines including IFN-inducible 10KD protein (IP-10; CXCL-10, which also has antiangiogenic activity), Mig/Crg-2 (CXCL-9), and I-TAC (CXCL11) [138,139,140,141]. These proteins may also have defensin-like activity, nonspecifically arming antimicrobial responses [142]. These chemokines recruit neutrophils, natural killer (NK) cells, monocytes, and T cells to the brain. They have been shown to be important in the host's response to LCMV, MHV, VSV, and TMEV infections [143,144,145,146,147,148].

There are many other ISGs that have antiviral activity, although they are less well studied. One of these is ISG12, which contributes to resistance to sindbis encephalitis [149]. Adenosine deaminase, which acts on dsRNA (ADAR1), is an IFN-γ-inducible antiviral enzyme that may be coupled with the PKR pathway [150].

Reactive nitrogen and oxygen species

The production of superoxide (O_2*), nitric oxide (NO), and peroxynitrite ($ONOO^-$), the reactive molecule, contributes to elimination of pathogens from a range of cell types throughout the body. There are three isoforms of the enzyme responsible for generating NO, nitric oxide synthase (NOS) [151]. In the CNS, NOS-1 is found in neurons, NOS-2 in microglia and inflammatory macrophages, and NOS-3 in astrocytes, ependymal and endothelial cells [152]. NO is not only involved in long-term potentiation in the CNS [153]. It also contributes to regulation of blood flow [154]. Astrocytes and endothelial cells constitutively express NOS-3 and the release of this small effector molecule that results in increased local perfusion [155].

NO has been associated with some inflammatory neurological disorders [156]. The mechanism of NO-mediated inhibition is modification of viral proteins at cysteine, serine, and tyrosine, resulting in inappropriate folding, assembly, and/or enzyme activity. NO-mediated inhibition and/or pathology in the CNS contributes to the host response for reovirus, TMEV, HIV-1, SIV, adenovirus, Junin, bornavirus, Venezuelan equine encephalitis, MAIDS, CMV, Murray Valley encephalitis, MHV, Sindbis, and VSV [157,158, 159, 160,161,162,163,164,165,166,167,168,169,170,171,172, 173,174,175,176,177, 178].

NOS-2 is not constitutively expressed but is rapidly induced when macrophages or microglia are exposed to inflammatory cytokines. Microglia produce reactive oxygen species, as well, contributing to neurotoxicity [60]. However, unlike most of the IFN-regulated effector molecules described above, NOS-1 mRNA is not induced in neurons by IFNs, although the enzyme accumulates and is more active [64, 179,180,181,182]. This is one instance where IFN induction of antiviral activity is at a posttranscriptional level.

Major histocompatibility complex (MHC) molecules

MHC molecules are expressed at very low constitutive levels by parenchymal cells in the CNS. Both class and MHC molecules are essential for presentation of viral peptides to $CD8^+$ and $CD4^+$ T cells for elicitation of the acquired immune response, and as tissue targets for effector cells. During inflammation, whether due to infection or to autoimmune responses, MHC molecules are detectable in glia, endothelial cells, meningeal, and ependymal cells [183,184]. Neurons rarely express these ligands of CD4 and CD8 T cells [185] due to differential expression of SOCS1 [186] and a failure to load peptides onto ectopically expressed transgenic MHC I molecules [187]. Exceptions include isolated regions such as the vomeronasal organ where it may contribute to recognition of pheromones [188,189,190] and developmental pruning of synaptic boutons [191] under experimental conditions [192,193,194]. However, IFNs are able to induce the expression of components of the immunoproteasome by neurons both *in vivo* and *in vitro* [182,195,196]. It is not known what cell physiological effect(s) result from expression of the immunoproteasome by neurons. Thus, virally infected neurons are not capable of being recognized by effector T cells, and elimination of virus

must be accomplished by other mechanisms. This is important since neurons are essentially postmitotic, and if they were killed by cytolytic effector cells their functional role would be irreversibly lost.

Neuropeptides, peptide hormones, and neurotransmitters

Neurons release a variety of peptides and hormones in order to "talk" to other neurons. Many of these proteins have activities outside the synaptic signaling and can regulate immune responses. Among these molecules are substance P, neuropeptide Y (NPY), vasoactive intestinal peptide (VIP/PACAP), neurokinin1 (NK1), α-melanocyte-stimulating hormone (α-MSH) [197,198,199,200,201,202,203]. NK1 and NPY are inflammatory and may have defensin-like activity. Defensins are discussed in section 6, below. Others like VIP/PACAP are negative modulators, which act principally on dendritic cell induction of regulatory T cells [204,205].

Adenosine signaling, through surface A_1 and A_{2A} adenosine receptors, has been shown to be beneficial in the lung [206] and neuroprotective [207,208,209]. The receptors are also regulatory for pain [210]. In HIV infection, these receptors have been shown to play an anti-inflammatory role [211]. However, A1 receptors may also contribute to neutrophil infiltration, although this is antagonized by A2 receptors [212]. Expression of A2B receptors is induced by HIF-1-α, a cytokine-inducible transcription factor [213]. ATP, released by cells, can attract neutrophils to tissues via engagement of A3 and P2Y2 receptors [214]. There is reciprocal modulation of cannabinoid receptor expression by adenosine [215,216], thus signaling by one neurotransmitter can alter the response of neurons to other neurotransmitters.

Cannabinoids are endogenously synthesized (endocannabinoids) lipid neurotransmitters and are also found in some plants (e.g., marijuana) or pharmaceuticals. Two serpentine 7-transmembrane receptors have been well-described: CB_1 expressed by neurons and CB_2 expressed by cells of the reticuloendothelial system including microglia [217,218,219]. The functions of these receptors are distinct, although the same signaling pathways are used: the serpentine 7-transmembrane receptor is G-protein coupled, which (1) negatively regulates Ca^{2+} channels inhibiting Ca^{2+} release, (2) activates Raf-1, MEK, and ERK, as well as (3) adenyl cyclase, which ultimately activates protein kinase A. The CB_1 receptor is associated with hypothermia, immobility, euphoria, and hyperphagia, while the CB_2 receptor is a negative regulator of monocyte and microglial activation, hence immunodampening. Thus selective receptor agonists can target either immune responses or neurons. However, this distinction is potentially murky when you consider the regulation of cell-autonomous innate immune responses to viral infections in neurons.

Cannabinoids have been shown to be neuroprotective in Alzheimer's, Parkinson's, stroke, and excitotoxicity [220,221,222,223,224] and may target microglial activation [217,218,225,226,227]. Cannabinoids may contribute to neurogenesis by antagonizing NO production [228] and in ischemia [229,230], and I speculate may be protective of BBB integrity during infections where NOS-2 is overactive, such as bornavirus.

An indirect anti-inflammatory effect of cannabinoids had been found with activation of the nuclear transcription factor PPAR family, described below [231,232,233]. CB_2 receptor activation may lead to release of endogenous opioids, which inhibit inflammatory pain [234,235]. Somewhat unexpectedly, the antinociceptive and antipyretic effects of acetaminophen (Tylenol) may be due to binding CB_1 receptors.

Δ^9-tetrahydrocannabinol treatment decreases host resistance to HSV-2 infection in both mice and guinea pigs [236,237], probably by inhibiting host inflammatory immune responses against the virally infected cells. In several models where inflammation contributes to pathology, such as TMEV, the synthetic cannabinoid WIN 55,212–2 ameliorates clinical disease [171,238]; WIN 55 may also induce PGE_2 [239]. However, cannabinoids may contribute

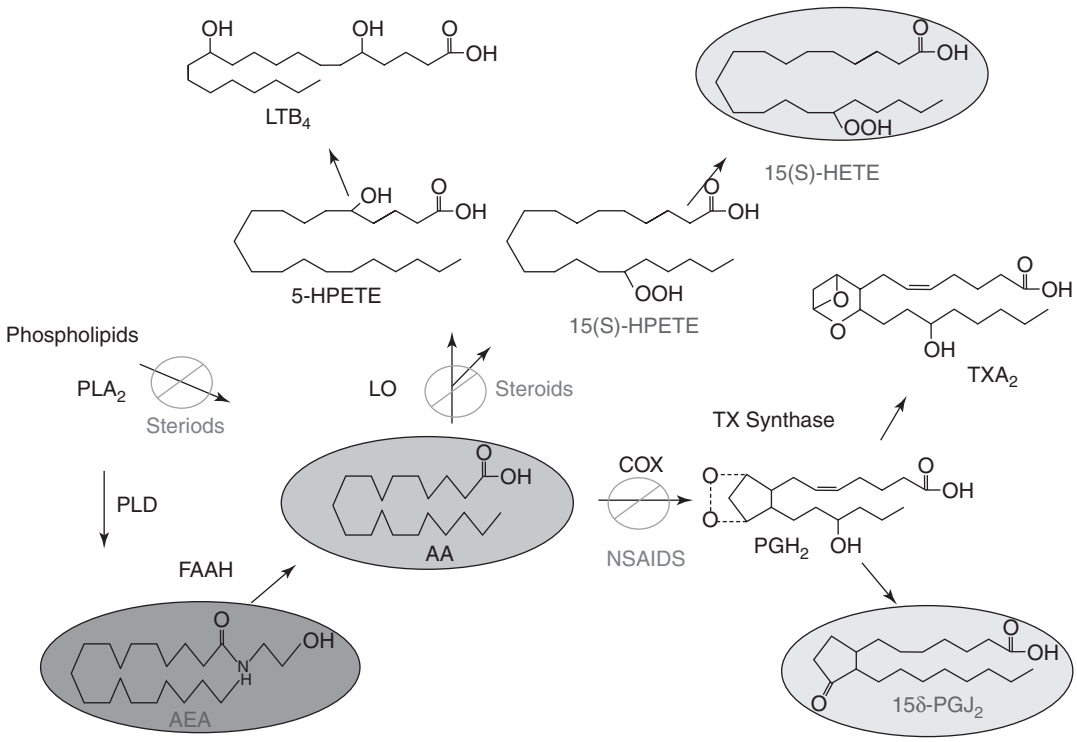

Figure 14.1. Biosynthetic pathways of eicosinoids important to innate immunity. The compounds discussed in this chapter include many products that originate in membrane phospholipids that are released by the action of phospholipase A_2 (PLA_2) to yield arachidnoic acid (AA) or anandamide (AEA), an endogenous cannabinoid that can be converted to AA by fatty acid amide hydroxylase (FAAH). AA can be metabolized by many different enzymes to yield potently bioactive compounds. Cyclooxygenases (COX) result in the production of prostaglandin H_2 (PGH_2), which is converted to many proinflammatory prostaglandins (PGE_2, for instance), thromboxane A_2 (TXA_2) by tissue-specific synthases, and anti-inflammatory 15δ-PGJ_2, a PPAR agonist, in the presence of aspirin. Lipoxygenases (LO) lead to the production of proinflammatory leukotrienes (LT) and anti-inflammatory 15(S)-hydroxyleicosatetraenoic acid [15(S)-HETE]. Not shown, for simplicity, are lipoxins (LX), which are also produced by LO, resolvins, protectins, and epoxyeicosatreinoic acids (EET). Some inhibitors are shown, and there are many others that are in clinical use.

to syncytia formation in HIV-E [240], which results in pathology. In VSV infection of neuronal cells, activating the CB_1 receptor leads to ~15-fold enhanced viral replication via inhibition of Ca^{2+}-flux and thus impairing the activity of constitutive NOS-1 [241]. Therefore, there is no hard and fast rule about the outcome of cannabinoid activity. Caution is urged when considering use of these drugs; the effect(s) may be on reticuloendothelial cells or on neurons.

Lipids in innate immunity in the CNS

The first part of this section will be devoted to *eicosanoids*, lipid mediators derived from arachidonic acid, liberated from cell membranes by phospholipase A_2 (Figure 14.1). These include prostaglandins (PG), leukotrienes (LT), lipoxins, epoxides, and other by-products. The second part of the section will include exogenous sources of these metabolites and lipid modification of cellular proteins.

Cannabinoids were discussed. *PPAR agonists* will be described below transcription factors. *Sex hormones* are discussed below, HPAI axis and neuroendocrine regulation are included here and in Chapter 16.

Cyclooxygenase (COX)

COX 1 and 2 are the enzymes responsible for the pathway from arachidonic acid leading to the formation of distinct prostaglandins (PGs) and thromboxane [242]. PGJ_2 will be discussed below as an agonist for the nuclear transcription factor PPAR. The family of receptors for PGs is among the 7-transmembrane serpentine surface molecules [243]. The end products have many biologic effects ranging from platelet aggregation (TXA_2) to inflammation and fever (PGE_2) [243] but also a profound consequence is immunoregulation, modulating dendritic cell maturation, differentiation, cytokine secretion and antigen presentation [244]. The importance of these molecules in physiological processes and pathology has led to drug discovery efforts [245]. PG production antagonizes innate host cell ability to respond to HIV, CMV, HHV-6, RSV, EMCV, HCV, MAIDS, rotavirus, and HBV infection in peripheral tissues [246,247,248,249,250,251,252,253,254,255,256]. In the CNS PGs compromise host responses to VSV, TMEV, JEE, Bornavirus, HSV-1, and EMCV infections [239,257,258,259,260,261,262,263,264,265,266,267]. The mechanism of interference involves suppression of NO production by NOS isoforms [257,259]; the role of NO was discussed at length earlier. Therefore NSAIDs and COXIBs are beneficial not only to prevent fever, aches, and pains, but also to promote recovery from viral infection [257,264].

5-Lipoxygenase (5-LO)

5-LO is the enzyme responsible for LT formation. In general, there is a dynamic yin-yang relationship between the balance of COX and 5-LO activity, since they both use the same initial substrate, arachidonic acid. There are two groups of LT which contribute to pathophysiology based on the receptors used and whether the LT contains cysteine. The CysLT (LTA_4, LTC_4, LTD_4) are associated with fluid production, fibrosis, and airway inflammation in asthma and other pulmonary diseases, while LTB_4 is a potent chemoattractant of neutrophils [268,269]. High levels of LT are seen secondary to mast cell infiltration or Th2-biased host responses patients infected with RSV, HIV, and CMV viruses [270,271,272,273,274,275]. More important, in the CNS, rather than contribute to pathology and BBB disruption, LT plays a beneficial role in recruiting neutrophils [144] and promoting recovery from VSV encephalitis [276].

Lipoxins (LX)

LX are anti-inflammatory LO products of arachidonic acid; 15-epi-LXA_4 is produced in the presence of aspirin. They are produced at temporally and spatially distinct sites from the inflammatory LT [277,278,279,280,281]. These molecules signal through SOCS2 [280]. LXA_4 and 15-epi-LXA_4 were associated with attenuation of neural stem cell proliferation and differentiation, in contrast to the activity of LTB_4, which induced proliferation [282]. The literature is sparse concerning the contribution of LXs in the resolution of inflammation associated with viral infection, however, it is possible that LX are produced in the CNS.

11,12-epoxyeicosatrienoic acid (EET) and hydroxyleicosatetraenoic acid (HETE) are the products of cytochrome p450 epoxygenase and are also anti-inflammatory, probably through activation of the PPAR nuclear transcription factor family (discussed later) [283,284]. 15- and 20-HETE regulate cerebral blood flow, enhancing perfusion [285]. Additional anti-inflammatory lipid molecules are resolvins and protectins, which are produced late in inflammation and promote resolution [286].

Omega-3 fatty acids, consumed in diets rich in cold-water fish (or by capsules) are also anti-inflammatory. They attenuate cytokine production and COX activity, downregulate adhesion molecules, and promote recovery from spinal cord injury [287,288,289,290]. In infections the data is mixed [291]

with benefit in HIV [292] and RSV [246], but more rapid death in lymphoma associated with the MuLV RadLV [293].

Protein isoprenylation

Statins (HMG CoA inhibitors) were licensed to block cholesterol biosynthesis and diminish inflammation. Bisphosphonates block bone resorption by acting on osteoblasts. Farnesyl transferase (motif CAAX, found in some proteins with an unpaired Cys) inhibitors were hoped to inhibit cellular Ras family activity and thus be cancer therapeutics. These three classes of drugs block distinct enzymes in the same branched lipid biosynthetic pathway and therefore contribute to regulation of cell autonomous and systemic innate immunity.

These inhibitors of lipid modification can be anti-inflammatory, in part, because protein isoprenylation is contributory to production of monokines like IL-1 [294]. TLR4 signaling is impaired by statins [295], as is LPS-induced AKT phosphorylation [296]. Cytokine activation of microglia is negatively regulated by RhoA, which prevents NF-κ B activation. RhoA negatively regulates COX-2 expression, leading to more PGs when isoprenylation is inhibited [297]. Chemokine production and chemokine receptor expression are dampened by the drugs [298].

However, bisphosphonate treatment results sustained activation of Rac, Cdc42, and Rho [299], possibly because isoprenylation of phosphatases (PTPases) including the PRL family (phosphatase found in regenerating liver), regulate the activity of Rac [300]. Rho/Rho-kinase activity modifies actin cytoskeletal proteins and results in dynamic cellular shape changes as well as the activation of NOS-3, resulting in the production of NO and thus, endothelial cell relaxation; inhibition of protein isoprenylation inhibits this cytoskeletal plasticity and changes in blood-flow dynamics [301]. These drugs may diminish inflammation by inhibiting diapadesis of inflammatory cells [302].

Protein isoprenylation is essential for formation of functional clusters of proteins tethered to cellular membranes [303]. Among the functional complexes which require lipid modification for effective enzymatic activity are the small GTPase activating proteins (GAPs) including RhoGAP [304]. Monocyte antibacterial activity associated with NADPH oxidase, activated by Rac guanine nuclear exchange factor, is negatively regulated by statins [305]. Ras must be farnesylated to interact with phosphoinoside-3-kinase [306].

With respect to viral infections, there have been several reports that protein isoprenylation is essential to replication of HBV, HCV, HDV, RSV, and HIV [307,308,309,310,311]. VSV replication in neurons is inhibited about 15-fold by one of the drugs [312]. But membrane fusion, which is important to initiate many virus infections or to release enveloped virus progeny, is inhibited by isoprenylated SNAREs [313]. Isoprenylated proteins are not incorporated into lipid rafts [314]. An IFN-inducible antiviral protein, human guanylate-binding protein-1 (hGBP-1), a GTPase, is isoprenylated, and located at the Golgi, thus its activity is impaired in the presence of pathway inhibitors [315,316]. Thus, statins/bisphosphonates/isoprenyl transferase inhibitors may inhibit viral replication but may also suppress the host IFN-dependent antiviral pathway(s) and inflammation.

Apoptosis and autophagy

Cells under stress from viral infection, TNF-family cytokines, and cytoxic T lymphocyte (CTL) recognition can undergo programmed cell death (apoptosis) [317]. I will not review the cellular pathways that lead to genome fragmentation and membrane inversion, but will focus, instead, on associations between viral infection and apoptosis. Cells that commit suicide in this manner may spare the host from continued viral replication, a benefit, especially since most cells can be replaced by stem cells in that organ; however, if the infected cell undergoing apoptosis were a neuron, significant consequences might ensue [318]. Among the viruses associated with cellular apoptosis are influenza [319,320], coronaviruses [321], hemorrhagic fevers (Ebola, Junin,

Marburg) [322], adenovirus [323], reovirus [324], and HCV [325]. Neurotropic viruses which elicit this outcome include alphaviruses [326], poliovirus [327,328] (see also Chapter 1, Neurotropic picornaviruses), VSV [329,330,331,332], rabies [333,334], HIV-1 [335,336,337], and HTLV-1 [338]. In fact, apoptosis is such an important cellular defensive response to viral infections that poxviruses have developed an evasive pathway, using serine protease inhibitors (serpins) [339,340]. But scientists are clever and have selected apoptosis as a tool for viral oncolysis (which is discussed in detail in Chapter 22 of this book).

At other times, viral infections or cellular stress from starvation can lead to recycling of large volumes of cytoplasmic contents by generation of vesicles that fuse with lysosomes (autophagy, self-eating) [341,342,343]. This pathway can become dysregulated and result in neurodegenerative diseases [344,345,346]. In the periphery, influenza virus [347], EBV [348], and HBV [349] infections have autophagy-associated pathology. Some picornaviruses and swine fever virus use this cellular response to develop additional membranes on which to replicate [350,351,352]. In the CNS infections caused by HIV [353], LCMV [354], and HSV [355], autophagy-associated pathology has been reported. Thus, in general, autophagy is an innate host cellular response to suppress viral infection, but this can be manipulated by some viruses to their benefit.

Protein players in innate immunity

Many different classes of proteins are critically involved in innate immunity in the CNS. This section will briefly describe the roles of defensins, lactoferrin, complement cascade components, and cytokines. IFNs and chemokines were discussed earlier.

Defensins

Defensins are small, conserved antimicrobial peptides, produced by many cell types including epithelial and leukocytes, which are found in both very primitive species which lack adaptive immunity and mammals [356,357]. While inflammatory infiltrating cells contribute, in the CNS parenchyma, defensins are synthesized by astrocytes, the choroid plexus, and the hypothalamus [106,202,358,359]. Peripherally synthesized defensin molecules can cross the BBB [360]. They have been shown to contribute to elimination of both bacteria and to virus infections. In viral infections of the CNS, these include HIV, HSV, and adenovirus [361,362,363,364]. The release of defensins may be regulated by LTB_4 [273].

Lactoferrin

Lactoferrin is a small, secreted, iron-complexed protein which has both antibacterial and antiviral activity. Lactoferrin is found in milk and plasma and secreted by neutrophils [365,366,367]. Recent studies suggest that it may contribute to innate immune inhibition of CNS infections associated with picornaviruses, alphaviruses, papovaviruses, and herpesviruses [368,369,370,371,372].

Complement

Compelment cascade components and their receptors are expressed both constitutively and can be induced during immune responses in the CNS [373,374,375]. Of course, in the classical cascade, specific antibody must first be synthesized and engage its epitopes, inducing conformational changes in IgG which result in exposure of the cryptic C1q binding site and the initiation of the cascade. The alternative and lectin pathways can also induce activation of complement. IgG can cross the BBB, and can, under circumstances of persisting immune responses, be synthesized in the CNS in tertiary lymph nodes. But, that reflects adaptive and not innate immune responses.

The small anaphylatoxins, C3a, C4a, and C5a, which are proteolytic products of the zymogens, are potently active as activators of vascular permeability and chemoattractants for neutrophils. These molecules are produced in the CNS by astrocytes and microglia [376,377,378]. C5a is a potent recruiter

of polymorphonuclear leukocytes (PMNs). We asked whether it was essential for the host response to VSV encephalitis and found that C5a was not required [379], the redundance of chemokines and LTB_4 were sufficient to promote recovery.

Complement receptors include both serpentine 7-transmembrane molecules (which bind C3a, C4a, and C5a) G-protein coupled and transmembrane glycoproteins (such as CD21, the EBV receptor). Some complement receptors, including CD46, a measles virus, and human herpes virus-6 (HHV-6) receptor, are expressed by neurons and endothelial cells [380,381,382]. Complement activation has also been shown to be critical for the development of the adaptive Ab response for WNV [383,384]. Complement contributes to the neurovirulence of Sindbis, HIV, SIV, and Bornavirus infections [376,378,385, 386,387]. HSV, vaccinia, and the murine gamma herpes virus MHV-68 [388], have developed evasive proteins that prevent complement activity and contribute to their disease pathogenesis. The vaccinia virus proteins have been isolated and have been proposed as a therapeutic where host complement activation is pathogenic in the CNS [389]. Recombinant HSV-1 deficient in the complement-interacting gamma134.5 gene product has been proposed as an effective vector for viral oncolysis [390]. Similarly, herbal proteins are also able to block complement and have been suggested as potential neuroprotective therapeutics [391].

Cytokines

Cytokines are comprised of dozens of different protein mediators that are generally secreted by one cell and act on the secreting cell (autocrine) locally (paracrine) or systemically (endocrine) to either activate and differentiate another cell type or to regulate the activity of another cell. Generally, the receptors are heterodimers of surface glycoproteins. Although many investigators have tested the effects of individual molecules in experimental systems, in real life, cytokines are secreted as part of a coordinated program with many different molecules released at the same time; it is the composite of these mediators' signal-transduction pathways which leads to the outcome. The differentiation state of the downstream cell, the quantity, and duration of exposure determine the response. Cytokines can downregulate their receptors by internalization, leading to desensitization, or induce enhanced expression of their own (and other) receptor and second messengers, which can positively regulate subsequent responses.

In the brain these molecules can be produced by both parenchymal cells and by infiltrating inflammatory cells. Two excellent reviews of cytokines and the CNS are a book edited by Richard Ransohoff and Tika Benviste and an article by Iian Campbell [392,393]. It is the balance between proinflammatory cytokines such as TNF-α or IL-23 and the anti-inflammatory molecules such as TGF-β or IL-10 which regulates the outcome.

Systemically produced cytokines can lead to CNS consequences ranging from the fever response to IL-1 (inducing the production of PGE_2) or TNF-α secretion, resulting in transient disruption of the BBB. Excessive peripheral release of cytokines has been associated with "sickness behavior" [199,394,395, 396,397,398]. Additional studies indicate that IL-16 has been associated with neurodegeneration in EAE [399,400,401]; however, it has not as yet been associated with pathology viral infections. In infections, proinflammatory cytokines can lead to beneficial outcomes, such as IL-12, TNF-α, or IFN-γ induction of NO, which leads to elimination of VSV infections [144,166,181,402,403,404,405,406,407]; however, IL-12/IL-23 and IL-18 are not necessarily critical even if synthesized or administered [408,409]. These are important in TMEV and MHV [410,411,412,413,414]. Excessive expression of proinflammatory cytokines, in other circumstances, may contribute to pathology (neurovirulence or neurodegeneration) in bornavirus, JEV, HTLV-1 (see Chapter 8), HIV/SIV (Chapter 9), MHV (Chapter 4), TMEV (Chapter 1), enterovirus 71 (Chapter 1), LCMV (Chapter 5), canine distemper, rabies (Chapter 3), and VEE (Chapter 6) [95, 178,415,416,417,418,419,420,421,422,423,424]. Cytokines are essential mediators in viral infections of the CNS. Some cytokines are neuroprotective,

promoting recovery [425]. The timing, quantity, and balance of these bioactive molecules determine the outcome: recovery or pathology.

Transcription factors regulating inflammation

Many transcription factor families regulate responses, and the roles of IFN-inducible STATs have been discussed above. In this section, three classes of transcriptional facors will be described: hypoxia-inducible factor, peroxisome proliferation activating receptor, and high mobility group protein.

Hypoxia-inducible factor-1α (HIF-1α)

HIF-1α is a transcription factor whose expression is triggered by transient hypoxia (or ischemia/stroke) and induces the expression of a number of genes including defensins, the adenosine A_2B receptor, vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), NOS-2, and NOS-3 [213,426, 427]. VEGF regulates not only BBB permeability but also angiogenesis. HIF-1α is also induced by IL-1 and TNF-α [426,428] and possibly TLR agonists. It is induced during inflammatory and demyelinating diseases [429]. In studies with viral vectors for oncolysis in the CNS, HIF-1α expression was enhanced [430,431,432]. Thus, HIF-1α expression, whether elicited by transient vascular compromise or cytokine expression, enhances the innate antiviral (and antibacterial) gene expression and leads to increased BBB perfusion of the local area.

Peroxisome proliferating activating receptors (PPARs)

PPARs, nuclear hormone transcription factors, have three isoforms α, δ, and γ, each with distinct activity and expression. PPAR-γ, the canonical nuclear hormone receptor involved in muscle glucose uptake and lipid homeostasis and cell differentiation, is translated as two splice variants, γ1 and γ2. PPAR-γ2, 30 amino acids longer than γ1, is expressed in high levels of adipose tissue. PPAR-γ ligands are polyunsaturated fatty acids, eicosinoids, FA oxidation products (13-HODE and 15-HETE), J-series prostaglandins (15-deoxy-D12,14-prostaglandin J_2), some nonsteroidal anti-inflammatory drugs (NSAIDS), and insulin-sensitizing thiazolidinediones (TZDs). PPAR-γ functions as an obligate heterodimer with retinoic X receptor (RXR) to activate transcription by binding to 5′ promoters of target genes.

PPAR-γ agonists modulate inflammatory responses in the CNS, resulting in the reduction of iNOS in cerebellar granule cells. PPAR-γ signaling has anti-inflammatory function in EAE, PPAR-γ agonists alleviate symptoms with antagonists performing the opposite, indicating regulation of autoreactive Th1 cells. PPAR-γ also can regulate immune responses within the CNS; they are in clinical trial for MS. Alzheimer's disease is a severe neurodegenerative disease characterized by the accumulation of amyloid plaques accompanied by activated microglia. TZD treatment in *in vitro* experiments with microglia and monocytes attenuated the secretion of proinflammatory cytokines. Medium from TZD-treated microglia was neuroprotective for neurons [433,434,435].

In vitro treatment of cells with PPAR agonists inhibited replication of RSV, HHV8, HCV, HIV [436, 437,438,439], and VSV [unpublished], although the mechanisms by which this inhibition occurred have not been elucidated. However, HBV X-associated protein 2 complexes with PPAR and inactivates it [440], an evasive pathway. Thus, treatment with PPAR agonists, such as TZDs, may be beneficial for viral encephalitis both as potential antiviral compounds and as anti-inflammatory compounds in infections where pathology is associated with inflammation, such as bornaviral disease.

High mobility group protein B1 (HMGB1)

HMGB1 is unique among transcription factors as it is found not only in the nucleus but also the cytoplasm associated with α-synuclein filaments [441] and is actively released. Its expression may be

upregulated by IFN [442]. HMGB1 engages either AMIGO receptors of neurons where it regulates neurite outgrowth [443,444,445,446], TLR 2 and TLR 4 [447], or the receptor for advanced glycation end-products (RAGE), which results in a proinflammatory response by microglia, macrophages, and dendritic cell maturation. HMGB1 release has been shown to be neurotoxic in ischemia, Alzheimer's disease [448,449], and in both bornavirus disease and WNV [450,451]. HMGB1 is a Janus molecule with both regulatory transcriptional activity and signaling of tissue damage; it may be important in eliciting innate immunity during viral encephalitis.

BBB

Within the CNS, there are anatomically distinct regions which have some constitutive vascular permeability (e.g., circumventricular organ, choroid plexus), but most areas are highly restricted in access to circulating cells and proteins. Astrocytes regulate the perfusion of the parenchyma by controlling vasodilation of the cerebrovascular capillaries through the activity of NOS-3 [452,453,454]. The BBB is associated with the immune privilege of the brain and separates the CNS from peripheral circulation and immune surveillance, which is characteristic of the periphery [455,456,457]. Entry of cells requires adhesion to the brain microvascular endothelium, release of MMPs to degrade tight junctions and the extracellular matrix, as well as migration along a gradient of chemoattracting molecules [458]. The chemoattracting molecules for circulating cells range from LTB_4 to complement products, chemokines, cytokines, FLT3L, and even ATP (all discussed above).

A hallmark of many viral infections including lethal LCMV (Chapter 5), HIV (Chapter 9), polio (Chapter 1), HSV-1 (Chapter 11), MHV (Chapter 4), JE (Chapter 7), and VSV is the breakdown of the BBB [259,453,459,460,461,462,463,464,465]. However, the global breakdown of the BBB seen in fatal LCMV (Chapter 5) and in fatal VSV infections is extreme and unusual. In most cases, the overall integrity of the BBB is maintained, but in discrete regions, there is increased perfusion leading to entry of normally excluded proteins from circulation.

The BBB breakdown associated with infection results from excessive normal physiological process regulating blood flow within the CNS [466,467,468]. Activation leads to NOS-3-expressing astrocytes to release NO, which induces guanadyl cyclase to produce cGMP, leading to endothelial and smooth muscle cell relaxation [154]. Other mediators, such as the small complement cascade mediators C3a, C4a, and C5a, VEGF, and PGE_2, can also lead to the relaxation and increased permeability of the BBB.

Parenchymal and inflammatory cells in innate immunity in the CNS

Infiltration of peripheral circulating cells

Normally, there are very few lymphocytes, neutrophils, and NK cells in brain parenchyma. Infiltration of inflammatory cells ranging from PMNs to NK cells to macrophages and finally lymphocytes takes place in response to a series of signals from both chemoattractant molecules and orchestrated binding to microvascular endothelial cell surface molecules [415,469,470,471,472,473]. For cells to cross the endothelial vessel wall, they must diapedese and then digest the basement membrane with MMPs. This review will not discuss the infiltration of antigen-specific T cells or B cells, as it is limited to innate immune responses.

Chemoattractants for **neutrophils** include adenosine, ATP, f-MetLeuPhe, C5a, LTB_4, and chemokines. For VSV encephalitis, LTB_4 and chemokines, but not C5a, are essential [144,276,379]. PMN infiltration is also characteristic of Murray Valley encephalitis, MHV, HSV-1, TMEV, Western equine encephalitis, and adenovirus infections [474,475,476,477,478,479,480,481].

NK cells are generally the second cell type to diapedese in response to viral infections of the CNS in response to both chemokines and IL-12. NK cells nonspecifically recognize patterns of receptor

expression on cells and are sensitive to low levels of MHC molecules, and, when activated, release IFN-γ, perforin, and granzymes, like $CD8^+$ CTL. NK cells have been associated with the host response to WNV, sindbis, MHV, bornavirus, HSV-1, VSV, SIV, CMV, TMEV, and enterovirus 71 [477,482,483,484, 485,486,487,488,489,490,491,492,493,494,495]. NK cells contribute not only lytic activity against virally infected cells, but they also are a significant source of IFN-γ secretion, both of which regulate viral replication.

Antigen processing and presentation

Pioneering work by the late Helen Cserr and her colleagues, including Paul Knopf, explored lymphatic drainage of soluble antigens and their ability to evoke an acquired immune response [1]. This drainage is polarized from rostral to caudal and is modest and does not include the hallmarks of peripheral tissue dendritic cells bearing antigens to the draining lymph nodes.

There is little constitutive expression of class MHC molecules in the undisturbed CNS; however, both **astrocytes and microglia** readily express these molecules in response to inflammatory cytokines, especially IFN-γ [496,497,498,499,500,501,502]. Infection indirectly induces the expression of MHC and enhances the expression of MHC I by parenchymal cells [335,404,503,504,505,506]. There was controversy for many years about which resident parenchymal cell type(s) were competent and effective at eliciting host T cell responses to experimental antigens, pathogens, and autoantigens [507,508].

Perivascular macrophages have been shown to be an important player in antigen presentation for the brain. Bill Hickey's group has documented their recruitment from circulation and function in infections and autoimmune disease [487,509, 510,511,512].

Dendritic cells are the principal antigen-presenting cells in the periphery. They are extremely difficult to detect in undisturbed brain tissue. During immune responses in the CNS, it is possible to find cells expressing dendritic cell markers [513,514,515, 516,517,518,519,520,521]. There was (and remains) controversy as to whether dendritic cells differentiate from a resident precursor or are recruited from circulation, but recent reports are consistent with infiltration of these circulating cells [513,522,523, 524,525,526,527]. In addition to any chemokines, Flt3L has been shown to recruit dendritic cells to the CNS [528].

HPAI axis and neural-endocrine regulation

In Chapter 16 of this book, a more extensive discussion of neuron-endocrine-immune networks can be found. *The hypothalamic-pituitary-adrenal-immune (*HPAI*) axis* controls not only fight-or-flight in response to stressors but also critical control of immune responses to infections. There are short-term and chronic stress manifestations of this [529,530,531,532,533,534,535,536], with long-term compromise of immune responses to viral infections [537]. This may be manifest as alterations in the humoral response to viral infection [538] or as increased susceptibility and/or progression of disease [539]. Acute stress may also alter the integrity of the BBB [540], thus potentially permitting entry to otherwise excluded viruses.

Sympathetic nervous system

Chemical sympathectomy, achieved by infusion of 6-hydroxydopamine, has profound effects on the peripheral immune response, as there is sympathetic innervation of the spleen and lymph nodes [541]. Hosts are more susceptible to bacterial and HSV-1 infections [542,543,544], but when hosts are already immune suppressed, whether by malnutrition or by lentivirus infections, they are not further compromised [545,546].

We tested whether chemical sympathectomy altered the ability of peripheral plasmacytoid dendritic cells to produce IFN-β in response to VSV infection of the CNS and found no contribution of innervation of secondary lymphoid organs in this response [21]. Thus, it does not appear that

the sympathetic nervous system regulates antiviral responses.

Cholinergic pathways have been shown to be anti-inflammatory in bacterial model systems and ischemia-reperfusion injury [547], inhibiting cytokine production and tissue injury, but there are no reports in the literature on the impact of this regulatory arm of the CNS on viral infections.

Leptin was originally identified as the gene product deficient in obese mice and was found to regulate energy balance, but like so many other effector molecules, it has many other activities. Recent evidence suggests that this adipocyte-produced protein is also immunoregulatory and is, in fact, a proinflammatory cytokine [548,549,550,551]. Its production is controlled by NF-AT [552]. Leptin is pathogenic in EAE [553] by virtue of its action on dendritic cells, resulting in the induction of Th1 responses [554] and its inhibition of thymic apoptosis [555]. Therefore, leptin is a potential target for therapeutic intervention in persistent inflammatory infections of the CNS, such as HIV-E and bornaviral disease.

Sex hormones regulate more pathways than just those in secondary sexual organs and hair growth. **Estrogen** is neuroprotective in infection, Alzheimer's disease, traumatic injury, and ischemia [556,557,558,559,560,561,562]. Estrogen has profound immunomodulating effects ranging from induction of NOS-3, and thus increased vascular perfusion [563,564]. Estrogen positively regulates expression of IFN-γ [565]; this is clearly linked with the increased frequency of females who have Th1-associated autoimmune diseases such as EAE/MS. Additionally, sex hormones regulate PPARs and can influence the severity of EAE [566].

There is an effect of sex in viral infections of the CNS. Female mice are more resistant than males to lethal VSV infections [567,568] and show improved clearance of MHV and Semliki Forest virus infection from oligodendrocytes [569,570]. However, female mice undergo more severe demyelination in TMEV infections [571]. Thus, where inflammation is beneficial to clearing virus and resolving infection, females are at an advantage. In contrast, where inflammation contributes to viral disease pathology in the CNS, females are disproportionately affected.

Summary and speculation

Innate immunity in the CNS is complex and includes protein effectors (IFNs, cytokines, chemokines, defensins, complement, lactoferrin, and other molecules), lipid mediators (PGs, LTs, cannabinoids, etc.), small diffusible molecules (NO, ONOO-), and both parenchymal and inflammatory cells. These responses are highly regulated and are triggered, in part, by receptors that bind common pathogen-associated or damage-associated molecules. Combined, these responses provide a critical barrier, controlling viral replication until adaptive immune responses are marshaled. Inflammation is essential but must be carefully controlled to prevent tissue damage and pathology. Virtually every pathway has regulation (e.g., kinases and phosphatases) that ultimately determine the magnitude and then the quashing of responses. We are still learning about the essential pathways and their controls, about how drugs may alter the dynamic equilibrium, and in which situations responses need to be ramped up or downregulated. I predict we will make many future advances that will benefit the health of the populations who are infected with neurotropic viruses.

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15

Role of Toll-like receptors in neurotropic viral infections

Robert W. Finberg, Shenghua Zhou, and Evelyn A. Kurt-Jones

Introduction: TLRs and viruses

Toll-like receptors and their specificity

Toll-like receptors (TLRs) are pattern recognition proteins found both on cell surfaces as well as within intracellular compartments. Originally defined on the basis of their homology to the Drosophila protein Toll, which is important in the fruit fly defense against fungal infections [1], mammalian TLRs were first demonstrated to be critical in determining whether animals develop shock after challenge with bacterial lipopolysaccharide (LPS). The interaction between E.coli LPS and TLR4 leads to a series of events resulting in the production of cytokines and inflammatory mediators that affect vascular permeability and that ultimately cause a decrease in blood pressure and death of the animal. Subsequent studies have revealed a role for TLRs in the immune responses not only to bacteria but also to fungi, parasites, and viruses.

TLRs are a family of proteins with a structure including an N-terminal pattern recognition domain composed of leucine-rich repeats which form a molecular scaffold and a cytosolic C-terminal Toll-interleukin-1 receptor (TIR) domain that interacts with a series of adapter proteins. Engagement of TLR adapters ultimately leads to intracellular signaling events that induce the production of chemokines and cytokines (Figure 15.1). The human genome encodes 10 different TLR proteins, all of which are homologous to the interleukin-1 receptor (IL-1R) protein in their TIR domain. While TLRs are pattern recognition proteins (recognizing viruses and bacteria predominantly through their leucine-rich repeat regions), they initiate the production of cytokines and chemokines that directly (through activation of other cells) or indirectly (through stimulating migration of immune cells) result in the initial host response to infection. The secretion of cytokines is ultimately achieved through a cascade of signaling proteins (Figure 15.1). For most TLRs, the adapter protein, MyD88, is critical for production of interferon or inflammatory cytokines. The TLR3 protein is an exception to this rule and signals through an intracellular adapter protein called TRIF. Notably, TLR4 activates both MyD88 and TRIF (via an intermediate adapter TRAM). Polymorphisms or mutations in both TLRs and adapter proteins can (and do) affect immune responses to viruses. Other, non-TLR cytosolic viral sensing proteins, including the helicases RIG-I and MDA-5, have crucial roles in recognition and containment of virus in the cell (Figure 15.1).

Mammalian TLR-mediated signaling is triggered not only by bacterial and viral lipids and proteins but also by nucleic acids. The TLR response to viruses is complicated by the fact that viruses initially bind to the extracellular surface (like bacteria), and later they replicate intracellularly and then they may bud from the cell surface again, allowing them potentially to interact with TLRs at multiple stages in their life cycle. While initial experiments revealed the ability of cell surface TLRs to recognize viruses, subsequent studies have defined the fact that TLRs also recognize nucleic acids

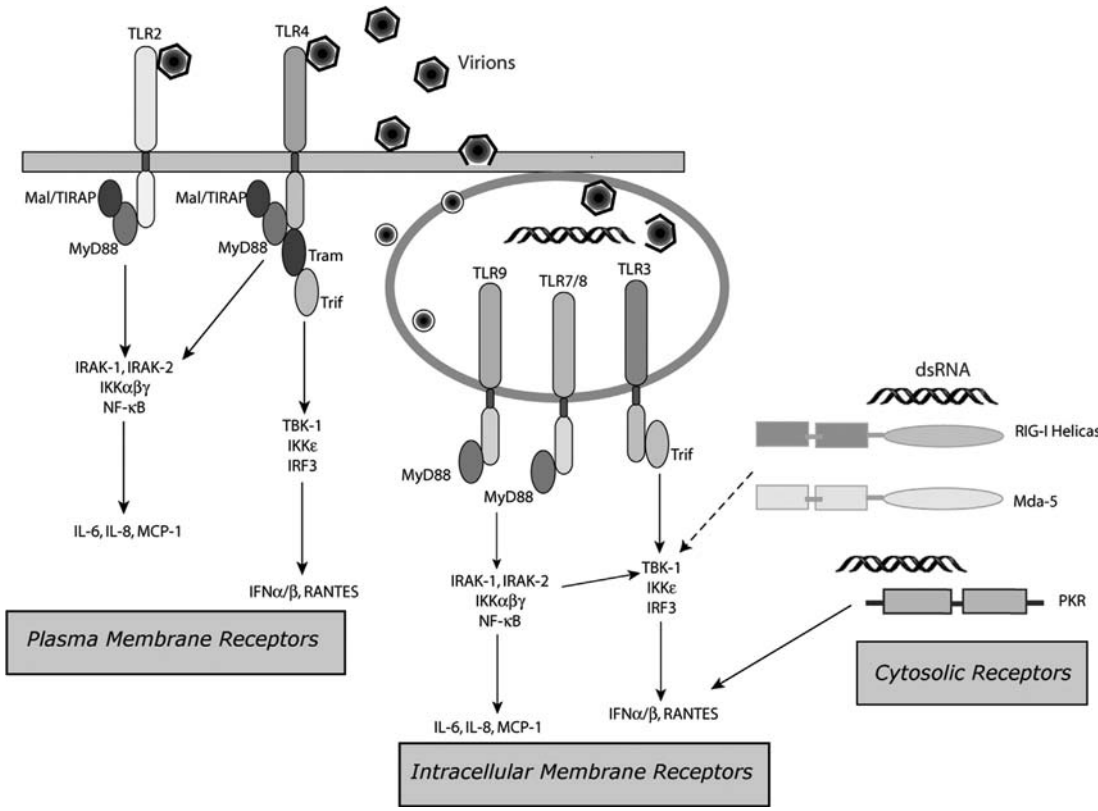

Figure 15.1. TLR and RIG-I/Mda5 activation pathways, Toll-like receptors, and the cytosolic helicases activate type I IFN and inflammatory cytokine production via IRF and NF-κB. These receptors share downstream intracellular signal-transducing proteins including IRAK/IKKαβγ and TBK-1/IKKε/IRF3-signaling cascades. (Reviewed in [1].)

generated by replicating viruses. Of interest is the fact that different TLRs recognize different components of pathogens. For example, TLR2, a protein expressed on the cell surface, triggers cytokine release when herpes simplex virus (HSV) first interacts with the cell surface, while TLR9, an intracellular protein, is stimulated to produce cytokines by HSV DNA (Figure 15.1). Viral nucleic acids also trigger TLR3, which recognizes double-stranded RNA (dsRNA), as well as TLR7, which recognizes single-stranded RNA (ssRNA).

Not surprisingly, multiple TLRs may be involved in the response to a single virus. For example, on attachment to the cell surface, herpes simplex virus (HSV) may activate cytokine secretion that is mediated via TLR2 [2]. Once inside the cell, HSV DNA is capable of stimulating interferon secretion, which is activated through TLR9 [3,4]. Furthermore, many, if not all viruses, have been demonstrated to have ssRNA or dsRNA replication intermediaries, which in turn activate TLR7 and TLR3, respectively. Murine cytomegalovirus (CMV), a DNA virus closely related to human CMV, has been demonstrated in animal models to stimulate cytokine production through both TLR7 and TLR3 [5,6]. This presumably occurs through RNA intermediaries in the virus cycle, but the precise mechanism and viral target has yet to be elucidated. Murine CMV also interacts with TLR2,

and this contributes to the outcome of infection [7]. It would be anticipated on the basis of its life cycle that CMV DNA stimulates interferon secretion through TLR9. HSV has been shown to stimulate interferon via TLR9 [6], but it is not clear that this interaction is important in the outcome of disease such as encephalitis. Both TLR2 and TLR9 play a role in herpetic keratitis [25]. TLR-virus interactions occur at many stages during the virus life cycle and may have different effects depending on when the TLR is engaged (Figure 15.2).

TLRs: their role in innate and adaptive immunity

As pattern recognition proteins that are stimulated by components of infectious organisms, TLRs may initiate events that result in the production of cytokines that lead to inflammation. These events can result in capillary leakage, decreased blood pressure, and death of the host. The absence of TLR4 protein makes mice resistant to lipopolysaccharide (LPS)-mediated shock [8]. In this instance the presence of the TLR protein appears to be acting in opposition to the interest of the host. However, mice lacking TLR4 are extremely sensitive to infection with Salmonella. While wild-type animals die when challenged with high doses of E.coli LPS, mice with a mutant TLR4 gene do not respond to E.coli but die of Salmonella infection. This is the "double-edged sword" of TLRs. On the one hand, they may induce host responses that result in protection against infection, while on the other hand they can be associated with inflammatory events that lead to death of the host.

The mammalian immune system consists of two separate but interactive units: (1) the innate immune system, and (2) the adaptive immune system. The cells of the innate immune system include the polymorphonuclear leukocytes (PMNs or neutrophils), monocytes, NK cells, and tissue macrophages that are critical in the initial responses to pathogens and without which the host cannot protect itself from infection. TLRs which are expressed on neutrophils, macrophages, and other cells, are important because they initiate the production of cytokines. These cytokines may activate macrophages (e.g., interferon gamma, IFNγ) or induce the production of antiviral proteins in other cells (e.g., interferon α,β). In addition, TLRs lead to the production of chemokines that control migration of cells to areas of tissue destruction. The innate immune system also includes serum components like the complement system and other proteins that may bind to pathogens. The adaptive immune system allows mammals to react to pathogens by stimulating responses based on recombination events. Somatic recombination, mediated through recombination activating genes, leads to the generation of T and B cell responses that recognize the specific pathogen, generation of "second set" antibody and T cell expansion, and protection of the host from future encounters via the generation of long-lived memory cells which are capable of rapid reactivation when the same pathogen is encountered again.

Although TLRs are not directly involved in recombination events and are not an essential part of the adaptive immune system (T and B cell responses can be generated in the absence of TLRs), the cytokines and chemokines produced by TLRs have a major effect on the initiation and augmentation or suppression of the immune response. The type of cytokine response induced by the initial interaction with a virus will determine the type of T and B cell response produced. Induction of interferon gamma is likely to lead to a Th1 type of T cell response (characterized by cytolytic T cells (CTLs) and in mice, an IgG2a antibody response) while production of IL-4 is likely to lead to the IgG1 and IgE antibodies being produced and a lack of CTLs (a Th2-type response). Since TLRs are expressed on antigen-presenting cells, the presence or absence (or even an allelic polymorphism) of TLRs may define the type of response to an infecting pathogen.

In the case of leprosy, polymorphisms of TLR2 determine whether a patient is likely to develop tuberculoid leprosy (characterized by a Th1 response) or disseminated lepromatous disease (characterized by a Th2 response) [9].

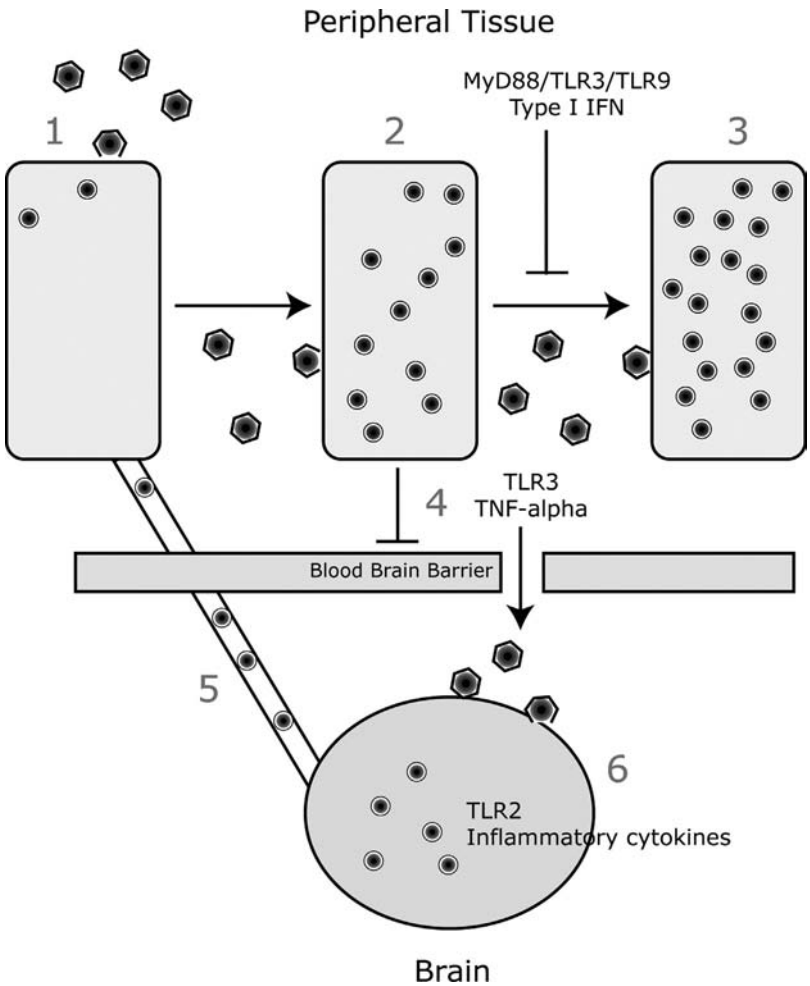

Figure 15.2. Viral encephalitis and TLR receptors. (1) Viruses such as herpes simplex virus (HSV) or West Nile virus infect cells at sites in the periphery. (2) Virus replicates within infected cells and (3) Spreads to adjacent cells or is released and transported to distal cells via blood or interstitial fluids. TLR activation can limit viral replication and spread via type 1 interferon. (4) Blood-borne virus is prevented from entering the brain by the blood-brain barrier (WNV). TLR activation can open the blood-brain barrier via TNF-alpha. (5) HSV enters the brain directly via peripheral neurons and infection of central neurons. (6) Virus within the brain replicates in neuron cells as well as CNS glial cells and triggers inflammation via TLRs

Pathogenesis of viral encephalitis

The development of encephalitis (i.e., inflammation of the brain) requires two steps: (1) virus or viral antigens must cross the blood-brain barrier, and (2) inflammatory cells must accumulate in the brain (after crossing the blood-brain barrier). TLRs and their associated adapter proteins have been demonstrated to influence both steps.

Wang *et al.* have demonstrated that TLRs may be required in the first step of infection [10]. While wild-type mice challenged intraperitoneally with West

Nile virus developed encephalitis, mice deficient in TLR3 (TLR3 knockout mice) did not develop disease. Both wild-type and TLR3 knockout mice succumbed to lethal encephalitis after intracranial challenge indicating that the difference in susceptibility related to penetration of the blood-brain barrier [10]. The ability of the virus to cross the blood-brain barrier correlated with the ability of the virus to induce inflammatory cytokines (present in wild-type but not TLR3 knockout mice). In this case, the failure of a TLR-mediated inflammatory response prevents the virus from crossing the blood-brain barrier and causing disease. The innate immune response system can have exactly the opposite effect as well. In the case of vesicular stomatitis virus (VSV), an animal model reveals that mice deficient in the adapter protein MyD88 are susceptible to intranasal challenge, while wild-type mice do not succumb. In this case, the production of interferon appears to prevent virus spread to the brain [11]. In animal models, Zhou *et al.* have demonstrated that mice deficient in MyD88 have defective virus-specific CD8 T cells [12]. Such a defect results in virus persistence when the virus challenge is in the periphery, but it could also result in a lack of a CD8 T cell response in the brain.

Pathogenesis of encephalitis

Virus-associated inflammation of the brain can be caused by direct lytic effects of virus or by the virus inducing swelling that in turn causes inflammation. Since the brain is in a closed space, swelling alone can lead to mortality. In the case of HSV encephalitis, a lack of cytokine production (mediated through TLR2) can lead to a lack of symptoms. In an animal model, wild-type mice succumb to intraperitoneally administered HSV while TLR2-deficient mice do not die at the same dose of virus. Death in this case is associated with levels of cytokines in the brains of infected mice, but there is no association with levels of virus [2].

Another way that encephalitis can occur relates to the involvement of the adaptive immune response. Encephalitis can be caused by a T cell response that recognizes viral antigen in the brain. In this case, it is the interaction between the T cell and the antigen-expressing cells in the brain that leads to encephalitis, which may be accompanied by low levels of virus or no live virus at all. The inflammatory response in this case, typically occurs 1 to 2 weeks after the viral infection at a time when viral titers are minimal and is due to virus-specific T cells. In this case, elimination of T cells will prevent or ameliorate the signs and symptoms of encephalitis.

Evidence for involvement of TLRs in encephalitis

Herpes simplex virus

HSV is the most common cause of sporadic encephalitis in humans (see Chapters 11 and 17). In mouse models, injection of the virus intraperitoneally leads to lethal encephalitis, with adult mice being much more resistant to disease than neonatal animals. Experiments using a mouse model demonstrated that mice deficient in TLR2 are much more resistant to viral challenge than wild-type mice. TLR2 knockout mice survived HSV challenge that was uniformly lethal in wild-type mice. This did not correlate with viral replication, as viral titers in the brain were the same or lower in wild-type mice compared to TLR2 knockouts. Survival was inversely correlated with the ability of mice to produce cytokines in the brain [2]. Thus, wild-type but not TLR2-deficient mice had high levels of the monocyte chemoattractant protein MCP-1 and concomitant brain inflammation while TLR2-deficient mice did not produce brain MCP-1 and lacked inflammatory cell infiltrates despite the presence of virus.

The opposite result was seen in mice impaired in their ability to produce interferon through TLRs. Mice with the UNC93b1 mutation (so-called 3D mice) do not signal via TLR3, 7, or 9. Not surprisingly UNC93b1 mutation results in abnormalities in both *in vitro* and *in vivo* responses to viruses. Mice with this mutation die when challenged with murine cytomegalovirus (MCMV) at doses much lower than

Table 15.1. How does the immune response to viruses contribute to encephalitis?

Mechanism	Result	Example of TLR response
Lack of IFN response to virus	Increased viral replication	HSV-Unc93b deficiency leads to lack of TLR signaling and increased susceptibility to HSV encephalitis in humans [13]. Lack of MyD88 leads to increased replication of LCMV and VSV in mouse models [11,12]
Lack of cytokine response to virus	Decreased host inflammatory response	Mouse models of West Nile virus and HSV, where deficiency in TLRs leads to decreased cytokine responses and lack of encephalitis in TLR3 [10] or TLR2 [2] knockout mice
Intrinsic T cell deficiency	Lack of MyD88-associated T cell activation leads to lack of CD8 T cells in brain	T cell-deficient mice survive LCMV infection [14]

the doses needed to develop symptoms in wild-type mice [15]. More surprising is the fact that humans with a defective UNC 93-B gene seem to be uniquely susceptible to HSV encephalitis. The susceptibility to HSV correlates with a defect in the production of interferon in response to HSV *in vitro* [13]. It is thought that the failure to produce interferon allows the virus to spread to the brain. A similar defect has been described in a mouse model of VSV (discussed later). Surprisingly, patients with this gene abnormality have not been noted to be susceptible to other infections.

Vesicular stomatitis virus

VSV is a member of the *Vesiculovirus* genus in the *Rhabdoviridae* family. VSV is able to access the mouse brain following intranasal infection and cause encephalitis, characterized by hind limb paralysis [16,17]. It has been demonstrated that type 1 IFN and neutralizing antibody play an important role in the protection of mice from this fatal infection [18]. Recent studies suggested that the host response to VSV is controlled by several different TLRs. Both TLR4 and TLR7 have been reported to be involved in VSV-induced type 1 IFN production [3,19]. Most likely, TLR-dependent induction of cytokines may also be cell-type dependent. *In vivo*, a role for the TLR adapter protein MyD88 has clearly been demonstrated [11]. Intranasal challenge with VSV leads to a lethal encephalitis and defective IFN production in MyD88 KO mice but not wild-type animals. This effect is not seen when mice are challenged intravenously or intraperitoneally rather than

Table 15.2. Animal models of neurotropic viruses and TLRs

Virus	TLR involved	Effect on disease	Mechanism
HSV	TLR2	Encephalitis is absent without a TLR-mediated cytokine response	TLR2-mediated cytokine induction causes disease in brain
West Nile	TLR3	Encephalitis does not occur without a cytokine response that allows virus spread to the brain	TLR3-induced cytokines allow virus to cross to brain
LCMV	MyD88	Encephalitis requires a T cell response	Required for CD8 T cells
VSV	MyD88	Encephalitis occurs in the absence of an interferon response that prevents virus spread	MyD88 required for interferon production

intranasally. Mice deficient in either IL-1R1 or IL-18R, which also use MyD88 as the adapter molecule, are not susceptible to intranasal VSV challenge. The significantly lower levels of type 1 IFN in the initial infection stage and the inability to maintain neutralizing antibody in MyD88 knockouts could be responsible for the susceptibility of MyD88-deficient mice to intranasal VSV infection [11,12].

Lymphocytic choriomeningitis virus

LCMV is another useful model to evaluate the regulation of protective immune responses versus immunopathogenesis [14,20,21] (see Chapter 5). LCMV is a noncytopathic virus. LCMV replication rarely causes tissue damage, rather, LCMV-associated diseases in mice are mediated by both innate and acquired immune responses [14,20,21]. The murine response to LCMV involves the pattern receptor CD14 in association with TLR2 and TLR6 [12]. Moreover, the TLR adapter molecule MyD88 is involved in the activation of LCMV-specific CD8 T cells. In addition, although LCMV might be predicted to stimulate an immune response through TLR3 via a dsRNA intermediary, no role for TLR3 has been demonstrated.

In the case of LCMV infection, T cells migrate to the brain and can cause fatal disease. The elimination of T cells can prevent fatality in this model [22]. Both $CD8^+$ and $CD4^+$ T cells contribute to the clearance of acute as well as chronic LCMV infection. However, these virus-specific T cells might also cause severe tissue damage, even animal death under certain circumstances [14,20]. It has been well-documented that intracranial infection with LCMV in immunocompetent adult mice induces fatal meningitis, with death occurring between day 7 and day 9 post-infection [14]. Studies have found that intracranial LCMV infection up-regulates several chemokine genes, including MCP-1, TNF-α, and RANTES, as well as interferon-stimulated genes in the brain [23,24]. More studies are needed to dissect if TLRs are involved in the regulation of the activation and migration of both $CD8^+$ and $CD4^+$ T cells following LCMV infection and if TLRs play a role in the regulation of LCMV-induced chemokine and cytokine responses within the brain.

Conclusions

Viral encephalitis, like all inflammatory events in the mammalian host, is a result of the responses of both the innate and adaptive immune systems. Several TLRs have been demonstrated *in vitro* to be critical to the production of host cytokines in response to viruses. These critical receptor proteins include TLRs expressed on the surface of the cell as well as several intracellular receptors. The role that these proteins have on the outcome of disease relates not only to their ability to induce the production of interferon, which may control local disease and prevent spread to the brain, but also their ability to induce inflammatory cytokines, which can cause edema and migration of neutrophils and result in fatal disease. The adapter proteins associated with the generation of TLR-mediated signal transduction events are also important in the pathogenesis of encephalitis. MyD88, a protein required for signaling by most TLRs (all except TLR3), may be associated with as-yet-undefined TLRs or TLR-like proteins and is critical to the development of T cell responses to virus and migration of T cells. T cells are critical in the late stages of viral encephalitis in that they are often essential for viral clearance. At the same time, they can be responsible for virus-induced disease by recognizing viral antigens in the brain and causing inflammation.

It is obvious from the mouse models of encephalitis as well as from the few human "accidents of nature" that susceptibility to encephalitis depends on both initial entrance of the virus as well as subsequent early (innate immune), and late (adaptive immune) responses. These events have been demonstrated in both humans and mice to be dependent upon TLRs and their associated signal transduction proteins. Further definition of the precise pathways involved and the time course of these events should result in a better definition of the pathogenesis and lead to better ways to intervene. The development of

drugs that specifically augment or inhibit the actions of the TLRs should be very useful in treatment of viral encephalitis in the future.

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16

Neuroendocrine-immune interactions in neurotropic viral infections

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Historical aspects of the neuroendocrine-immune connection

In order to understand the connection between the neuroendocrine and immune system, it is important to discuss the historical aspects of this relationship and the formulation of the concepts of homeostasis and stress. Claude Bernard in the 1860s developed the concept of "the milieu interne" to describe the balance of the internal milieu. In 1927, Cannon defined the fight or flight response to a threat and the concept of homeostasis as the physiological process by which an organism maintains a stable internal environment [1]. Then in 1936, Hans Selye observed that sick patients all had similar nonspecific symptoms: malaise, fever, and loss of appetite [2]. He proposed the general adaptation syndrome that states that when threatened by a threat or infection, the central nervous system (CNS) diverts the organism's energy reserves from nonessential functions (reproduction, growth) to functions that allow the organism to cope with the insult. Selye borrowed the term "stress" from the physical sciences to describe factors that upset homeostasis. He observed that stressed animals developed atrophy of the thymus, spleen, and lymph nodes and enlarged adrenal glands. Eventually, these effects were discovered to be the result of activation of the hypothalamic-pituitary-adrenal axis (HPA). Recently, McEwen has proposed the concept of allostatic load, which describes the cumulative effects of chronic stess that can result in dysregulation of multiple integrated physiological systems [3].

The HPA-immune axis was demonstrated by Besedovsky and his colleagues who observed that physiological concentrations of glucocorticoids facilitated antigenic specificity [4] and conversely, that an immune response induces glucocorticoid production [5]. He later implicated the brain in immune-neuroendocrine networks by measuring hypothalamic electrical activity during an immune response [6].

Although it has long been known that immune cells have receptors not only for antigenic determinants derived from pathogens but also for hormones, neurotransmitters, and others, it was not until the 1980s that the work of Blalock and colleagues established the bidirectional communication between the immune and neuroendocrine systems. Blalock also developed the concept of the immune system as a "sensory system" mediating communication between multiple body systems [7,8].

Stress and the immune system

Although it is self-evident that chronic stress renders individuals more susceptible to infections, it is only recently that the underlying mechanisms have begun to be investigated [9]. A stressor could be considered a stimulus that induces reactions in the brain (stress perception) that in turn activates the HPA axis and/or the sympathetic nervous system (SNS) [10]. Cortico-limbic structures are involved in the perception of an event as threatening or challenging and beyond the organism's capacity to cope,

which in turn engages the HPA and SA outflow pathways. Stress initiates production of corticotropin-releasing hormone (CRH) and activation of the locus coeruleus-noradrenaline (LC-NA)/autonomic (sympathetic) neurons of the hypothalamus and brain stem. In turn, these systems regulate the activities of the HPA axis and the systemic/adrenomedullary SNS, respectively [11]. Activation of these systems results in the production of corticoid hormones and catecholamines that can directly modulate the activity of various immune effector cells since immune cells have receptors for these substances [12]. The immune system in turn can affect the CNS via cytokine production. For instance IL-1 induces the production of corticotropin-releasing hormone by the hypothalamus [13].

There is convincing evidence in the literature associating chronic stress with the onset and progression of infectious diseases (e.g., influenza, herpes), and also autoimmune diseases (e.g., multiple sclerosis, rheumatoid arthritis, lupus, insulin-dependent diabetes) [14]. Stressful life events and poor social support play a role in the onset and exacerbation of autoimmune diseases such as rheumatoid arthritis [15,16,17]. In addition, intervention studies indicate that emotion-focused [18,19] and cognitive-behavioral stress management decreases the clinical signs of infectious and autoimmune disease [20,21,22,23,24].

Stress may have differential effects on the immune system depending on whether it is acute or chronic. Acute stress is considered to be a stressor of less than 2 hours duration and chronic stress is endured for days to years and is typified by serious life events and chronic illness. Seyle's general adaptation syndrome may explain the immunological outcomes that occur following acute stress. Both the fight or flight response and the immune responses are enhanced following acute stressors. Acute stress has been shown to enhance antigen-specific cell-mediated immunity [25], alter populations of T-cell subsets [26], and modulate mononuclear cell trafficking [27]. Acute stress also causes redistribution of immune cells from the bone marrow into the blood, lymph nodes, and skin [10,25]. The increased numbers of immune cells in these compartments will allow for heightened responsiveness in the event of a skin wound, a natural consequence of an encounter with a predator as the acute stressor. In contrast, chronic stress suppresses the ability of the immune system to respond to challenge and thus increases susceptibility to infectious diseases and cancers.

Interactions between the neuroendocrine and the immune systems have been demonstrated to be critical in the development and control of the immune response to infection. The experimental approach to investigating these complex interactions has involved the use of stress. For the purposes of this discussion, we will concentrate on the effects of stress on CNS infections induced by two neurotropic viruses, namely: herpes simplex and Theiler's virus.

Herpes simplex virus infections and the neuroendocrine-immune connection

Herpes simplex virus-1 (HSV-1) mainly affects the orofacial areas and HSV-2 the genital areas and persists in a latent state within the nervous system (see also Chapter 11). It has been well-recognized for many years that stress provokes the iridescence of HSV infections but the molecular basis of this phenomenon has only recently been unraveled. In 1957, Rasmussen described the effects of stress on herpes infection in mice [28]. More recently, the impact of stress on immune responses to HSV has been thoroughly investigated most notably by Bonneau, Sheridan, and Glaser [reviewed in 29]. It is also interesting to note that another herpes virus, human herpes virus-6, has been implicated in the pathogenesis of multiple sclerosis (MS), and so these findings may have implications for autoimmune diseases such as MS [30]. In considering persistent viral infections, the immunosuppressive effects of stress at the initial time of infection would allow increased viral replication of HSV and perhaps increased ability to establish a persistent infection. Once the latent state is established, stress-induced immunosuppressive may lead to reactivation but perhaps also reduced immunopathology.

Stress-induced changes in the innate immune response to HSV

The early immune response, as measured by type I and II interferon production, has been shown to be reduced by stress in a dermal HSV-1 infection model and a corresponding increase in viral titers was noted [31]. Hyperthermic stress was shown to increase IL-6 expression in the trigeminal ganglia of latently infected mice [32]. The increase in IL-6 was mediated by glucocorticoids because cyanoketone (a corticosterone synthesis inhibitor) blocked this effect. In contrast, restraint stress applied during the primary footpad HSV infection was shown to decrease splenic IL-6 production [33].

Products of the SNS also affect the innate response to HSV. Epinephrine and norepinephrine have been shown to reduce macrophage killing of HSV-infected cells [34] and also restraint stress reduces splenic natural killer (NK) cell lysis [35].

Interestingly, stress effects within the CNS appear to have differential effects on microglial cells as compared to macrophages in the periphery. Stress-induced glucocorticoids resulted in increased numbers and activation of microglia in the CNS [36].

Stress-induced changes in the adaptive immune response to HSV

Dendritic cell activation is required for the generation of an adaptive immune response, and stress levels of glucocorticoids have been shown to suppress the formation of peptide-MHC (major histocompatibility complex) class I complexes on the membrane of HSV-infected dendritic cells [37]. This, in turn, will result in the reduction in HSV-specific CTLs.

Restraint stress has also been demonstrated to delay the recruitment of $CD4^+$ and $CD8^+$ T cells into the brains of HSV-infected mice [38] and also suppresses lymphadenopathy via glucocorticoid-dependent mechanisms [39]. Following footpad inoculation of HSV, restraint stress also reduced the lymphoproliferative response to HSV in the popliteal lymph nodes [35,40]. Restraint stress (RST) also suppressed the differentiation and maturation of HSV-specific CTL precursors. The lytic ability of these cells could be restored by nadalol – a nonspecific adrenergic receptor antagonist [41]. Memory T cell response to HSV is also impaired by stress and is thought to be due to decreased cytokine production of the cytokines responsible for memory cell activation [42].

Studies on the effect of stress on the generation of antibodies to HSV are variable. Some studies suggest that there is no change in IgM or IgG antibodies [43] whilst others show an increase in IgM antibodies following stress [44]. In contrast, yet another study showed decreased antibody titers in HSV-infected mice following footshock stress [45].

Perhaps the most intriguing aspect of the effects of stress in herpes viral pathogenesis is the reactivation of HSV. Hyperthermic stress has demonstrated the importance of the HPA axis in reactivation in the trigeminal ganglia associated with increased IL-6 in the ganglia. Interestingly, social stress applied by altering the social hierarchy resulted in the reactivation of HSV in 40% of the mice latently infected with HSV [46]. Counterintuitively, the dominant mice were more likely to suffer from a reactivation than the subordinate animals.

Theiler's virus infection and the neuroendocrine-immune connection

A viral etiology for multiple sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS occurring at a prevalence of 250 000–350 000 in the United States [47] and an incidence approaching 1/1000 [48]. The national annual costs of this disease are estimated to be $6.8 billion [49]. The etiology of MS is unknown although epidemiological studies have implicated an infective agent as a probable initiating factor [50]. There is an increased risk of developing MS associated with late infection with mumps, measles, and Epstein-Barr virus [51]. Furthermore, exacerbations of MS are frequently preceded by viral infections [52].

Interferon-beta (IFN-β) has been reported to have a beneficial effect on relapsing/remitting MS [53]. IFN-β has a number of immunomodulatory properties but is also an effective antiviral agent and therefore may be mediating its therapeutic effects by interfering with viral replication. Further evidence that viruses are implicated in MS comes from the frequent isolation of viruses such as measles, mumps, parainfluenza type I, from the brains of MS patients at postmortem [54]. The most recent viral culprit: human herpes virus simplex type 6 [30] has been isolated from MS brains. However, this virus has also been detected in the majority of non-MS brains, suggesting that there is a "normal brain flora."

In animals, the following viruses are also known to cause demyelination: measles virus in rats; JHM mouse hepatitis virus; Semliki Forest virus and Theiler's virus in mice; visna in sheep; and herpes simplex in rabbits [55]. Theiler's virus infection in mice represents not only an excellent model for the study of the pathogenesis of MS but also a model system for studying disease susceptibility factors, mechanisms of viral persistence within the CNS, and mechanisms of virus-induced autoimmune disease. The fact that Theiler's virus only causes demyelinating disease in genetically susceptible strains of mice suggests that it is a genopathogen (i.e., a pathogen that is only capable of causing disease in certain strains of the species). This concept, if applied to MS, would mean that this disease could be caused by an infective agent that is only pathogenic to individuals with an MS-susceptible genotype.

Stress and multiple sclerosis (MS)

Since the earliest descriptions of MS, stress has been considered a controversial but potentially important factor in the onset and course of the disease [56]. Anecdotal accounts suggest that significant stressful life events frequently trigger the development of MS symptoms [57]. Psychological stress has been shown to precede both the onset and recurrence of MS symptoms in 70–80% of cases, using standardized assessment of life stressors measures [58]. More recently, acute life stressors have been shown to be correlated with relapses in MS [59,60]. A meta-analysis of 14 papers concerning stress and MS concluded that there was "a significantly increased risk of exacerbation associated with stressful life events" [61].

Theiler's virus-induced demyelination as a model for MS

There are two main types of animal models for studying MS: experimental autoimmune encephalomyelitis (EAE) and virus-induced demyelination. EAE involves generating autoreactivity to myelin components and is useful for studying the autoimmune aspects of MS. One of the most researched virus models of MS is Theiler's virus-induced demyelination (TVID).

Theiler's murine encephalomyelitis virus is a picornavirus with biological similarities to poliovirus although classified in the cardiovirus genus (see also Chapter 1, Neurotropic picornaviruses). Theiler's virus causes an asymptomatic gastrointestinal infection and occasionally paralysis [62]. The persistent TO strains of Theiler's virus (BeAn, DA, WW, Yale) cause a primary demyelinating disease in susceptible strains of mice that is similar to MS [63]. Theiler's virus must establish a persistent infection in the CNS in order to cause demyelination [64]. TVID-resistant strains of mice are able to clear the infection effectively from the CNS. A number of studies have reported that viral persistence and demyelination in susceptible strains of mice are under multigenic control. Genes coding for MHC class I and the T cell receptor [65] and a gene locus on chromosome 6 not linked to the T cell receptor locus [66] have been implicated in susceptibility to demyelination. Two additional loci, one close to Ifnγ on chromosome 10 and one near Mbp on chromosome 18, have been associated with viral persistence in some strains of mice [67]. Immune recognition of Theiler's virus is clearly an important element in susceptibility to demyelination, as indicated by the genetic association with MHC and the T cell receptor, although other undefined factors are also involved.

The neuroimmunological mechanisms of restraint stress-induced mortality and neuropathogenesis of acute Theiler's virus infection

In order to investigate the interactions between the neuroendocrine and immune systems in Theiler's virus infection, we employed a restraint stress model originally described by Sheridan and colleagues [68]. RST is considered both a physiological and psychological stressor and involves placing mice in well-ventilated tubes overnight. Our restraint stress studies involve four strains of mice with differing susceptibility to TVID (shown in Table 16.1).

Our first study into the effects of stress on the neuropathogenesis of Theiler's virus involved male CBA mice subjected to 5 nights of restraint stress per week for a total of 4 weeks (for experimental groups, see Table 16.2). Stress had a profound effect on survival; 80% of the stressed-infected mice died during the first 3 weeks of infection. RST increased glucocorticoid (GC) levels, increased signs of sickness behavior, increased viral titers in the CNS, adrenal hypertrophy, thymic atrophy, decreased numbers of circulating lymphocytes and increased circulating neutrophils [69,70,71]. Similar results were found in another study with male and female SJL and CBA mice: chronic RST stress administered in the first 4 weeks of Theiler's virus infection decreased body weights, increased clinical symptomatology of infection, and increased plasma GC levels during the acute viral infection. Although all RST-stressed mice displayed significantly increased GC levels, female SJL mice showed higher basal and stress-induced increases in GC [72].

Table 16.1. Differing susceptibility of mouse strains to Theiler's virus-induced demyelination

Mouse strain	Susceptibility to Theiler's virus-induced demyelination
SJL/j	High susceptibility
CBA	Intermediate susceptibility
BALB/c	Resistant
C57Bl/6	Resistant

Table 16.2. Experimental design

Group	Infection status	Restraint stress
I	Infected	Restrained
II	Infected	Nonrestrained
III	Noninfected	Restrained
IV	Noninfected	Nonrestrained

Five-week-old mice were randomly assigned to one of two groups, 10 mice per group according to a previously reported protocol [68,69] and treated as follows on day 1: (1) A control group where mice remained undisturbed in their home cages; (2) A group in which each mouse was placed in a well-ventilated restraining tube for 8–12 hours overnight. On day 0, following isoflurane inhalation anesthesia, half the mice in each of the groups were either infected intracerebrally with 5×10^5 pfu of Theiler's virus (BeAn strain obtained from Dr. H. L. Lipton, Department of Neurology, Northwestern University, Chicago, IL) or similarly inoculated with virus-free BHK cell supernatant. Restraint began 1 day prior to infection and 5 days per week for 1 month postinfection.

Clearly stress has a profound impact on the neuropathogenesis of Theiler's virus infection. We proposed the following mechanisms for this phenomenon: stress activates the HPA axis resulting in adrenal hypertrophy and increased production of corticosterone. Corticosterone in turn causes thymic atrophy and immunosuppression by decreasing the number of circulating lymphocytes and reducing both the innate and adaptive immune response to Theiler's virus, thus reducing the effective clearance of virus from the CNS. Therefore, we began to systematically dissect the effects of stress on the various components of the immune response to Theiler's virus.

Effects of stress on the early disease and innate immune response to Theiler's virus

Interferon and NK cells in Theiler's virus infection

The early events that occur during Theiler's virus infection are crucial in the effective clearance of

virus from the CNS. Failure to clear virus results in the establishment of persistent infection of the CNS and subsequent demyelination [73,74]. The first response to viral infection is the production of type I interferons, which are critical in the early clearance of Theiler's virus from the CNS as demonstrated by experimentation with IFN-α/β receptor knockout mice. These mice die within 10 days of infection with severe encephalomyelitis [75].

NK cells are activated early in viral infections and play an important role in natural resistance to certain viruses, tumor surveillance, and regulation of hematopoiesis. In Theiler's virus infection, susceptible SJL mice were found to have a 50% lower NK cell activity when compared to resistant C57BL/6 mice [76]. The low activity of NK cells in the SJL mice is due to a differentiation defect in the thymus that impairs the responsiveness of NK cells to stimulation by IFN-β [77]. When resistant mice were depleted of NK cells by monoclonal antibody to NK 1.1 or anti-asialo-GM1 and then infected with Theiler's virus, they developed severe signs of gray matter disease. Thus, NK cells are critical in the early clearance of Theiler's virus from the CNS.

We examined the effects of stress on the NK cell response to Theiler's virus infection in male CBA mice using the experimental design shown in Table 16.2. Restraint stress applied 1 day prior to infection with Theiler's virus resulted in 50% reduction in splenic NK cell activity in CBA mice 24 hours postinfection [70]. Similar results have been obtained with TVID-resistant strains of mice C57Bl/6 and BALB/c [78]. RST did not alter the NK cell response in SJL/J mice infected with Theiler's virus since this strain has a deficiency in NK cell response as previously described [77]. However, RST did impact the neuropathogenesis of Theiler's virus infection in SJL/J mice, and therefore stress must mediate its immunosuppressive effects on the other components of the immune response. Stress-induced NK cell suppression may contribute to but is not sufficient to observe the stress-induced exacerbation of acute and chronic Theiler's virus infection.

The effects of RST on chemokine/cytokine expression

CBA mice were subjected to the RST protocol for 7 nights and then sacrificed and RNA isolated from the brains and spleens. Ribonuclease protection analysis indicated that infection with Theiler's virus increased the following chemokine expression: lymphotactin (Ltn), interferon-induced protein (IP-10), macrophage inflammatory protein-1 (MIP-1), monocyte chemoattractant protein-1 (MCP-1), and TCA-3, in the spleen but not the brain at day 2 p.i. The fact that chemokine expression was increased first in the spleen provides evidence that the immune response to Theiler's virus is initiated in the periphery. Ltn, normal T cell expressed and secreted (RANTES) and IP-10 were elevated in both the spleen and the brain at day 7 p.i. and were significantly decreased by RST in the brain. These chemokines are responsible for the recruitment of $CD4^+$ and $CD8^+$ T cells, macrophages, and NK cells and thus may account for the diminished inflammatory cell infiltrate in the CNS of RST-stressed mice and subsequently the reduced viral clearance and increased mortality in virus-infected RST-stressed mice [69,71].

The effects of stress on cytokine production in both the spleen and brain were measured in CBA mice following seven RST sessions. Theiler's virus infection elevated IFN-γ, LT-β, IL-12p40, IL-6, and IFN-β in the brain at days 2 and 7. Importantly, restraint attenuated the increases in IFN-γ, LT-β, IL-12p40, and IL-6, but elevated IFN-β. The increased mRNA IFN-β levels may be as a result of the increased viral titers stimulating the production of this interferon. Interestingly, stress increased the anti-inflammatory cytokine IL-10 in the spleen that may contribute to the decrease in proinflammatory cytokine production [79]. In further experiments examining cytokine expression, mice were subjected to the RST paradigm and, at sacrifice, half the brain taken for measurements of virus load and the other half for RPA analysis of cytokine mRNA levels. mRNA levels of IFN-γ, LT-β, and TNF-α negatively correlated with viral titers in the CNS such that mice with higher cytokine levels

had lower virus levels. TNF-α protein levels, as measured by western blots, gave similar results to the RPA data for this cytokine [79].

Enzyme-linked immunosorbent assay (ELISA) examined the effects of RST stress on IL-1β and TNF-α levels in serum. No detectable levels of IL-1β were observed in any of the groups of mice, but interestingly restraint stress induced high levels of TNF-α in the serum of both infected and uninfected mice [70].

Effects of stress on the acute neuropathology induced by Theiler's virus

Restraint stress had a profound effect on the development of early brain lesions in mice infected with Theiler's virus. RST significantly reduced the inflammatory cell infiltrate into the CNS at day 7 p.i. [69,80]. This was particularly evident in the hippocampus. Interestingly, microglial activation was also severely diminished in infected/RST mice. By day 24, p.i. RST mice had increased levels of inflammation in the CNS, which may be due to recovery of the immune system and increased activation due to the increased viral titers in the CNS [69]. The early stress-induced decreases in CNS inflammation may be related to the effects of stress on downregulation of chemokines as noted above.

Restraint stress facilitates dissemination of Theiler's virus during early disease

The RST stress model was used to investigate the effect of stress on the systemic dissemination of Theiler's virus during the early stage of disease [80]. Repeated RST stress remarkably facilitated the spread of virus from the CNS to such systemic organs as the spleen, lymph nodes, thymus, lungs, and heart and compromised the ability of viral clearance within those tissues. RST stress also altered the tropism of Theiler's virus, enabling it to become cardiotropic, resulting in higher myocardial infectivity, accompanied by a granular degeneration of the myocardium. These results demonstrate the profound impact that RST stress has upon both the tissue and organ dissemination of the virus and the organ tropism of Theiler's virus. An additional finding associated with stress was hepatic necrosis in the restrained animals, regardless of whether they were infected or not.

These profound effects, including the liver injury induced by RST, may explain the exacerbated clinical symptoms and higher mortality in stressed and infected animals. However, the viral distribution within the diverse tissues and the mechanisms by which the virus enters these cells require further investigation. The fact that stress alters the pathogenesis of Theiler's virus infection may have important implications for other disease processes. Stress may render a virus pathogenic for diverse organs and result in novel diseases.

Restraint stress reduces adaptive immune responses to theiler's virus

$CD4^+$ and $CD8^+$ T cells in Theiler's virus infection

The role of T cells in TVID is a good example of the "double-edged sword" nature of the immune system. In early infection both $CD8^+$ and $CD4^+$ T cells have been shown to play an important role in viral clearance [81,82,83], but in later disease, these T cell subsets mediate in the demyelinating process [84,85,86]. In early disease, $CD4^+$ T cells assist B cells in the production of antibodies, which are important mediators of picornavirus clearance [81,87]. In addition, $CD4^+$ T cells produce IFN-γ, a potent inhibitor of Theiler's virus infection *in vitro* [88] and *in vivo* [89,90]. $CD8^+$ T cells also mediate viral clearance as demonstrated by *in vivo* depletion experiments [82] and studies with gene knockout mice [91,92]. Mice depleted of $CD8^+$ failed to clear virus from the CNS and developed more severe demyelinating disease than the immunocompetent controls [82]. β_2-microglobulin knockout mice were constructed on a TVID-resistant background and these mice were shown to lack functional cytotoxic T cells [91,92]. Histological evidence of demyelination developed in the knockout mice following intracranial infection

with Theiler's virus. Introduction of resistant H-2D^b [93] or H-2D^d transgene [94] into susceptible strains of mice render these animals resistant to TVID. CD8$^+$ T cells also provide protection against TVID when adoptively transferred to a TVID-susceptible BALB/c substrain, BALB/cAnNCr [95]. In addition, Mendez-Fernandez and colleagues have shown that a heightened CD8 response to the immunodominant viral peptide VP2$_{121–130}$ early in infection is essential for Theiler's virus clearance from the CNS. In this study, naturally susceptible FVB (H-2^q) mice that were transgenically altered to express the class I D^b molecule (previously shown to code for a heightened cytotoxic T lymphocyte (CTL) response to VP2$_{121–130}$) became resistant to demyelination after i.c. inoculation with the DA strain of Theiler's virus [96]. Taken together, these investigations clearly implicate CD8 T cells in viral clearance and resistance to demyelination. Indeed cytotoxic T lymphocyte (CTL) activity has been detected in Theiler's virus-infected SJL/J mice [97,98] and C57BL/6 mice [99]. The CTLs may be important either by recognizing viral determinants or by inhibiting delayed type hypersensitivity (DTH) responses [82].

The immunodominant Theiler's virus-specific T cell epitopes have been identified in SJL/J mice [100,101,102,103]; therefore we examined the effects of stress on the T cell response to Theiler's virus in this strain of mice. SJL mice were assigned to the experimental groups shown in Table 16.2 with the stressed mice being subjected to 8 nights of RST stress. At sacrifice, splenic and CNS T cell responses to Theiler's virus were measured using an enzyme-linked immunosorbent spot (ELISPOT) assay. Theiler's virus infection increased the number of IFN-γ-producing cells in response to either CD8 epitope (FNFTAPFI corresponding to VP3$_{159–166}$) or a CD4 T cell epitope (QEAFSHIRIPLPH corresponding to TMEV VP2$_{74–86}$). RST stress significantly decreased both the splenic virus-induced CD4 and CD8 T cell response. Furthermore, there was a significant interaction between stress and infection with restraint significantly decreasing the infection-related increase in CD8$^+$ T cell responses within the CNS [104].

There are reports in the literature that stress inhibits Th1 responses and increases Th2 responses. Therefore, we examined the expression of both Th1 and Th2 cytokines in the serum of the experimental animals. RS significantly decreased both type 1 {IL-12(p40), IL-12(p70), IFN-γ} and type 2 (IL-4 and IL-5) serum protein concentrations as measured by Bioplex. The transcription factors T-bet and GATA-3 are the drivers of Th1 and Th2 polarization, respectively [105,106]. Therefore, we also measured splenic mRNA expression levels of these factors in the experimental mice and found significant decreases in both Th1 and Th2 responses on day at day 8 postinfection [104].

RS also caused decreased serum concentrations of RANTES and MCP-1 but increased IL-6, KC, and G-CSF protein concentrations. The chemokines RANTES and MCP-1 are involved in the chemoattraction of both memory T cells and monocytes to the site of infection to mediate early viral clearance from the CNS [104].

Interestingly, the chemokine KC (CXCL1) and growth factor G-CSF increased in stressed mice. KC plays a major role in the trafficking of neutrophils, and the hematopoetic factor G-CSF is, in part, responsible for the maturation of neutrophils from the bone marrow. Considering this data in the context of our previous findings [69,70], the stress-induced increases in KC and G-CSF may explain the increase in neutrophilia.

Restraint stress fails to render TVID-resistant mice susceptible to TVID

Strains of mice that are resistant to TVID mount an effective immune response to the virus and clear the infection during the first month. We were interested in whether chronic RST stress applied during the acute phase of Theiler's virus infection would render the genetically TVID-resistant C57BL/6 strain, susceptible to TVID. Despite the fact that we have shown chronic RST stress decreases virus-specific antibody and both CD8 T cell and NK cell activity in Theiler's virus-infected C57Bl/6 mice, RST stress does not render resistant C57BL/6 mice susceptible

to persistent infection with Theiler's virus [107]. Thus stress-induced immunosuppression is unable to overcome the genetic resistance barrier.

The effects of restraint stress during acute infection on the later demyelinating disease

Failure to clear Theiler's virus during the acute disease leads to viral persistence in the CNS and subsequent demyelination. Demyelination induced by Theiler's virus is partly mediated by viral lysis of oligodendrocytes [108]; immune mechanisms including bystander demyelination mediated by virus-specific DTH T cells [84,101]; cytotoxic T cell reactivity [85]; autoimmunity [81,86,109,110,111]; and epitope spreading [111]. The autoimmune reactivity seen in TVID may result from viral damage to oligodendrocytes, myelin uptake by macrophage/microglial cells, and subsequent presentation to and activation of autoreactive T cells. Recently, these autoimmune T cells have been shown to be pathogenic and are able to demyelinate *in vitro* [112].

Susceptibility to TVID is correlated with increased MHC class II expression *in vitro* on astrocytes [113] and cerebrovascular endothelial cells [114] following treatment with IFN-γ. Increased MHC class II expression on cells within the CNS may lead to increased antigen presentation and inflammation. Astrocytes and cerebrovascular endothelial cells derived from TVID-resistant mice failed to express MHC class II following treatment with IFN-γ. Thus, the TVID-susceptible CNS is more reactive following inflammatory insults than the TVID-resistant CNS.

Life stressors have been implicated in the onset of MS, and we have shown that chronic stress during acute infection with Theiler's virus leads to decreased viral clearance from the CNS. Other studies have shown that increased viral load during acute disease leads to increased later demyelinating disease [82]. Thus, we speculated that stress applied during the acute viral infection results in higher viral load in the CNS and would subsequently lead to increased demyelination. To test this hypothesis we subjected SJL/j mice to RST for 4 weeks (8 hours per night) and then monitored the course of disease and assessed the spinal cord lesions 14 weeks postinfection. During early infection, both male and female stressed mice displayed decreased body weights and locomotor activity, with increased behavioral signs of illness and plasma GC levels. During the subsequent demyelinating phase of disease, previously stressed mice had greater behavioral signs of demyelination, worsened rotarod performance, and increased inflammatory demyelinating lesions of the spinal cord [72].

Interestingly, correlational analysis of all of the dependent variables found that in the acute phase of disease in SJL mice, plasma GC levels, clinical symptomatology, and body weight loss were all highly correlated. GC levels during RST stress in the acute phase were also highly correlated with histological indications of meningitis, rotarod performance, and clinical symptomatology in the chronic phase of disease. Thus, plasma GC levels during stress in the acute phase may be a good predictor of disease course in the chronic phase. Acute-phase clinical symptomatology had similar predictive value with chronic phase clinical symptomatology, rotarod performance, and histological indications of meningitis. A follow-up experiment with RST-stressed SJL mice indicated that they developed higher viral loads in the CNS at day 25 p.i. as compared to non-RST Theiler's-infected mice. Thus we propose that mice developing high GC levels as a result of RST develop more severe immunosuppression and therefore higher viral titers and consequently more severe demyelinating disease.

The effects of stress on the development of autoantibodies to myelin were also investigated in this experiment. We had previously detected autoantibodies to whole myelin membranes during the late phase of TVID [81]. In SJL mice infected with Theiler's virus (restrained and nonrestrained) we detected antibodies to proteolipid protein (PLP), myelin basic protein (MBP), and myelin oligodendrocyte glycoprotein (MOG) [72]. To the best of our knowledge this was the first report of antibodies to specific myelin components and demonstrates the value of TVID as a model for MS. Female SJL mice had higher antibodies to MOG 33–55 than males at

day 69 p.i., and previously stressed female mice had decreased antibody titers to MBP when compared to non-RST infected mice. Although antibody titers to Theiler's virus and PLP were no different between the infected/restrained and infected/nonrestrained mice [72], other evidence suggests that repeated exposure to social disruption stress alters autoantibody development [115].

Chronic restraint stress applied during the late demyelinating disease has no effect on disease course

Immunosuppressive therapies such as cyclophosphamide or treatment with rabbit antithymocyte serum [116] or antibody to $CD4^+$ T cells [81] have been shown to improve the late demyelinating disease induced by Theiler's virus. Previous experiments have shown that RST stress, as well as GC treatment, suppress relapsing EAE [117,118]. Therefore, chronic RST stress in the late phase of TVID should have a protective effect due to stimulation of the HPA axis, increase in GC, and subsequent downregulation of the damaging autoimmune response. However, three independent experiments showed that although RST stress elevated GC levels, it did not alter the clinical score or histological signs of inflammation [unpublished observations].

The effect of naturalistic stressors on the neuropathogenesis of TVID

To evaluate the generality of our effects, we examined the impact of two naturalistic stressors: social disruption stress and maternal separation. For these studies we used BALB/cJ mice that are susceptible to TVID [119] and develop early clinical signs of Theiler's virus infection in the form of hind limb impairment/paralysis [115,120,121]. Social disruption stress (SDR) was found to have similar effects on the neuropathogenesis of Theiler's virus infection as RST stress. However, unlike restraint stress, SDR caused glucocorticoid resistance and increases in CNS inflammation consistent with the literature [122,123]. SDR applied to BALB/cJ mice prior to infection with Theiler's virus was associated with elevated IL-6 levels at day 9 p.i. that highly correlated with measures of motor impairment in the chronic phase (vertical activity, overall activity, hind limb impairment) and to immunological measures such as antibody to myelin and Theiler's virus. Overall, SDR caused greater motor deficits in both the acute and chronic disease as well as elevated autoimmune indicators (Ab to myelin components) and Ab to virus in the chronic phase [115]. IL-6 has been implicated as one of the mediators of the aversive effects of SDR on Theiler's virus infection [121].

Neonatal stress was found to have long-term effects on adult immune, endocrine, and behavioral responses to Theiler's virus infection. BALB/cJ pups were subjected daily to either 180-minute maternal separation (MST), 15-minute MST, or control conditions during postnatal days 1–14. As adults, mice were inoculated with Theiler's virus and sacrificed at days 14, 21, or 35 pi. Prolonged 180-minute MST decreased viral clearance in the spinal cords of males and females, whereas brief 15-minute MST increased clearance in females. The 15-minute and 180-minute MST mice exhibited blunted corticosterone responses to infection, behavioral alterations, and enlarged adrenals, spleens, and lymph nodes. Histological analyses of spinal cord sections indicated that both the 15-minute and 180-minute MST conditions reduced signs of meningitis and microgliosis in spinal cord [124,125]. Similar to the effects of RST stress, the decrease in CNS inflammation observed in maternally separated mice may be attributable to alterations in chemokine and cytokine expression during early infection.

Conclusions

RST stress has a profound global impact on the immune response to Theiler's virus. Figure 16.1 shows the effects of stress in TVID that we have examined to date: (1) RST affects immune cell development by inducing high levels of GC that reduce circulating lymphocyte numbers and cause thymic atrophy. Stress increases KC and G-CSF that leads to increased neutrophils in the circulation. (2) RST

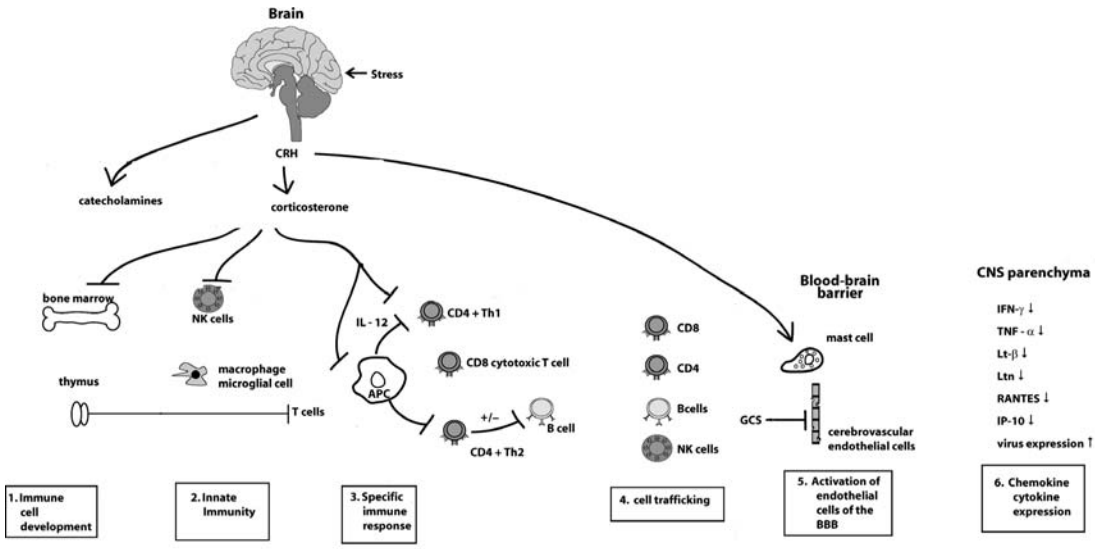

Figure 16.1. Diagrammatic representation of the effects of restraint stress in TVID. (1) RST affects immune cell development by inducing high levels of GC that reduce circulating lymphocyte numbers and cause thymic atrophy. Stress increases KC and G-CSF that leads to increased neutrophils in the circulation. (2) RST affects the innate immune response to Theiler's virus by reducing NK cell activity and IL-12 production. Also, chemokine production is decreased in both the circulation, the spleen, and CNS. (3) RST reduces virus-specific CD4 and CD8 T cell responses, increased antibody production, and decreased virus-induced proinflammatory cytokines TNF-α, IFN-β, and LT-β. (4) RST reduces immune cell trafficking to the CNS and (5) alters the permeability of the blood-brain barrier. (6) RST also reduces the expression of chemokines in the CNS: Ltn, RANTES, and IP-10, which are responsible for the recruitment of $CD4^+$, $CD8^+$, T cells, macrophages, and NK cells to the site of infection.

affects the innate immune response to Theiler's virus by reducing NK cell activity and IL-12 production. In addition, chemokine production is decreased in both the circulation RANTES and MCP-1 and the spleen and CNS. (3) RST also reduces the acquired immune response to Theiler's virus as evidenced by reduced virus-specific CD4 and CD8 T cell responses, increased antibody production, and decreased virus-induced proinflammatory cytokines TNF-α, IFN-β, and LT-β. These cytokines have pleiotropic effects on the immune system and also have antiviral effects. Mice subjected to RST also developed high levels of the anti-inflammatory cytokine IL-10 that further suppress the immune response to Theiler's virus. (4) RST reduces immune cell trafficking to the CNS as evidenced by reduced inflammatory cell infiltrates into the CNS. (5) RST also affects the permeability of the blood-brain barrier (data not shown). (6) RST reduces the expression of chemokines in the CNS: Ltn, RANTES, and IP-10, which are responsible for the recruitment of $CD4^+$, $CD8^+$ T cells, macrophages, and NK cells to the site of infection. The net effect of these changes is to reduce the ability to clear virus from the CNS. As a result of increased viral levels in the CNS, stressed mice subsequently develop early onset and worse demyelinating disease.

Summary

The findings presented in this chapter review the current research into stress and HSV and Theiler's virus. However, there is also increasing research on the effects of stress on human immunodeficiency

virus (HIV) infections, which deserve consideration. HIV is considered a neurotropic virus since it infects the CNS shortly after infection. HIV has a predilection for the hippocampus and basal ganglia, brain regions associated with the HPA axis, autonomic nervous system, and cognition. Studies of HIV+ patients have shown that they suffer from hyporeactivity of the autonomic nervous system and HPA axis, and thus HIV infection could be considered a model of chronic stress [126]. Interesting studies by Cole and colleagues [127] have demonstrated that socially inhibited gay men have elevated autonomic nervous system activity and accelerated HIV disease progression. Their studies indicated that T cells treated with catecholamines increased HIV replication rates. Furthermore, studies with simian immunodeficiency virus-infected macaques have shown that social stress alters glucocorticoid regulation and results in reduced survival times [128].

Research investigations into the role of the neuroendocrine-immune axis in neurotropic viral infections have clearly shown the importance of the psychological status of the host's response to infection. Generalizing these findings to the development of autoimmune diseases, stressful events that occur prior to or during infection, may result in immunosuppression and failure to eliminate the pathogen. Persistent infection then may lead to the development of autoimmune diseases such as multiple sclerosis. Stress-induced immunosuppression may also facilitate the generation of pathogens with enhanced and altered pathogenicity, giving rise to novel disease processes.

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17

Epidemiology of viral encephalitis

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Introduction

The epidemiology of encephalitis is a composite of the epidemiologic features of the multiple individual etiologies associated with this syndrome. Understanding the epidemiology is confounded by difficulties in both clearly defining a case and determining its etiology. Encephalitis, or inflammation of brain tissue, can occur from various insults to the central nervous system (CNS) including direct invasion by an infectious agent; an abnormal immune response to infection, vaccines, or antigens present at sites outside the CNS; or a combination of factors. In addition, the symptoms of brain dysfunction, the clinical hallmark of encephalitis, can also be associated with a number of noninfectious entities including autoimmune diseases, tumors, paraneoplastic syndromes, metabolic disorders, and toxins.

The many causes of encephalitis and nonencephalitic brain disease, the fact that the many different causes often cannot be distinguished clinically, and the difficulty in defining its etiology have frustrated our understanding of this syndrome. Encephalitis is, however, associated with substantial morbidity, long-term disability, and death and, consequently, is an important problem for which better treatment and prevention strategies could be of substantial benefit. This chapter will focus on overall epidemiologic patterns. Information on specific agents can be found in more detail in the virus-specific sections.

Definitions

Various case definitions of encephalitis in general and for encephalitides due to specific etiologies such as smallpox vaccination, arbovirus infections, and rabies infection, have been developed [1,2,3,4]. These definitions include clinical signs and symptoms that indicate involvement of the parenchyma of the brain (e.g., altered consciousness, personality changes, focal neurologic findings such as weakness or paralysis, and seizures) and results of laboratory studies (e.g., examination of cerebral spinal fluid [CSF]), brain imaging, and/or electroencephalography (EEG) studies that suggest brain disease and inflammation. Histologic evidence of inflammation in brain tissue is the most definitive indicator of encephalitis, but brain tissue is usually not available for testing. Since death may occur late in the course of the illness, postmortem studies of brain tissue may not be helpful in understanding the acute or precipitating disease process.

Etiologies

Encephalitis has been associated with a large number of infectious agents or processes including viruses, bacteria, fungi, and parasites; infection- or vaccine-related autoimmune responses; and noninfectious causes such as autoimmune diseases (e.g., lupus, rheumatoid arthritis, Hashimoto thyroiditis); cancer and leukemia; paraneoplastic syndromes;

Table 17.1. Selected etiologies of encephalitis

Viruses	**Viruses** *continued*	**Parazites**
Herpesviruses	Rubivirus	Toxoplasma gondii
Herpes simplex virus 1	Rubella	Free-living amoebae
Herpes simplex virus 2	Lyssaviruses	Naegleria fowleri
Varicella-zoster virus	Rabies	*Acanthamoeba*
Epstein-Barr virus	Arenaviruses	Balamuthia mandrillaris
Cytomegalovirus	Lymphocitic choriomeningitis virus	Malaria
Human herpesvirus 6	Reoviruses	Neurocysticercosis
Herpes B virus	Colorado tick fever virus	*Echinococcus*
Picornaviruses	Poxviruses	Baylisascaris procyonis
Enteroviruses	Smallpox virus	*Trichinella*
Parechoviruses	Retroviruses	*Gnathostoma*
Flaviviruses	HIV	*Shistosoma*
West Nile virus	Poliomaviruses	
St. Louis encephalitis virus	JC virus	**Vaccines**
Japanese encephalitis virus	Rotaviruses	Smallpox vaccine
Dengue virus	Erythroviruses	Rabies vaccines
Tick-borne encephalitis virus	Parvovirus B19	Oral poliovaccine (OPV)
Murray Valley encephalitis virus		Yellow fever vaccine
Bunyaviruses	**Bacteria**	
California serogroup	Mycoplasma pneumoniae	**Prions**
La Crosse virus	Bartonella henzelae	Creutzfeldt-Jakob disease
Jamestown Canyon virus	Rickettsia Prowazekii	New variant Creutzfeldt-Jakob disease
California encephalitis virus	Rickettsia rickettsii	Kuru
Rift Valley fever virus	Coxiella burnetii	
Toscana virus	*Ehrlichia sp*	**Encephalitis "mimickers"**
Alphaviruses	Mycobacterium tuberculosis	Neoplasms
Eastern equine encephalitis virus	Brucella melitensis	Paraneoplastic syndrome
Western equine encephalitis virus	Tropheryma whippelii	Autoimmune diseases
Venezuelan equine encephalitis virus	Spirochetes	Rheumatoid arthritis
Chikungunya virus	Treponema pallidum	Lupus
Paramyxoviruses	Borrelia burgdorferi	Hashimoto thyroiditis
Nipah virus	Listeria monocytogenes	Stroke
Hendra virus	Leptospira	Inborn errors of metabolism
Measles virus		Epilepsy
Mumps virus	**Fungi**	Infections (non-encephalitis)
Human parainfluenza viruses	Cryptococcus neoformans	Neisseria meningitides
Orthomyxoviruses	Histoplasma capsulatum	Haemophilus influenzae
Influenza virus	*Candida*	Streptococcus pneumoniae
Adenoviruses		Toxins

stroke; metabolic disorders; inborn errors of metabolism; and toxins [Table 17.1]. In a high percentage of cases, no etiology is determined. The wide range of infectious causes of encephalitis and the lack of a dominant cause are illustrated in data from a number of studies including several summarized in Table 17.2. The study by Glaser et al. of >1500 encephalitis cases in the United States illustrates that, in addition to infectious agents, a variety of non-infectious causes also present as encephalitis (8% of cases in this study) [5]. The differences in results among these studies (e.g., between 31–66% of cases with no etiology identified) can be explained by regional differences in causative agents and

Table 17.2. Range of encephalitis causes identified in selected etiologic studies of hospitalized cases of encephalitis in North America, Europe, and Asia

Source: Glaser *et al.* [5]
Prospective, all ages, California, USA, 1998–2005[a]

Etiologies	No. (%)
Conf./prob. infectious	248 (16)
HSV	45 (3)
EV	43 (3)
VZV	23 (2)
WNV	19 (1)
EBV	17 (1)
Measles SSPE	6 (<1)
Other viral	17 (1)
TB	19 (1)
Pyogenic bacteria	14 (<1)
Bartonella species	13 (<1)
Other bacterial	4 (<1)
Fungal	3 (<1)
Parasitic	7 (<1)
Prion	18 (1)
Possible infectious	204 (13)
M. pneumoniae	96 (6)
Influenza A, B	22 (1)
Adenovirus	14 (<1)
HSV	13 (<1)
Chlamydia species	10 (<1)
Other	49 (3)
Noninfectious	122 (8)
Unknown	996 (63)
Total	1570 (100)

Source: Kolski *et al.* [4]
Prospective, pediatric, Toronto, Canada, 1994–1995[b]

Etiologies	No. (%)
Confirmed/probable	20 (40)
M. pneumoniae	11 (22)
HSV	4 (8)
EV	2 (4)
HHV-6	2 (2)
Influenza A	2 (2)
EBV	1 (2)
Powassan	1 (2)
Possible	13 (26)
M. pneumoniae	9 (18)
Influenza A	1 (2)
Parainfluenza 3	1 (2)
EBV	2 (4)
Unknown	17 (34)
Total	50 (100)

Source: Iff *at al.* [8]
Retrospective, pediatric, Switzerland, 1980–1991[c]

Etiologies	No. (%)
Known	43 (54)
Rubella	7 (8)
VZV	6 (7)
EBV	5 (6)
B. burgdorferi	5 (6)
HSV	4 (5)
EV	4 (5)
TBE	3 (4)
Adenovirus	3 (4)
Measles	3 (4)
M. pneumoniae	2 (3)
RSV	1 (1)
CMV	1 (1)
Unknown	37 (46)
Total	80 (100)

Source: Kupila *et al.* [14]
Prospective, adults, Finland, 1999–2003[d]

Etiologies	No. (%)
Known	15 (36)
Confirmed	10 (24)
Probable	5 (12)
Identified agents	
HSV	4 (9)
VZV	5 (12)
TBE	4 (9)

Source: Xu *et al.* [18]
Prospective, pediatric, Beijing, China, 1991–1994[e]

Etiologies	No. (%)
Known	35 (36)
HSV	2 (2)
EV	15 (15)
Rubella	6 (6)
EBV	1 (1)
Mumps	7 (7)
JE	5 (5)

Source: Lee *et al.* [11]
Prospective, all ages, Taiwan, 2000–2001[f]

Etiologies	No. (%)
Known	86 (69)
Confirmed	21 (17)
Possible	65 (52)
Identified agents	
HSV	45 (36)
VZV	16 (13)
TB	10 (8)

(*Continued*)

Table 17.2. (*Continued*)

Source: Kupila *et al.* [14] Prospective, adults, Finland, 1999–2003		**Source: Xu *et al.* [18]** Prospective, pediatric, Beijing, China, 1991–1994		**Source: Lee *et al.* [11]** Prospective, all ages, Taiwan, 2000–2001	
Etiologies	**No. (%)**	**Etiologies**	**No. (%)**	**Etiologies**	**No. (%)**
Mumps	1 (2)	HHV-6	2 (2)	CMV	8 (6)
M. pneumoniae	1 (2)	Unknown	62 (64)	Adenovirus	5 (4)
Chlamydia	1 (2)	Total	97 (100)	EV	1 (<1)
Unknown	27 (66)			Influenza A	1 (<1)
Total	42 (100)			Unknown	38 (31)
				Total	124

Notes: The following case definitions of encephalitis were used in these studies:
[a] Encephalopathy (a depressed or altered level of consciousness lasting >24 h, lethargy, or a personality change) with >1 of the following: fever, seizure, focal neurologic findings, CSF pleocytosis, or EEG or neuroimaging findings consistent with encephalitis. Immunocompromised patients excluded.
[b] Encephalopathy (depressed or altered consciousness lasting >24 h, lethargy, extreme irritability, or personality change) with ≥2 of the following: fever, focal neurologic findings, CSF pleocytosis, EEG findings consistent with encephalitis or abnormal neuroimaging. Obvious bacterial infections, viral exanthems, noninfectious disorders excluded.
[c] Febrile illness at admission or in previous 4 weeks and ≥1 of the following: somnolence, stupor, coma, mental deterioration, motor and/or sensory disturbances (paralysis, ataxia, impaired speech or oculomotor dysfunction), and epileptic seizures. Excluded: neonatal infections, febrile seizures, noninfectious conditions.
[d] Acutely altered consciousness or personality, epileptic seizures, or focal neurologic signs, and either CSF pleocytosis or neuroradiologic and EEG findings compatible with encephalitis; immunocompromised and noninfectious conditions excluded.
[e] Acute fever, vomiting, headache, CSF pleocytosis, normal glucose; and altered consciousness or behavior (seizure, coma lethargy, agitation), motor disturbance (paraparesis, ataxia, weakness), and abnormal EEG or brain CT scan. Bacterial meningitis and neoplasms excluded.
[f] Acute and severe neurologic dysfunction including acute mental dysfunction, memory impairment, loss of consciousness, pareses, abnormal behavior, convulsions, involuntary movements; CSF findings and EEG and/or CT scans of the brain compatible with encephalitis. Acute meningitis and noninfectious conditions excluded.
The percent values are rounded to whole numbers. The sum of subgroups may not always be 100% because of rounding and, in some cases, mixed infections with >1 agent.
HSV, herpes simplex virus; EV, enterovirus; VZV, varicella-zoster virus; WNV, West Nile virus; SSPE, subacute sclerosing panencephalitis; TB, tuberculosis; M, Mycoplasma; HHV-6. herpesvirus 6; EBV, Epstein-Barr virus; B, borrelia; TBE, tick-borne encephalitis; RSV, respiratory syncytial virus; CMV, cytomegalovirus; JE, Japanese encephalitis virus.

differences in diagnostic testing, case definitions, exclusion criteria, etc.

Viruses are the agents most often linked to acute encephalitis, but no single virus has been associated with a large proportion of cases. The most common viral cause varies by region but usually is either herpes simplex virus (HSV) or one of the arthropod-borne viruses. Enteroviruses, various members of the *Paramyxoviridae* (e.g., measles, mumps, and nipah virus), rabies virus, varicella-zoster virus (VZV), and HIV are often important contributors as well [4,5,6,7,8,9,10,11,12,13,14,15,16,17,18]. The above noted viruses usually account for a substantial percentage of cases with known etiology in prospective studies. A number of other viruses have occasionally been shown to cause encephalitis, e.g., adenoviruses [19,20,21,22], lymphocytic choriomeningitis virus (LCMV) [23] (see Chapter 5), and parainfluenza virus

3 [24,25]. Some viruses (e.g., cytomegalovirus [CMV] and polyomaviruses, such as JC virus) are associated with encephalitis but primarily in patients with compromised immune systems [26,27] (see Chapter 10). A number of viruses have been detected in patients with encephalitis including Epstein-Barr virus (EBV) [28], human herpesvirus (HHV)-6 and HHV-7 [29,30,31], influenza virus [32,33], rotaviruses [34], respiratory syncytial virus (RSV) [35,36], parvovirus B19 [37,38], hepatitis C virus [3], and Chandipura virus [39,40], but a causal relationship has yet to be established. In some cases, dual infections with these viruses and another agent are observed [41,42], which adds difficulties to determining their etiologic role.

Both live and inactivated viral vaccines have also been associated with encephalitis. Oral live poliovirus vaccine can revert to wild-type and cause bulbar poliomyelitis, particularly in immunodeficient persons [43,44,45], and yellow fever vaccine has been associated with infectious encephalitis in young infants [46]. Other vaccines such as older rabies vaccines and smallpox vaccine have induced an abnormal or autoimmune response leading to a clinical picture of acute disseminated encephalomyelitis (ADEM) [47,48]. Cessation of widespread smallpox vaccination programs and improvements in rabies and other vaccines have resulted in marked decreases in vaccine-associated ADEM [16,49].

Challenges of etiologic diagnosis

Etiologic diagnosis of encephalitis is difficult under the best of circumstances. In prospective etiologic studies, even those with extensive testing [4,5,14,15,50] and hospitalization and mortality data [6,7,8,10,16,17,18], the cause remains unknown in 35 to >80% of cases. A number of factors contribute to the difficulty determining the etiology, including the type, timing, and quality of specimens, limited sensitivity and/or specificity of diagnostic tests, and potential presence of novel pathogens (see Chapter 18). Probably the single greatest diagnostic challenge is the lack of a specimen from the active site of the disease, the brain. Brain tissue is usually not available, and more easily available CSF is only linked to the disease process if there is accompanying meningitis (i.e., meningoencephalitis). In the absence of meningeal involvement, CSF may be too separated from the affected site to contain the etiologic agent and reflect the disease process. Demonstration of the local antibody production in CSF provides another way to detect active infection in the CNS and can be a highly sensitive and specific diagnostic way to identify some infections, e.g., West Nile virus (WNV), other arboviruses [51], and HSV [52, 53] (see Chapters 7, 11, and 18). Detecting infection at other sites may or may not be sufficient to indicate a causal relationship to the disease depending on the previously established likelihood that the pathogen causes encephalitis, clinical and epidemiologic features of the disease, etc.

As with most infections, brain, CSF, and non-CNS specimens (e.g., respiratory, urine, blood, and stool specimens) are most likely to yield the pathogen if collected early in the course of the illness. Similarly, acute- and convalescent-phase serum specimens are most likely to detect a rise in antibody titers if the acute specimen is collected early in the infection and the convalescent-phase specimen sufficiently late (e.g., 3 to 4 weeks into the illness) to allow time for the antibody response to develop. Finally, even if the right types of specimens are collected at the right time, suboptimal handling and storage conditions may affect the ability to detect agents.

The sensitivity and specificity of individual tests and breadth of available tests are important determinants for the ability to diagnose the cause of encephalitis. Development and increasing availability of molecular methods of pathogen detection, particularly sensitive polymerase chain reaction (PCR) assays, have made it increasingly likely that if present, we will be able to detect a pathogen in a specimen (see also Chapter 18). Many of these assays can also be performed quickly and with good sensitivity and specificity and, thus, provide information in a clinically relevant fashion (e.g., diagnosing HSV to guide treatment).

The availability of better assays for the large number of known causative pathogens, the recent discovery of new pathogens [54,55,56], and likely future discovery of additional pathogens provide both opportunities and challenges. The clinicians and laboratories are left with a large number of possible agents to look for, none of which causes a large percentage of all cases in many settings. The clinical and epidemiologic features of the illness, however, can often provide a focus for diagnostic testing. The clinical presentation, time of year, or other information may be suggestive of a specific etiology (e.g., HSV and temporal lobe involvement, enterovirus 71 [EV71], and bulbar encephalitis, Japanese encephalitis (JE) and time of year, etc.). However, usually there is too much overlap in clinical features associated with the many possible agents to make a pathogen-specific diagnosis based on clinical features alone.

Last and sometimes not appreciated is the fact that detecting a pathogen does not necessarily indicate it is the causative agent. The level of confidence that a detected pathogen is the causative agent of the encephalitis varies by pathogen, the type of specimen, and clinical and epidemiologic features of the illness. For some pathogens (e.g., many arboviruses, rabies virus, and Nipah virus), the link is well-established and their detection in the appropriate clinical and epidemiologic context indicates causality (see Chapters 3, 6, 7, and 21). For others (e.g., HSV and enteroviruses), the link is clear, provided the pathogen is detected in CSF specimens or brain tissue (see Chapters 1 and 11). Some (e.g., influenza virus, rotavirus, and HHV-6) are suspected, but not confirmed to cause encephalitis, and their detection, even in CSF, suggests but does not clearly indicate causality.

A set of criteria developed for the Unexplained Encephalitis Studies conducted by California, Tennessee, and New York state health departments and Vanderbilt University in collaboration with the Centers for Disease Control (CDC) is outlined in Table 17.3. These criteria consider the certainty of previously established links between the pathogen and encephalitis, the type of specimen, and clinical and epidemiologic features of the illness to define confirmed, probable, or possible causes of encephalitis [5]. A link between a pathogen and disease is further confounded if the infection triggers a host response (e.g., immune response leading to ADEM) or the pathogen releases a toxin that causes the disease. In these instances, the link between the pathogen and disease may be obscured by a time delay between the infection and the encephalitis or by a non-CNS infection causing the encephalitis.

Disease burden

Although encephalitis is not a common illness, it is associated with substantial morbidity and mortality. Since most encephalitis cases are hospitalized, hospital discharge data should provide a good approximation of the disease incidence. In the United States, studies of hospitalizations by discharge diagnoses between 1988 and 1997 identified 7.3 encephalitis hospitalizations per 100 000 population/year for a total of about 19 000 hospitalizations and 230 000 hospital-days costing approximately $650 million each year [10]. The highest rates of encephalitis-associated hospitalizations in the United States were observed among children <1 year of age (13.7 per 100 000), followed by persons aged 65 years or more (10.6 per 100 000), and the lowest – among persons aged 5–19 years (4.1 per 100 000) [10]. An earlier study estimated that there were 7.4 hospitalized cases of encephalitis per 100 000 population/year in the United States during 1950–1981 [57].

Estimates of encephalitis hospitalization rates in countries vary. For example, studies in Finland and Switzerland found about 1.4 encephalitis hospitalizations per 100 000 population in adults aged ≥15 years [7] and 8.8 to 10.5 encephalitis hospitalizations per 100 000 population in children aged <15 years [8,58]. In England and Japan, the estimated rates for all ages are lower, 1.5 per 100 000 [17] and 1.8 per 100 000 [59], respectively. The reasons for these differences in rates are not clear but could include a number of factors such as true differences in rates or differences in case finding, case definition, etc. There are few estimates of hospitalization

Table 17.3. Diagnostic category definitions used in California encephalitis project (Adapted from [5])

Category	Definition	Examples
Confirmed		
1a	Agent is a well-established cause of encephalitis,	HSV-1, VZV, EV, WNV;
1b	agent is detected in CSF or brain specimens OR results of antibody testing are positive in instances in which PCR is not the diagnostic test of choice, AND	measles causing SSPE; rabies
1c	clinical presentation and epidemiological profile are consistent with infection.	
Probable		
1a	Agent is a well-established cause of encephalitis,	HSV-1, EBV
1b	agent is not detected in CSF or brain specimens, but there is strong serological or culture-based evidence of infection, AND	
1c	clinical presentation and epidemiological profile are consistent with infection; OR	
2a	agent is not a well-established cause of encephalitis, AND	Hepatitis C virus, HHV-6,
2b	agent is detected in CSF or brain specimens by PCR.	rotavirus
Possible		
1a	Agent is a well-established agent of encephalitis,	EV, HSV-1, influenza A and B
1b	clinical presentation and epidemiological profile are consistent with infection, AND	viruses, VZV
1c	serologic evidence of infection was suggestive but not conclusive or a positive culture result was noted for a specimen obtained from a site other than the CNS site; OR	
2a	agent is not well-established or the diagnostic method has not been developed	Adenovirus, RSV
2b	clinical presentation and epidemiological profile are consistent with infection, AND	
2c	there is strong serologic or culture-based evidence of infection at a site other than the CNS site.	

Note: EBV, Epstein-Barr virus; EV, enterovirus; HHV-6, human herpesvirus 6; HSV-1, herpes simplex virus 1; RSV, respiratory syncytial virus, SSPE, subacute sclerosing panencephalitis; VZV, varicella-zoster virus; WNV, West Nile virus

rates for individual agents. In one study in France, 2.3 HSV-associated encephalitis hospitalizations per 1 000 000 population/year were identified [60].

The impact of encephalitis on each patient's life is very high. In one study, 71% of adult patients with HSV encephalitis received care in an ICU and 45% required mechanical ventilation [61]. In another study, 65.9% patients with WNV encephalitis required physical, 50% required occupational, and 30.8% required speech therapy after their illness [62]. Only 37% of patients had recovered fully 12 months after acute WNV infection [63]. Additional studies are needed to further define the full medical and societal burden of encephalitis. Outbreaks of encephalitis, such as those associated with WNV, may incur substantial control costs in addition to patient-related costs. For example, during a WNV outbreak in Louisiana, it was estimated that the cost of a public health response was about one-half the medical costs for WNV-infected patients [64].

Encephalitis is associated with a substantial mortality. In the United States, encephalitis was listed

as a cause of death on approximately 1300 death certificates each year from 1979 through 1998 [16] and 7.4% of all encephalitis-associated hospitalizations resulted in a fatal outcome [10]. Studies from several European countries have found 5–30% of encephalitis cases resulting in death [6,7,8,65,66]. Long-term studies of encephalitis demonstrate that a substantial portion of the deaths occur after the acute illness has resolved, especially during the first year after the onset of illness and would not have been included in mortality rates reported in many of the above noted studies [61,67].

The overall mortality rate for encephalitis represents a composite of that for individual agents, and the death rate associated with individual agents can vary substantially (Table 17.4). For example, the death rate for rabies is nearly 100% while that for

Table 17.4. Fatal outcome of encephalitis by etiology

Etiology	Fatal outcomes,%	Reference
All causes combined	2–30%	[4, 6, 7, 8, 10, 15, 16, 17, 57, 65, 117, 165, 166]
All causes combined in HIV infected	19.1%	[10]
All causes combined in HIV uninfected	5.4%	[10]
Unknown cause	2–6.4%	[10, 166]
Rabies	100%	[68]
Nipahvirus	40–74%	[56, 81, 82]
Herpes simplex virus, treated	7–28%	[13, 85, 167]
Herpes simplex virus, untreated	50–70%	[85]
Varicella-zoster virus	0	[166]
Enteroviruses	0–7%	[87, 166]
Enterovirus 71 (EV71)	14%	[168]
Enteroviruses other than EV71	4%	[87]
Arboviruses		
Eastern equine encephalitis virus	50–75%	[112]
Japanese encephalitis virus	20–40%	[52, 69]
West Nile virus	9–17.5%	[137, 160, 169]
Saint Louis encephalitis virus	10–20%	[52]
Powassan virus	10–15%	[140]
Western equine encephalitis virus	3–4%	[112]
Far Eastern tick-borne encephalitis virus	5–20%	[119]
Western tick-borne encephalitis virus	<0.5%	[119]
Venezuelan equine encephalitis virus	<1%	[52]
La Crosse virus	<1%	[52]
Adenovirus	3.7–20%	[117, 166]
Influenza	31.8%	[89]
Measles		
Measles, SSPE	100%	[170]
Measles, inclusion body encephalitis	100%	[170]
Measles, ADEM	20%	[170]
Rubella	20%	[166]
Mumps	2%	[166]
LCMV	0	[23]

VZV, mumps virus, most enteroviruses, and some arboviruses is estimated to be <5% (see Chapters 10, 3, 6, 7, and 12). The agent-specific contribution to encephalitis mortality varies by region. For example, herpes encephalitis was the most commonly etiologic category for encephalitis deaths in the United States during 1979–1998, with approximately 140 to 200 deaths reported each year [16]. Globally, rabies is an important cause of encephalitis deaths leading to an estimated 55 000 deaths and 1.74 million disability-adjusted life years (DALY) in Asia and Africa each year [68]. JE is associated with an estimated 50 000 cases resulting in 10 000 to 15 000 deaths worldwide, mostly in Southeast and South Asia [69,70]. Treatment, prevention, and emergence or re-emergence of pathogens can change agent-specific disease patterns. For example, the availability of effective HSV antiviral therapy likely contributed to a decrease in the estimated rate of HSV encephalitis-associated deaths in the United States from 0.9 per 1 000 000 during 1979–1988 to 0.5 per 1 000 000 during 1989–1997 [16]. The emergence of WNV in North America has made it an important cause of encephalitis-associated deaths in the United States, in some years approaching and/or surpassing HSV-associated deaths (e.g., 284 deaths reported in 2002) [40]. JE vaccination programs can decrease disease associated with this virus, and dog control programs and vaccination of dogs and cats can nearly eliminate rabies encephalitis.

Surviving encephalitis patients often need prolonged rehabilitation and suffer significant neurologic or neuropsychiatric lifelong sequelae. The disability associated with individual agents can vary substantially. In a prospective study in Toronto, neurologic abnormalities were present in 67% of patients at the time of the discharge from the hospital [4]. A multicenter study of outcomes of adult herpes simplex encephalitis in France found that 6 months after onset of the illness, 15% of patients had died, 20% had severe disability, and only 14% had recovered completely [61]. In recent studies, persistent neurologic and cognitive problems at 6 and 9 months after onset were reported in about one-half of WNV encephalitis cases [62,71], while 34% experienced new onset depression, which was still present at 1 year after onset in 24% [72]. In a variety of other studies, permanent sequelae in encephalitis patients were reported in 10% to 50% of cases [6,8,65,66]. The variation in the above rates and types of complications likely result in part from differences in study populations, case definitions, types of outcome measures, diagnostic criteria, etc.

A few studies have also looked at longer-term sequelae of encephalitis (i.e., years after the acute illness). A long-term follow-up study of 74 children who survived acute encephalitis of various etiologies in Switzerland found that 1 to 12 years after the acute illness, 8 cases had severe, 6 cases moderate, and 14 cases, mild sequelae [8]. In a Finnish study of 70 children who had encephalitis 2 to 13 years earlier, 34% of cases vs 9% of their age- and sex-matched controls had some neurologic abnormality including 3% with serious sequelae [73]. In a study of childhood HSV encephalitis in Israel, at up to 5 years after acute illness, 36% of children were noted to have significant deficiencies (cognitive dysfunction, personality changes, speech abnormalities, motor skill disturbances, and epileptic seizures) [31]. A study of long-term follow-up of children in Taiwan who had EV71 encephalitis 1 to 7 (median, 3) years earlier, reported 75% of 28 children with EV71 encephalitis complicated by cardiopulmonary failure having serious sequelae, including limb weakness and atrophy, facial nerve palsy, dysphagia necessitating tube feeding, central hypoventilation requiring ventilator support, etc. [74].

As with most other infectious diseases, encephalitis in immunocompromised patients is associated with both increased morbidity and mortality from the usual agents of encephalitis (e.g., enteroviruses and WNV [22,75,76]). In addition, some viruses (e.g., CMV, HHV-6, polyomaviruses such as JC) [26,27,30] and other pathogens (e.g., toxoplasma) [77] that rarely cause CNS disease in a normal host can cause encephalitis in immunocompromised persons. HIV has been the single most important cause of immunosuppression-associated encephalitis as well as an important cause of encephalitis

by itself (i.e., HIV encephalitis [77,78]) (see Chapter 9). In a recent study of hospitalized cases of encephalitis in the United States, HIV-infected persons accounted for a disproportionately high proportion of encephalitis-associated hospitalizations (15.6%) [10] and even higher proportion of deaths (28%) [16]. The risk of dying from encephalitis was several hundred-fold higher in the HIV-infected population, compared to non-HIV-infected population [16]. During the mid 1990s, just before the introduction of HAART, HIV-infected persons accounted for almost 40% of all encephalitis deaths in the United States [10].

Transmission modes

The agents of encephalitis have various routes of transmission, including fecal-oral (e.g., enteroviruses), respiratory (e.g., measles, VZV, mumps, rubella, and adenoviruses), vector-borne (e.g., WNV, Eastern equine encephalitis [EEE], and St. Louis encephalitis [SLE], etc.) (see Chapter 20), via fomites (e.g., adenovirus and enteroviruses), animal bites (e.g., rabies) (see Chapters 3 and 21), bloodborne (HIV, WNV), tissue transplantation (rabies, LCMV), or sex (HIV). The increase in tissue and organ transplantation and related medical procedures is likely to correspondingly increase the frequency of outbreaks of encephalitis associated with such procedures. It is also likely that the list of agents transmitted by this route will grow.

Risk factors for disease

A variety of environmental, agent, and host factors affect the likelihood that an individual will develop encephalitis. For some agents, such as rabies virus, henipaviruses, and some arboviruses, the risk of being infected is the most important factor. For others, such as most enteroviruses and adenoviruses, infection is common but only rarely leads to encephalitis, and agent and/or host factors are probably the key to the disease manifestations.

Exposure

The recent addition of Nipah virus to encephalitic agents illustrates the risk of chance infection of humans with an animal pathogen leading to new and unsuspected risks for encephalitis. Nipah virus normally infects fruit-eating Pteropus bats (flying foxes) but can infect a wide range of mammals. Nipah virus and closely related viruses have caused a large outbreak of encephalitis in pig farmers in Malaysia and abbatoir workers exposed to pigs in Singapore in 1999 [79,80]. Subsequently, it was identified as the cause of several clusters of cases in villages in India and Bangladesh [56,81,82]. In these outbreaks humans were infected after a chance introduction from the animal reservoir either via intermediate amplifying host (pigs in the Malaysia and Singapore outbreaks) or directly from bats to humans with some human-to-human transmission (in the India and Bangladesh village clusters). The cases in Bangladesh have often occurred among young men, who apparently were infected while harvesting fruit from trees where the Pteropus bats had roosted. The risk of acquiring many arboviruses is increased with occupational or leisure activities that increase outdoor exposures to the arthropod vector [83]. Crowding and poor hygiene increase the risk of respiratory and fecal-oral transmission and may increase the risk of encephalitis associated with agents transmitted in this fashion (e.g., poliovirus and other enteroviruses, measles, etc.). For poliovirus, in some instances crowding and poor hygiene were actually associated with a lower risk of poliomyelitis because, in such settings, infection occurred very early in life when maternally acquired antibody protected from disease but virus still replicated enough to induce a protective immune response [84].

Host factors

A variety of host factors have been associated with encephalitis in an agent-specific fashion. Age is often an important factor, with the very young and elderly often being at highest risk [10,12,13,16,17]. For HSV encephalitis, the greatest risk occurs after 20 years

of age in whom 70% of cases occur, with more than 50% occurring among persons over 50 years of age [85]. Encephalitides due to enteroviruses [86,87], JE [69,70,88], La Crosse virus [83], and influenza [89] primarily affect children, while the elderly are at the highest risk of encephalitis due to WNV and SLE [83].

Differences in overall risk of encephalitis by sex are less pronounced. Some studies report slightly higher risk for males (male:female ratio, 1.3–1.4:1) [10,11,13,16], while others do not reveal any considerable differences [12,17]. Males appear to be at a higher risk of encephalitis due to enteroviruses [86,87], mumps [13], VZV [13], henipahviruses [81], and rabies virus [90,91]. No differences by sex were reported for other causes.

Underlying illnesses also contribute to the risk of encephalitis. The elevated risk and increased severity of encephalitides associated with various agents in immunocompromised patients, whether it is due to HIV, primary immunodeficiencies, or immunosuppressive treatment, is well known. In addition to older age, diabetes mellitus and kidney disease have been shown to be associated with the increased risk of developing WNV encephalitis and serious complications with encephalitis [62].

A variety of host immune response, metabolic, etc., differences are likely involved in the outcome for many infections including those causing encephalitis. Many of these factors are likely determined genetically [92,93], but which ones and how they affect disease outcome are by and large yet to be determined. A few genetic risk factors have been associated with some agent-specific encephalitides, including WNV and influenza virus. For WNV, the defective allele of the chemokine receptor CCR5 gene, CCR5Δ32, has been associated with greater risk of severe WNV infection [94,95,96]. In contrast, homozygocity for this allele (i.e., CCR5Δ32) has been linked with resistance to HIV infection [96]. Another genetic factor, single-nucleotide polymorphisms in the 2′-5′oligoadenylate synthetase (OAS) genes, has also been suggested to increase the risk of severe WNV disease [97,98]. Influenza virus-associated encephalopathy has been associated with a newly identified missense mutation (F303S) in the gene encoding the toll-like receptor 3. The toll-like receptor 3 mediates the production of proinflammatory cytokines [99].

Viral factors

Similar to host genetic factors, virologic factors that affect disease outcome are incompletely understood. It is, however, likely that genetically determined properties of viruses affect disease outcome in various ways. Genetic variations that may affect disease are most often noted for ribonucleic (RNA) viruses because of the high rate with which mutations occur. Large-scale outbreaks of CNS illness associated with specific enterovirus serotypes are usually linked with the emergence of new distinct genetic lineages that presumably facilitate transmission or infection in as yet undefined ways (e.g., recent large-scale outbreaks of echovirus 30 and echovirus 13) [100,102,102]. In other instances, specific mutations appear to attenuate or increase the virulence of a virus. A single amino acid substitution in non-structural protein NS2A was shown to result in attenuation of WNV by disabling its WNV ability to suppress interferon induction in mice [103]. Differences in replication, cellular tropism, and temperature sensitivity have been reported between strains of EV71 associated with bulbar encephalitis and those not associated with encephalitis [104]. A few mutations in oral poliovirus vaccine lead to reversion to wild-type phenotype resulting in viruses with regained neurovirulence capable of causing poliomyelitis [105,106,107].

Temporal patterns

Some viruses are associated with distinct temporal patterns of encephalitis including regular seasonal epidemics, periodic outbreaks or epidemics, and temporally and geographically restricted clusters or outbreaks. Other viruses such as HSV occur throughout the year without distinctive seasonal or geographic patterns. HSV is an extremely common infection; for example, a recent study in the United States

showed that approximately 60% of the population is seropositive for HSV-1 [108]. Primary infection with HSV leads to a lifelong latency with periodic reactivations. Encephalitis develops in a small proportion of infected individuals, is often associated with reactivation of latent infection, and may occur many years after the primary infection. Therefore, cases are rarely linked and are not associated with temporal or geographic clustering. Several purported clusters have been shown with molecular epidemiologic studies to be caused by unrelated strains of HSV, demonstrating that the infections were not actually linked [109,110].

Enteroviruses such as poliovirus and arboviruses such as WNV, SLE, and JE have consistent, yearly seasonal peaks in disease (mostly during summer and fall in the temperate climates and rainy season in tropical regions). The reason for these seasonal patterns are clear for the arboviruses (i.e., related to increases in the vector population and/or susceptible reservoir populations). The presence of the appropriate vector and large susceptible population of birds and humans led to large-scale summer-fall outbreaks of WNV each year following its introduction into the United States in 1999 [111]. Outbreaks of SLE have occurred approximately every 10 years during 1940–1970, with little intervening activity [83,112]. Periodic increases followed by several years of quiescence are also characteristic for enteroviruses with epidemic pattern of circulation (e.g., echoviruses 9, 13, 30) [113]. The peak years for these viruses have been correlated with periodic increases in CNS illnesses associated with enteroviruses [114]. This periodicity presumably reflects the time to accumulate sufficient susceptible population for these viruses to sustain an outbreak.

Geographic patterns

There are substantial geographic variations in causes of encephalitis (Table 17.2). The extent of geographic differences is less understood for many developing regions, especially Africa, where few studies of encephalitis have been reported. It is also important to remember that with some exceptions such as JE in some countries, no specific virus is responsible for a large proportion of cases. Some encephalitis-associated viruses (e.g., HSV, enteroviruses, VZV, respiratory viruses, measles virus, rabies virus, HIV) are present worldwide but at different levels relative to the overall encephalitis disease burden. For example, HSV is the most commonly detected viral cause of encephalitis in developed countries in Europe and North America [6,7,9,17,66,115] (see also Chapter 11) but found to be a relatively uncommon cause of encephalitis in studies from developing countries of Asia [15,18,116]. It is possible that lesser contribution of HSV to encephalitis cases in some Asian countries may be attributed to higher overall rates from JE or other encephalitis viruses. VZV has been reported to account, for unknown reasons, for a higher proportion of encephalitis cases in Scandinavia [12,14,115] than in other regions [15,18,50,59] (see also Chapter 12).

Enteroviruses are among the most common human infections worldwide, but their reported contribution to encephalitis cases varies substantially (from <1–15% of all encephalitis cases) [12,18,50,87,115,117,118] (see also Chapter 1). Some of this variation probably results from serotype differences in rates of encephalitis and variation in the predominant enterovirus serotypes circulating at the time and location of studies [113]. Some differences could also result from differences in transmission patterns associated with crowding, levels of sanitation, or other factors.

Arboviruses, the most common causes of epidemics of encephalitis, are a diverse group of viruses that usually have very specific geographic distributions. For example, the New World arboviruses (SLE, EEE, WEE, VEE, etc.) circulate in Western hemisphere (see also Chapter 6), JE circulates in South and Southeast Asia [83,112], tick-borne encephalitis (TBE) viruses are endemic to Central and Eastern Europe (Western TBE) and to Siberia and the Russian Far East (Far Eastern-type TBE) [119,120] (see also Chapter 7). The geographic distribution for individual viruses can change over time, as

demonstrated by the recent introduction and established circulation of WNV in North America. WNV had previously only been known to circulate in Africa, Southern Europe, and parts of Asia. Also, recent re-emergence of Chandipura virus (family, *Rhabdoviridae,* genus *Vesiculovirus)* in India [39] (see Chapter 3) and Chikungunya virus (an alphavirus) on the island of Reunion with subsequent spread to the surrounding region caused large-scale outbreaks, which also involved cases of encephalitis [121,121,123]. Finally, discovery of new agents of encephalitis (e.g., new lyssaviruses related to rabies virus, such as Australian bat lyssavirus [124] and Duvenhage virus in South Africa [55], both linked with human cases of encephalitis) also contributes to our understanding of the diversity of encephalitis causes worldwide (see also Chapter 3).

A good example of very circumscribed distribution of virus-associated encephalitis is Nipah virus (see also Chapter 21). The geographic pattern of this virus follows the range of its reservoir species, fruit-eating bats of the genus Pteropus (flying foxes) [56,81,82]. EV71 outbreaks associated with fulminant bulbar encephalitis in young children have been described in Malaysia in 1997 [125], Taiwan in 1998 and 2000–2001 [126,127], and Singapore in 2000 [128]. In contrast, EV-71-associated outbreaks of hand-foot-and-mouth disease occur on a regular basis throughout the world without clusters of fulminant bulbar encephalitis [129,130]. The reason for this geographic pattern is not understood but could result from genetic differences in the infected populations, differences in the infecting virus, simultaneous circulation and coinfection with another agent, simultaneous exposure to a toxin, or other environmental factors, etc.

Health care practices can also lead to geographic differences in risk for some viral-associated encephalitis. Procedures associated with blood donation, tissue transplantation, and nosocomial infections, etc., can markedly affect the risk of subsequent transmission of viral encephalitis (e.g., HIV, LCMV, WNV, etc.) [131,132,133,134,135]. Polio, measles, mumps, and rubella vaccination programs can decrease or essentially eliminate these viruses as causes of encephalitis depending on immunization rates (see Chapters 1 and 2). The high rates of immunization for these viruses have essentially eliminated them as causes of encephalitis in the United States and some other countries [40,38]. Rabies is another virus for which effective control programs can nearly eliminate the risk of disease. For rabies, however, it is control and immunization of cats and dogs that protects humans (Chapter 3). Mosquito control programs initiated in the 1950s are considered to have decreased transmission of arboviruses and the attendant encephalitis in the United States (see also Chapter 2).

Regional patterns

Although there is limited data on the etiology of encephalitis for most regions except for developed countries of Europe and North America, certain parts of Asia, and the Oceania, it is possible to identify some distinct regional patterns, especially for the arboviruses.

North America

Surveillance data and studies in the United States and Canada have most often detected herpesviruses, WNV, and other arboviruses, enteroviruses, and respiratory viruses in cases of encephalitis. A variety of other viruses are also detected in encephalitis cases (Table 17.2).

The most common arbovirus presently detected in North America is the mosquito-borne flavivirus, WNV (see also Chapter 7). WNV was introduced into North America in 1999 [136] and over a few years spread from Northeastern United States to the entire continent. WNV continues to cause yearly outbreaks of encephalitis throughout the United States and Canada [111,137]. In the past, a closely related mosquito-borne flavivirus, SLE, caused large urban outbreaks in the United States every few years, but after the last major outbreak, with 2800 cases in 1975, it has been much less common [83,138]. In the last decade, the highest number of reported

cases of SLE encephalitis in the United States in a single year has been 79 (in 2001) [139]. Other flaviviruses, including the tick-borne Powassan virus, have been associated with rare cases of encephalitis in the Eastern United States and Canada but their detection has been increasing lately [140,141]. EEE, the alphavirus, is primarily present in eastern parts of North America, while Western equine encephalitis (WEE) is predominantly found in Western United States and Canada. WEE has caused large-scale outbreaks in the past (e.g., 3000 cases in 1941 [72]) but has become uncommon in the past 20 years (<10 reported cases after 1987) [138,139,142]. The North American bunyavirus, La Crosse virus, mostly encountered in the Midwest and central regions of the United States, was the most commonly detected arbovirus in the United States during the years prior to the introduction of WNV [139,142,143]. Jamestown Canyon virus is present in the Eastern United States and rarely detected in sporadic cases of encephalitis [144].

Encephalitis due to polio, measles, and mumps viruses has been essentially eliminated from the United States and Canada due to successful immunization programs. On the other hand, the increase in immunocompromised populations from HIV, organ transplantation, and immunosuppressive therapy for cancer and leukemia have resulted in encephalitis associated with a wider range of viruses including some otherwise not associated with encephalitis [10,16,26,27,77,78].

Europe

The viruses most commonly identified in encephalitis cases in Europe include herpesviruses, arboviruses, respiratory viruses, and the vaccine-preventable viruses – measles and mumps. Herpesviruses (HSV and VZV) account for a greater proportion of encephalitis cases among adults in some parts of Europe (i.e., Scandinavia) than most other countries [5,6,7,8,9,11,12,14,17,58,66,115]. Flaviviruses, including TBE and WNV, are the most common arboviruses in Europe. TBE is most often detected in Northern and Central European countries and reported in up to 9% of encephalitis cases in studies in Finland, Lithuania, Switzerland, and Sweden [8,9,14,115,120]. WNV is most often detected in Southern Europe and reported as causing outbreaks of encephalitis in Romania in 1996 [145] and southern Russia in 1999 [146]. Toscana virus, a bunyavirus endemic to Mediterranean region, has been associated with cases of encephalitis, particularly in central Italy [147]. With suboptimal vaccination coverage, measles and mumps outbreaks continue to occur in some European countries, and with these outbreaks encephalitis associated with measles, mumps, and rubella continues to be reported [17,148].

Asia

Given its size, diverse geography, and large populations, Asia has a variety of distinct patterns of encephalitis. Arboviruses (JE, WNV, Dengue virus), enteroviruses (particularly, EV71), paramyxoviruses (henipaviruses), influenza viruses, and vaccine-preventable viruses are the notable viruses associated with encephalitis. The flavivirus, JE, is by far the most common cause of encephalitis in South and Southeast Asia. In some regions, it accounts for up to 30% of all childhood encephalitis [15,18,50,88,149,150]. WNV is endemic to parts of Asia [150,151]. Dengue viruses, extremely common in countries of South and Southeast Asia, have been identified in connection with acute encephalitis [15,152], but a causal relationship has yet to be established. Rift Valley fever virus, an African bunyavirus also found in the Arabian Peninsula, has been associated with encephalitis. For example, in an outbreak of Rift Valley fever in Saudi Arabia, meningoencephalitis was observed in up to 4% of cases [153,154].

Enteroviruses are frequently detected in encephalitis cases throughout Asia with detection rates as high as 5% of encephalitis cases in Thailand [50] and 15% in Beijing, China [18]. Outbreaks of EV71-associated bulbar encephalitis (Malaysia, Singapore, and Taiwan) in the late 1990s were notable for their high case fatality rates [130]. Nipah virus cases have, to date, only been detected in Asia.

Vaccine-preventable viruses continue to cause encephalitis in some parts of Asia. Bulbar poliomyelitis continues to occur in parts of India, Pakistan, and Afghanistan. Measles, mumps, and rubella-associated encephalitis continue to occur in many Asian countries [18,50,116], and rabies remains a substantial public health problem in the region. A recent estimate suggests that rabies accounts for nearly 24 000 cases each year in Asia [68].

Australia and Oceania

The spectrum of encephalitogenic viruses in the Pacific Rim includes the usual agents such as herpes viruses, enteroviruses, respiratory viruses, etc., and region-specific arboviruses. Kunjin virus, currently considered a subtype of WNV, and Murray Valley encephalitis virus – a flavivirus from the JE serologic complex [155], are endemic in this region. Hendra virus, closely related to Nipah virus [156,157], and the recently discovered Australian bat lyssavirus [158] have also been detected in cases of encephalitis in Australia.

Central and South America

Arboviruses are the best-studied causes of encephalitis in Central and South America. Epidemics of human encephalitis associated with the flaviviruses such as Rocio virus in Brazil in 1975 [83,159] and SLE in Argentina in 2005 [159] have been reported. Of note, SLE has been uncommon in the Americas over the past 20 years, and the recent outbreak in Argentina was associated with a new strain of SLE, genotype III [159]. It is possible that biologic differences of this new strain from previously circulating strains may facilitate its spread and reoccurrence of SLE as a more important cause of encephalitis. As elsewhere, Dengue viruses circulate widely in the tropical regions of the Americas and likely contribute to encephalitis cases. The alphavirus EEE has been detected in the Caribbean islands of Cuba and Hispaniola, and Venezuela, Brazil, Peru, and Argentina in South America [83]. Another alphavirus, Venezuelan equine encephalitis (VEE) virus, has been reported to be endemic in Peru, Ecuador, Venezuela, and Mexico, and likely present through the region resulting in periodic outbreaks in horses and humans [83,112].

Africa

The data on encephalitis causes in Africa is very limited. Arboviruses, vaccine-preventable viruses, and HIV appear to be the important contributors to encephalitis disease. WNV, named after the place of its first isolation – West Nile province in Uganda, is endemic to Africa. Recently, outbreaks of WNV encephalitis have been reported in Tunisia and Sudan [160,161]. Another important arbovirus, Rift Valley fever virus, causes periodic outbreaks throughout Africa, which likely include cases of encephalitis. Wild polioviruses type 1 and 3 continue to circulate in sub-Saharan Africa, with Nigeria being the most important reservoir [162]. With difficulties in maintaining high levels of immunization in many countries, some poliovirus-free countries have had reintroduction of virus and outbreaks of poliomyelitis. In Namibia, reintroduction of wild-type 1 poliovirus led to an outbreak with a high proportion of adult cases, which are at a greater risk of bulbar polio and death [163]. Measles is very common in most parts of Africa and is likely associated with a substantial number of cases of encephalitis. Rabies also continues to be an important cause of encephalitis and estimated to be responsible for approximately 31 000 deaths each year [68]. Africa has the highest rates of HIV infection, and with most HIV-infected persons being untreated, it is likely that HIV-associated encephalitis is an important contributor to the encephalitis burden there. An autopsy study of fatal cases of HIV infections among children in Africa identified encephalitis associated with HIV, CMV, and measles (13% combined) [164].

Comment

Although the epidemiology of viral encephalitis is a composite of a diverse group of viruses, it is worth

noting some overall themes for this disease. First, although much less common than some other illnesses, such as acute respiratory illness and gastroenteritis, the high mortality rate and frequent long-term disability make encephalitis a significant public health problem. Data on the overall burden associated with encephalitis is being developed and should encourage efforts to better understand this disease. Second, the diversity of etiologic agents, lack of a dominant causal agent in most settings, and lack of good data on etiologic agents for many settings confound our ability to prioritize efforts to develop and implement treatment and prevention strategies. New diagnostic assays are making it likely that, if present in a specimen, we will be able to detect most agents. Despite these improved diagnostic tools, it is likely that a substantial portion of cases will continue to go undiagnosed because specimens from the site of disease, the brain, are usually unavailable, and establishing a causal link when detected at other sites is often difficult. New, creative approaches to diagnosis are needed for many agents. Finally, the epidemiology of encephalitis will continue to change. The continuous changes in travel, social practices, commerce, agriculture, environment, health care practices, and others, have in the past and will no doubt in the future lead to emergence and re-emergence of viral agents of encephalitis throughout the world and challenge our ability to respond to known and new health threats.

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18

Pathogen surveillance and discovery

W. Ian Lipkin and Thomas Briese

Introduction

This chapter will describe methods and perspectives for pathogen discovery and surveillance, provide vignettes from our own experience that illustrate the complexity of pursuing research in this arena and the process that led to the implementation of particular strategies, and discuss challenges associated with proving causality.

Proof of causation

Discovery of an organism in association with disease is only the first step in understanding its role in pathogenesis. Many have wrestled with the challenge of codifying the process of proving causation. The germ theory of disease formulated by Pasteur, Koch, and Loeffler proposed precise criteria that define a causative relationship between agent and disease: the agent should be present in every case of a disease, it should be specific for a disease (i.e., present in none other); it should be propagated in culture and proven capable of causing the same disease upon inoculation into a naïve host. Known as Koch's postulates [1], these criteria were subsequently modified by Rivers for specific application to viruses [2] and by Fredericks and Relman to reflect the advent of molecular methods [3] (Table 18.1). Koch's postulates remain the ideal standard by which causality is considered to be proven. However, there are problems with holding to this standard. Some agents cannot be propagated in culture. Additionally, for many human viral pathogens there may be no animal model. In many acute viral diseases, the responsible agent can be readily implicated because it replicates at high levels in the affected tissue at the time the disease is manifest, morphological changes consistent with infection are evident, the agent is readily identified with classical or molecular methods, and there is evidence of an adaptive immune response. However, implication of viruses in chronic diseases may be confounded because persistence requires restricted gene expression, classical hallmarks of infection are absent, and/or mechanisms of pathogenesis are indirect or subtle. In the final analysis, investigators are occasionally left with what amounts to an assessment of strength of epidemiological association and biological plausibility based on analogy to diseases with related organisms where linkage is persuasive.

Molecular strategies for pathogen discovery

Methods for cloning nucleic acids of microbial pathogens directly from clinical specimens offer new opportunities to investigate microbial associations in chronic diseases [4]. The power of these methods is that they can succeed where methods for pathogen identification through serology or cultivation may fail due to absence of specific reagents or fastidious requirements for agent replication. Over the past decade, the application of molecular pathogen discovery methods resulted in the identification of novel agents associated with both acute and chronic

Table 18.1. Criteria for proof of causation

Robert Koch (1890)	Thomas R. Rivers (1936)	Fredericks and Relman (1996)
A microbe must be • Present in every case of a disease • Specific for that disease • Isolated, propagated, in culture, and shown to induce disease upon inoculation into an experimental host • Re-isolated from the experimental host wherein the original syndrome is replicated Note: This fourth postulate, though not required by Koch, logically follows his other conditions and so has been added by some reviewers.	• A specific virus must be found associated with a disease with a degree of regularity. • Note: The possibility of a viral carrier state was recognized and Koch's requirement of propagation in media or cell culture was abandoned. • The virus must be shown to be the causative agent of disease in the sick individual. Note: The pathogen should be present at the proper time in specific regions and the disease should be produced with some regularity by serial inoculation of infected material into a susceptible host.	• Candidate sequences should be present in most cases of disease and at sites of disease. • Few or no sequences should be present in host or tissue without disease. • Sequences should diminish in frequency with resolution of disease and increase with relapse. • Sequences should be present prior to the onset of disease.

diseases, including Borna disease virus, hepatitis C virus, Sin Nombre virus, HHV-6, HHV-8, *Bartonella henselae*, *Tropheryma whippelii*, West Nile virus, and SARS coronavirus [5,6,7,8,9,10,11,12].

Various methods are employed or proposed for cultivation-independent characterization of infectious agents. These can be broadly segregated into methods based on *direct analysis of microbial nucleic acid sequences* (e.g., cDNA microarrays, consensus polymerase chain reaction [PCR], representational difference analysis, differential display), *direct analysis of microbial protein sequences* (e.g., mass spectrometry), *immunological systems for microbe detection* (e.g., expression libraries, phage display), and *host response profiling*.

The decision to employ a specific method is guided by the clinical features, epidemiology, and spectrum of potential pathogens to be implicated. *Expression libraries*, comprised of cDNAs or synthetic peptides, may be useful tools in the event that large quantities of acute and convalescent sera are available for screening purposes; however, the approach is cumbersome, labor-intensive, and success is dependent on the presence of a specific, high-affinity humoral immune response. *Mass spectrometry* is an intriguing approach to pathogen discovery [13,14]; however, potential confounds include: mutations in flora that alter spectra without clinical correlation; the requirement for establishment of large libraries of spectra representing flora of thousands of organisms propagated *in vitro* and isolated *in vivo*; and the difficulties associated with extending this technology to viruses, where disease may occur without robust protein expression, and pathogenicity may be correlated with single base substitutions. The utility of *host response mRNA profile analysis* has been demonstrated in several *in vitro* paradigms and some inbred animal models [15,16,17]; nonetheless, a variety of organisms may activate similar cascades of chemokines, cytokines, and other soluble factors that influence host gene expression to produce what are likely to be convergent gene expression profiles. *Representational difference analysis (RDA)* is a subtractive cloning method for binary comparisons of nucleic acid populations [18,19]. Although ideal for analysis of cloned cells or tissue samples that differ only in a single variable of interest, RDA is less well-suited to investigation of syndromes wherein infection with any of several different pathogens results in similar clinical manifestations or infection

is not invariably associated with disease. An additional caveat is that because the method is dependent upon the presence of a limited number of restriction sites, RDA is most likely to succeed for agents with large genomes. Indeed, it is noteworthy that the two viruses detected by RDA were herpesviruses. *Consensus PCR (cPCR)* has also been a remarkably productive tool for biology. In addition to identifying pathogens, this method has facilitated identification of a wide variety of host molecules, including cytokines, ion channels, and receptors. One difficulty in applying cPCR to pathogen discovery in virology has been that it is difficult to identify conserved viral sequences of sufficient length to allow cross-hybridization, amplification, and discrimination in a traditional cPCR format. Although this may not be problematic when one is targeting only a single virus family, the number of assays required becomes infeasible when preliminary data are insufficient to permit directed, efficient analysis. To address this problem, we adapted cPCR to *differential display*, a PCR-based method for simultaneously displaying the genetic composition of multiple sample populations in acrylamide gels [20]. This hybrid method, known as domain-specific differential display (*DSDD*), employs short, degenerate primer sets designed to hybridize to viral gene domains that represent larger taxonomic categories than can be resolved in cPCR. While this modification allowed us to identify West Nile virus as the causative agent of the 1999 New York City encephalitis outbreak [21,22], it did not resolve issues of low throughput due to limitations in multiplexing.

To address the need for sensitive, facile, highly multiplexed pathogen surveillance, we recently established two new platforms for virus detection, *MassTag PCR* and *GreeneChips*, that will be described in detail below. MassTag PCR is a multiplex PCR method that can accommodate in excess of 20 genetic targets with sensitivity in the range of 10–1000 RNA copies (variability is a function of primer degeneracy). GreeneChips are comprehensive microarrays that address all vertebrate viruses (viral GreeneChipVR), as well as human bacterial, fungal, and parasite pathogens (panmicrobial GreeneChipPm). Both methods rely upon the presence of an agent related to one already known. In instances where agents are novel or sufficiently distant in sequence to related agents to confound hybridization it may be necessary to resort to subtractive cloning or high-throughput unbiased sequencing. In the next section, we will briefly discuss examples that illustrate the decision to develop multiple platforms that enable a staged strategy for pathogen detection.

Adventures and misadventures along the way

Borna disease virus and neuropsychiatric disease

In 1985, Rott and Koprowski reported that serum from patients with bipolar disorder reacted with cells infected with Borna disease virus (BDV), an unclassified infectious agent named after a town in Saxony (Eastern Germany) that had large outbreaks of equine encephalitis in the late 1800s. Intrigued both by the concept that infection might be implicated in a neuropsychiatric disease and that established methods for virus isolation had failed, we and others began to pursue characterization of this elusive neurotropic virus using molecular tools. BDV nucleic acids were isolated by subtractive hybridization in 1989, the first successful application of subtractive cloning in pathogen discovery [8]. This effort relied upon cDNA cloning with home brew kits as it preceded the advent of polymerase chain reaction and ready access to sequencing technologies. The correlation between cloned materials and disease was achieved by demonstrating that (1) candidate cDNAs competed with RNA template from brains of infected rats for transcription and translation of a protein biomarker present in brain (hybrid arrest experiments), (2) the distribution of candidate nucleic acid correlated with pathology in brains of experimentally infected rats and naturally infected horses (*in situ* hybridization), and (3) no signal was obtained in Southern hybridization experiments where normal brain was probed with candidate clones. Based

on Northern hybridization experiments the genome was variously reported as an 8.5 kb negative polarity RNA or an 11 kb positive polarity RNA. Over the next 5 years, the genome was cloned, and the virus was visualized and classified as the prototype of a new family of nonsegmented negative-strand (NNS) RNA viruses with unusual properties: nuclear replication/transcription, posttranscriptional modification of selected mRNA species by splicing, low-level productivity, broad host range, neurotropism, and capacity for persistence [23,24,25,26,27,28]. It was widely held that the introduction of specific reagents such as recombinant proteins and nucleic acid probes would allow rapid assessment of the role of BDV in human disease. However, in a classic example of the pitfalls of PCR diagnostics, particularly using nesting methods, BDV was implicated in a wide variety of disorders that included unipolar depression, bipolar disorder, schizophrenia, chronic fatigue syndrome, AIDS encephalopathy, multiple sclerosis, motor neuron disease, and brain tumors (glioblastoma multiforme) [29,30]. At the time of this writing, there is no conclusive evidence that BDV infects humans. BDV is nonetheless a fascinating virus, and its discovery has yielded intriguing models of viral pathogenesis and provided guidance regarding methods for rigorously investigating the role of infection in chronic disease with sensitive molecular tools. It is worth noting that the 2 years of molecular gymnastics required to identify BDV could be collapsed into a few weeks with current art. However, even with the explosion in vial sequence data over the past decade, BDV is sufficiently different that it could not be identified by consensus PCR or microarrays based on sequences other than those representing *Bornaviridae*. To our knowledge, it is unique in this respect.

West Nile virus encephalitis

In late August 1999, health officials reported an outbreak of encephalitis accompanied by profound weakness in Queens, New York. There was neither an apparent increase in the frequency in New York of encephalitis *per se* nor an automatic reporting event that resulted in detection of the outbreak. Thus, the recognition of the syndrome was due to the clinical acumen of Deborah Asnis, an infectious diseases physician at Flushing Hospital Medical Center, and Marcelle Layton, assistant commissioner, Communicable Disease Program New York City Department of Health, and their associates.

On September 3, serology for the presence of antibodies to North American arboviruses yielded results consistent with infection with St. Louis encephalitis virus (SLEV)[31]. SLE had not been previously reported in New York, although mosquito vectors competent for transmission of SLE were present. Investigation of the outbreak epicenter revealed sites of active mosquito breeding and early victims of the outbreak had histories consistent with mosquito exposure. Thus, on September 3, a mosquito eradication program was adopted by the state and city of New York. Concurrently, wildlife observers independently noted increased mortality of avian species including free-ranging crows and exotic birds housed in the Bronx Zoo. Tracy McNamara, a veterinary pathologist at the Wildlife Conservation Society, performed histologic analysis of birds and found meningoencephalitis, gross hemorrhage of the brain, splenomegaly, and myocarditis [32]. Although 70% of emerging infectious diseases are zoonoses and the coincidence between the human and nonhuman outbreaks was striking, McNamara was unable to persuade her colleagues in human infectious disease surveillance to review materials. She forwarded tissue samples from diseased birds to the United States Department of Agriculture (USDA) National Veterinary Service Laboratory in Ames, Iowa, where virus was cultured and electron micrographs reported to be consistent with the presence of either a togavirus or a flavivirus (see also Chapter 7). Thereafter, the avian virus was forwarded from the USDA to the CDC Fort Collins for molecular analysis [33].

On September 13–15, the CDC Encephalitis Project (comprised of centers in California, New York, and Tennessee) held its annual meeting in Albany, New York. Data emerging from both California and New York over an 18-month survey period indicated that an etiological agent was never identified

in 70% of cases of encephalitis despite culture, serology, and molecular analyses. In this context, our group was invited to discuss methods for identification of unknown pathogens and to consider application to project samples of a new method for amplifying viral nucleic acids, domain-specific differential display (DSDD). Sherif Zaki at CDC Atlanta had demonstrated the presence of flavivirus protein in brains of human victims of the New York City outbreak [34]; however, efforts to amplify SLEV or other flaviviral sequences by conventional reverse transcriptase (RT)-PCR had been unsuccessful. Employing several degenerate primer sets designed to target in DSDD highly conserved domains in the NS3, NS5, and 3′-untranslated regions of flaviviruses, we obtained positive results for four of the five New York patients in only a few hours. Sequence analysis confirmed the presence of a lineage 1 West Nile virus [21,22]. Concurrently, our colleagues at the CDC in Fort Collins reported West Nile-like sequences in cell lines infected with homogenates from New York birds. In concert, these findings confirmed that the outbreak in New York City was a zoonosis due to West Nile virus.

Subsequently, we established quantitative real-time PCR assays for sensitive high-throughput detection of virus in clinical materials and mosquito pools. Analysis of blood samples from infected humans revealed the presence of WNV sequences in late 1999 [35]; however, the significance of human-human transmission was not appreciated until 2002, when transmission through organ transplants and blood transfusion led to implementation of blood screening by nucleic acid amplification tests [36,37]. This outbreak illustrates the power of molecular methods for addressing the challenges of emerging infectious diseases and underscores the significance of enhancing communication between the human and comparative medicine communities.

Enteroviruses and amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a disorder characterized by progressive loss of motor neurons and muscle atrophy. An inherited form caused by mutations in the superoxide dismutase gene has been described; however, the majority of cases are idiopathic. In 2000, Berger and colleagues, using nested PCR, sequencing, and *in situ* hybridization methods, reported the striking finding that 15 of 17 French subjects with ALS, and only 1 of 29 subjects with other neurologic diseases, had sequences of a novel echovirus in spinal cord [38]. Although other enteroviruses such as poliovirus and human enterovirus 71 have been unequivocally implicated in acute motor neuron disease, this publication was the first to provide compelling evidence that enteroviruses could cause slowly progressive chronic neurologic disease. Given the potential utility of antiviral treatment of this devastating neurodegenerative disorder, we were encouraged by the National Institute of Neurological Disorders and Stroke (NINDS) to try to independently replicate the echovirus data. Our experience in the BDV field, where problems with PCR hygiene had led to spurious links to disease, was invaluable in directing experimental design. Whereas the Berger group had used RNA template extracted from sections cut on cryostats and analyzed by nested PCR in the same laboratory, we collected frozen tissues from two tissue banks, extracted RNA in a laboratory with no history of virus research, and performed blinded real-time PCR analyses in yet another laboratory. Real-time PCR is similar in sensitivity to nested PCR but is less sensitive to false positive results because assays are performed in a closed system wherein signal is read as fluorescent signal. Analysis of spinal cord and motor cortex from 20 subjects with ALS and 14 controls revealed no echovirus sequences [39]. These results were well-received by colleagues but elicited less salutory correspondence from some individuals who noted that our publication was foreclosing a promising research lead and clinical trials with antiviral drugs.

Bioinformatics: establishment of the Greene Pathogen Database

A critical early step in the development of the MassTag PCR and GreeneChip array tools for

analyzing acute as well as chronic infection on a broader basis was the establishment of a viral sequence database. This effort was facilitated in 2002 by the move of the ICTVdB (International Committee on Taxonomy of Viruses Database, http://phene.cpmc.columbia.edu) and its director, Cornelia Büchen-Osmond, from Biosphere 2 (Earth Institute) in Oracle, Arizona, to the Greene Laboratory; and the establishment of a Northeast Biodefense Center Biomedical Informatics Core. Because vertebrate viruses are highest priority for human disease, we focused on them first, with a plan to later extend the database to viruses of invertebrates, plants, and prokaryotes as resources permitted. To ensure comprehensive coverage, we included every vertebrate virus listed in the ICTV database, a taxonomic database that describes viruses at the levels of order, family, genus, and species (Figure 18.1). Efforts to identify cognate sequences for members of each of these taxa in the GenBank database (NCBI GenBank sequence database) proved to be more difficult than anticipated. The GenBank database is not exhaustively curated; thus, it contains many entries where annotation is missing, outdated, or inaccurate. An additional confound is that only incomplete sequence is available for most viruses. To circumvent limitations in curation and nomenclature in the GenBank database, and to eliminate the need for supercomputing in establishment of multiple alignments at the nucleotide level, we began construction of our Greene Pathogen Database by using the Protein Families database of alignments and Hidden Markov Models (Pfam, http://pfam.sanger.ac.uk). Eighty-four percent of all viral protein coding sequences in GenBank were represented in the Pfam database. The remaining 16% were mapped to this set using the Basic Local Alignment and Search Tool (BLAST). The Greene viral database represents 2011 vertebrate virus species, comprising at the time of this writing 382 512 sequences, that include not only complete genomic sequence but also all subgenomic sequence information available. In order to address also nonviral pathogens, we then supplemented this database with 48 525 16S and 18S rRNA sequences obtained from the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu <http://rdp.cme.msu.edu>) that represent 135 bacterial, 73 fungal, and 63 parasite genera.

MassTag PCR

Real-time PCR methods have changed diagnostic molecular microbiology by providing rapid,

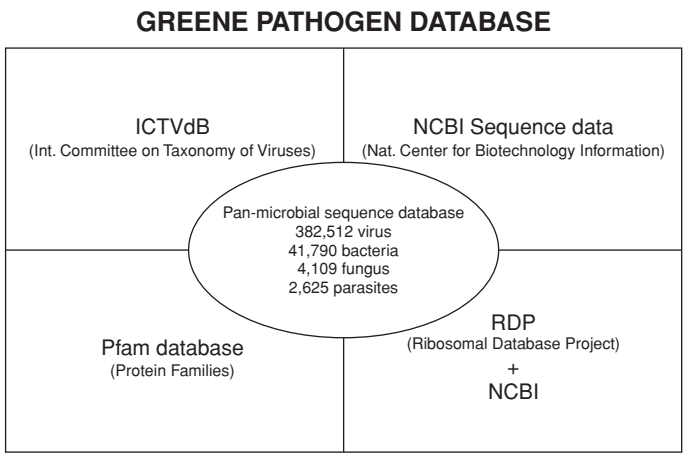

Figure 18.1. Greene Pathogen Database.

sensitive, and specific tools for detecting and quantitating genetic targets. Examples in common use include the management of HIV and HCV. However, the specificity of real-time PCR is both a strength and a limitation. Although the potential for false positive signal is low, so also is the utility of the method for detecting related but not identical genetic targets. Specificity in real-time PCR is provided by the hybridization of two primers combined with a specific reporter probe. The constraints of achieving hybridization at all three sites may confound detection of diverse, rapidly evolving microbial genomes such as those of single-stranded RNA viruses. These limitations can be compensated in part by increasing numbers of primer sets to accommodate various templates. However, because real-time PCR relies on fluorescent reporter dyes, the capacity for multiplexing is limited to the number of emission peaks that can be unequivocally separated. At present, up to four dyes can be identified simultaneously. Alternative, not dye-based multiplex assays rely mostly on formats in which products are distinguished by size, often in combination with secondary enzyme hybridization assays. Gel-based assays are reported that detect two to eight different viral targets with high sensitivity [40,41,42]. Multiplex detection of up to nine pathogens has been achieved in hybridization enzyme systems; however, the method requires cumbersome postamplification processing [43].

To address the need for enhanced multiplex capacity in diagnostic molecular microbiology, we have established a PCR platform based on molecular mass tag reporters that are easily distinguished in mass spectroscopy (MS) as discrete signal peaks. Advantages of the MassTag PCR system include: (1) hybridization to only two sites is required (forward and reverse primer) giving enhanced flexibility in primer design; (2) many published and validated consensus PCR primers can be adapted to MassTag PCR; alternatively primers can be selected from multiple sequence alignments derived from the Greene Pathogen Database using an in-house software program, SCPrimer (http://scprimer.cpmc.columbia.edu); (3) the current repertoire of more than 80 tags allows highly multiplexed assays; additional tags can be synthesized; and (4) sensitivity is comparable to other PCR applications. A limitation of MassTag PCR is that it is unlikely to provide more than a semi-quantitative index of microbe burden. Thus, we view MassTag PCR as a tool with which to rapidly screen clinical materials for the presence of candidate pathogens. Thereafter, targeted secondary tests, including real-time PCR, may be used to quantitate microbe burden and pursue epidemiologic studies.

In MassTag PCR, microbial gene targets are coded by a library of distinct mass tags. A schematic representation of the method is shown in Figure 18.2. Microbial nucleic acids (RNA, DNA, or both) are amplified by multiplex RT-PCR using primers labeled through a photocleavable link with tags of different molecular mass. After removal of unincorporated primers, tags are released through UV irradiation and analyzed by mass spectrometry. The identity of the microbe is determined by the molecular weights of the two tags that were incorporated into the PCR product by the microbe-specific primer pair.

The first description of this method was published in the context of a panel that distinguishes 22 different viral and bacterial respiratory pathogens [44]. It allowed us to identify viral and bacterial sequences in cultured materials as well as clinical respiratory samples and to readily recognize instances of coinfection not appreciated by reference laboratories. During the period October to December, 2004, an increased incidence of influenza-like Illness (ILI) was recorded by the New York State Department of Health that tested negative for influenza virus by molecular testing and negative for other respiratory viruses by culture. Concern that a novel agent might be implicated led us to investigate these clinical materials. MassTag PCR resolved 26 of 79 previously negative samples, revealing the presence of rhinoviruses in a large proportion of samples, about half of which belonged to a previously unrecognized genetic clade [45]. The 2004 New York ILI study confirmed the utility of MassTag PCR for surveillance, outbreak detection, and epidemiology by demonstrating its potential to rapidly query samples for the presence of a wide range of candidate viral and bacterial

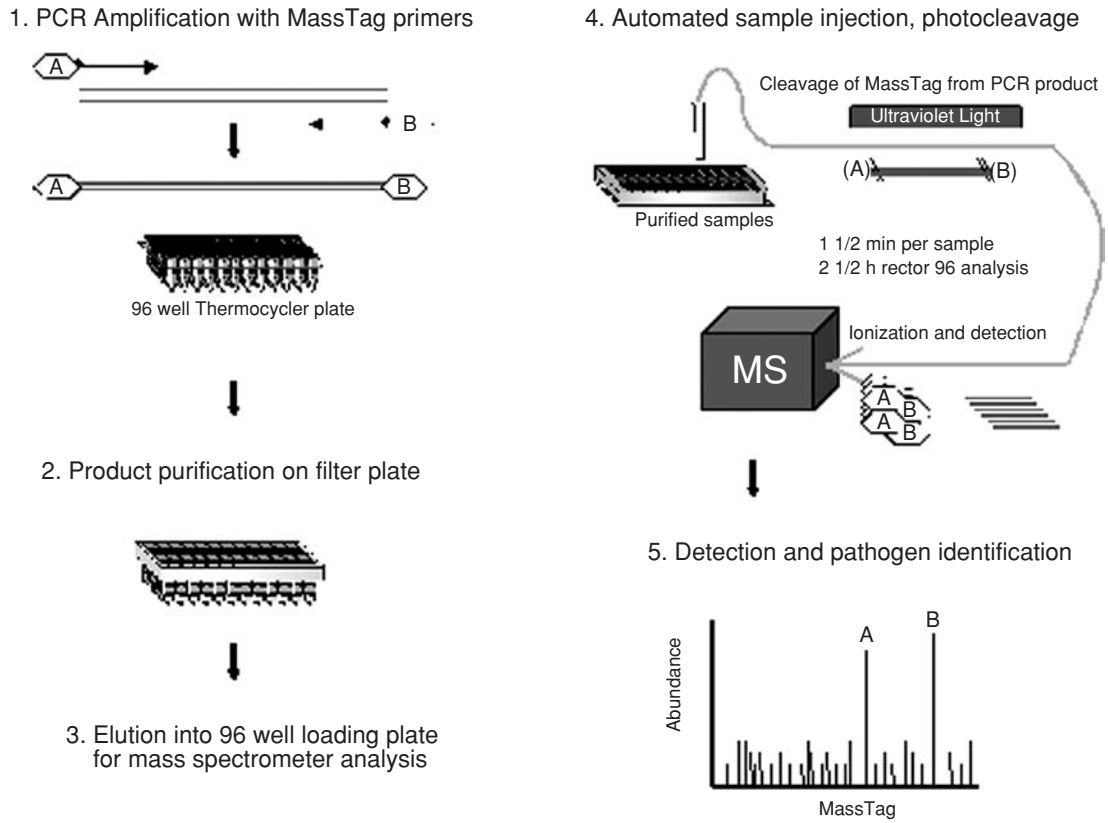

Figure 18.2. Schematic representation of MassTag PCR procedure.

pathogens that may act alone or in concert. We have expanded the repertoire of MassTag PCR primer panels to include causative agents of hemorrhagic fevers, meningoencephalitis, and enteric disease, and to subtype influenza viruses as a tool to expedite detection of new circulating strains and facilitate vaccine development.

GreeneChips

Although substantial viral coverage can be achieved by using several MassTag panels, there are nonetheless instances where larger numbers of known pathogens must be considered, new but related pathogens are anticipated, or there is risk that sequence divergence may impair binding of PCR primers. To address this challenge, we created GreeneChipVR1.5, a viral array uniquely suited to diagnostic use with clinical materials. The GreeneChip has distinct advantages over other array formats we explored.

We initiated viral array studies by spotting 50, 60, and 70 nt oligonucleotides representing a wide range of bunyaviruses and adenoviruses, with and without amino modifications at the 5′-end, onto poly-L-lysine- or epoxy-coated glass slides. No difference between poly-L-lysine or epoxy coatings, or between unmodified 60 or 70 nt oligonucleotide targets, was appreciated. However, hybridization signal improved with the increase in target length from 50 to 60 nt for unmodified, and further when amino-modified oligonucleotides were used. The enhanced

signal obtained with aminomodification is presumably due to a controlled binding of the target to the slide at one end of the molecule, such that the remainder of the target is free for hybridization. Unfortunately, the cost for amino modification becomes prohibitive with increasing size of the target library. Thus, we produced our first array using 60 nt oligonucleotides representing 1–2 sequences for each of 1710 vertebrate viruses in the ICTV database. This array of 3418 targets was used to establish conditions for amplification, labeling, and detection of viral sequences in clinical materials. A subset of targets in this slide was designed to include mismatches to bona fide viral sequences at the 5'- or 3′-end or interspersed throughout the target. Analysis of these chimeric targets revealed that under standard conditions, hybridization signal could be achieved with up to 8-nt mismatches randomly distributed throughout the target molecule. Tolerance was higher still with terminal mismatching. Based on these experiments, we now conservatively design oligonucleotides such that each array contains targets that address known viral sequences with no more than 4-nt mismatches.

Oligonucleotides for the next generation of arrays were selected to represent at least three genomic regions for every family or genus of vertebrate virus in the Greene Virus Database. Where possible, we chose highly conserved regions within coding sequence for an enzyme such as a polymerase and two other regions corresponding to structural proteins. Our reasoning was that RNAs encoding structural proteins may be present at higher levels than those encoding proteins needed only in catalytic amounts. Further, a use of targets representing noncontiguous sites along the genome might allow detection of naturally occurring or engineered chimeric viruses. We ultimately selected a total of 9066 oligonucleotides (60 mers) that cover all 2011 species of vertebrate viruses in 3 gene regions with 4 or fewer nucleotide mismatches to create GreeneChipVR1.1 in collaboration with Agilent Technologies. GreeneChipVR1.1 had several advantages: (1) at 9066 viral targets it was more complex than the first-generation array; (2) the Agilent printing technology allowed *in situ* synthesis of oligonucleotides at a right angle with respect to the planar slide surface to allow optimal exposure for hybridization; (3) the fidelity and reproducibility of printing density was markedly improved over simple oligonucleotide spotting; (4) the option to produce small batches of 20 slides facilitates easy modification of the target set to include new sequences.

Later versions of the array included Greene ChipVR1.5, GreeneChipPm, and GreeneChipResp. GreeneChipVR1.5 provides optimized coverage of all vertebrate viral sequences to allow improved detection of related but not yet characterized viruses. GreeneChipPm is a panmicrobial array that addresses both viral and nonviral pathogens. Greene ChipResp allows detection of all respiratory viruses and typing of all influenza strains.

GreeneChip probe preparation, hybridization, and labeling

Sensitivity is critical to implementation of arrays in pathogen detection and has long been a hurdle with investigating clinical materials. Thus, we invested considerable effort in establishing a robust universal amplification method for the GreeneChip platform. Efficiency of each individual step of the protocol has been monitored and optimized using spiked samples and quantitative real-time PCR. First-strand reverse transcription is initiated with a random octamer linked to a specific, artificial primer sequence in a two-step RT-PCR protocol using DNase treated RNA that, if necessary, can be subtracted for ribosomal RNA sequences (RiboMinus RNA). After reverse transcription and an RNase H digestion step, the cDNA is amplified in the second reaction using a 1:9 mixture of the above primer and a primer targeting the attached specific, artificial primer sequence. Second-strand synthesis is initiated at low annealing temperature (35°C) to allow priming via the random octamer during initial PCR amplification cycles; subsequent cycles use a stringent annealing temperature (55°C) to favor priming through the introduced specific, artificial primer sequence. Products of this PCR are then subjected to a "labeling" PCR reaction.

The "labeling" PCR employs the specific primer sequence to introduce into amplification products a capture sequence for 3DNA dendrimers that contain more than 300 fluorescent reporter molecules (Genisphere Inc.). The use of dendrimer labeling provides a 100x gain in sensitivity over microarray labeling methods where reporter molecules are directly incorporated into amplification products. In concert, these modifications allow for the detection of 2011 known viruses or related viruses with a sensitivity of less than 1000 molecules.

GreeneLAMP analysis software and GreeneChip validation

GreeneLAMP (Log-transformed Analysis of Microarrays using P-values) version 1.0 software was created to assess results of GreeneChip hybridizations. Common analysis software focuses on the differential two-color analysis used in gene expression arrays, which is not applicable to the GreeneChip. GreeneLAMP has a robust and generalized framework for microarray data analysis including: flexible data loading, filtering, and control experiment subtraction. Probe intensities are background corrected, $\log_2$-transformed, and converted to Z-scores (and their corresponding p-values). Where available, control matched experiments from uninfected samples are used and spots >2 standard deviations (SD) from the mean are subtracted. In instances where matched control samples are not available, the background distribution of signal fluorescence is calculated using fluorescence associated with 1000 random 60-mers (null probes). In both scenarios, positive events are selected by applying a false positive rate of 0.01 (the rate at which null probes are scored as significant) and a minimum p-value per probe of 0.1 (in cases with a matching control) and 0.023 (2 SD; in cases without a matching control). A map, built from a BLASTN alignment of targets to the Greene Pathogen Database, is used to connect probe sequences to the respective entries in the Greene Pathogen Database. Each of those sequences corresponds to an NCBI Taxonomy ID (TaxID). The individual TaxIDs are mapped to nodes in a taxonomic tree built based on ICTV virus taxonomy or the NCBI taxonomic classification for other organisms. The program output is a ranked list of candidate TaxIDs. Candidate TaxIDs are ranked by combining the p-values for the positive targets for that TaxID using the QFAST method of Bailey and Gribskov [46].

Specificity of the viral GreeneChip was assessed using extracts of cultured cells infected with adeno-, alpha-, arena-, corona-, entero-, filo-, flavi-, herpes-, orthomyxo-, paramyxo-, pox-, reo-, and rhabdoviruses (a total of 49 viruses). All were accurately identified by GreeneLAMP analysis. To assess the sensitivity of GreeneChip detection, viral RNA extracted from infected cell supernatants (adeno-, West Nile, St. Louis encephalitis, respiratory syncitial, entero-, SARS corona-, and influenza virus) was quantitated by real-time PCR, serially diluted, and subjected to GreeneChip analyses. The threshold of detection for adenovirus was 10 000 RNA copies; the threshold of detection for the other reference viruses was 1000 RNA copies per RT reaction. The respiratory GreeneChip was tested for detection and typing with 31 influenza virus A and B reference strains of human and animal origin and, because reference strains represent only a limited fraction of the genetic variability, with numerous circulating human influenza virus strains isolated worldwide since 1999. In summary, a total of 69 viruses comprising 54 influenza virus A and B isolates of human, avian, and porcine origin; and 15 noninfluenza virus human respiratory viruses were tested, identified, and subtyped.

GreeneChips were also validated with clinical samples from patients with respiratory disease, hemorrhagic fever, tuberculosis, and urinary tract infections, and were demonstrated to identify human enterovirus A, human respiratory syncytial virus A, influenza A virus, Lake Victoria Marburg virus, severe acute respiratory syndrome coronavirus, lactobacillus, mycobacteria, and gammaproteobacteria in various specimen types, including cerebral spinal fluid, nasopharyngeal swabs, sera/plasma, stools, and urine. Investigation by panmicrobial GreeneChip of blood collected during the 2005 Angola Marburg virus outbreak from an individual who died of

hemorrhagic fever but tested negative in MassTag PCR and viral GreeneChip analysis identified infection with *P. falciparum* as the probable cause of death [47].

Unbiased high throughput sequencing

The advent of high throughout sequencing technology affords unique opportunities for pathogen discovery. Unlike consensus PCR or array methods where investigators are limited by known sequence information and must make choices regarding the range of pathogens to consider in a given experiment, high throughput sequencing is unbiased. Several systems are in development. We have experience with the pyrosequencing system of 454 Life Sciences; however, the principles for sample preparation and data analysis are broadly applicable across platforms. Because all nucleic acid in a sample (whether host or pathogen) is amplified and sequenced, elimination of host nucleic acid can be critical to boosting pathogen signal toward the threshold for detection. Our approach is to apply a similar sample preparation and random PCR amplification protocol as developed for the GreeneChip, including extensive DNase I treatment of the RNA template to remove host chromosomal DNA. This process obviates the potential for detecting DNA genomes of pathogens; however, our reasoning is that an active infection should be associated with transcription. After amplification and sequencing, reads typically range in size from 40 to 400 base pairs. Raw sequence reads are trimmed to remove sequences derived from the amplification primer and filtered to eliminate highly repetitive sequences. After trimming and eliminating repeats, sequences are clustered into nonredundant sequence sets. Unique sequence reads are assembled into contiguous sequences that are then compared to the nonredundant sequence databases using programs that examine homology at the nucleotide and amino acid levels (using all six potential reading frames with adjustments for sequence gaps). Specific PCR tests are then designed to examine association with disease, measuring burden, and obtaining additional sequence for phylogenetic characterization.

A staged strategy for pathogen detection and discovery

We employ a repertoire of complementary platforms in a staged strategy for pathogen detection and discovery that balances costs, breadth, and sensitivity (Figure 18.3). Whereas multiplex MassTag PCR-based assays offer the highest sensitivity and throughput at lowest cost, array-based platforms offer unprecedented breadth in coverage. Samples are initially screened using MassTag PCR. GreeneChip analyses are used in the event that MassTag PCR is not informative or where larger numbers of pathogens must be considered. In the event that neither MassTag nor GreeneChip analysis results in identification of a candidate pathogen, samples are submitted for either unbiased high throughput sequencing or subtractive cloning. Candidates identified by any of these methods are further characterized by standard dideoxy sequencing and the deployment of specific assays (quantitative PCR, serology) for epidemiology and surveillance. New sequences are added to the database and queued for primer and target design.

Future perspectives

Technologies will continue to evolve, allowing faster, more sensitive and less expensive methods for pathogen surveillance and discovery. Although multiplex PCR is relatively mature, microarray technology is still in its infancy; near-term modifications already in development include microfluidic sample processing and direct measurement of conductance changes associated with hybridization. We have only touched the surface of proteomics and host response profiling. It is conceivable that biomarkers will be found that are specific for classes of infectious agents and/or provide insights that can guide clinical management. In chronic diseases the most

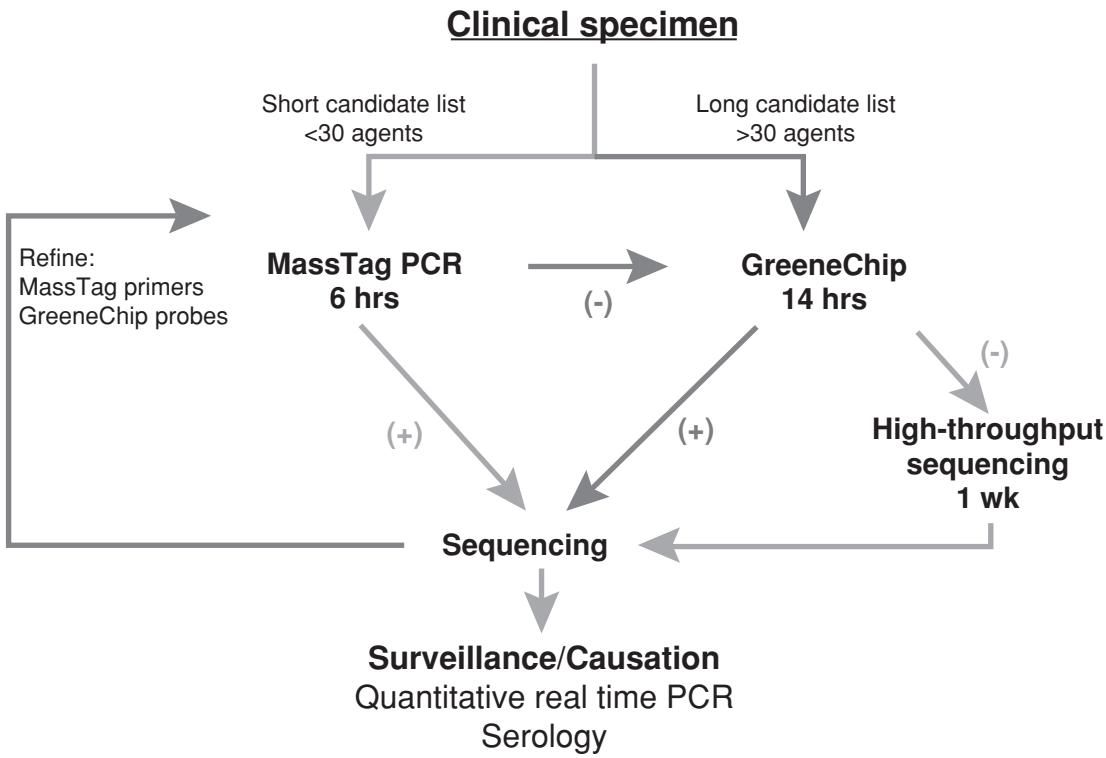

Figure 18.3. A staged strategy for pathogen detection and discovery.

substantive advances are likely to come not from technical improvements but from investments in prospective serial sample collections and an appreciation that many diseases reflect intersections of genes and environment in a temporal context.

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19

Clinical management of viral encephalitis

Kenneth L. Tyler and Donald H. Gilden

Introduction

Most cases of viral encephalitis are acute, although a few viruses can cause chronic progressive encephalitis. Rarely, systemic virus infection may trigger postinfectious encephalomyelitis. Viral encephalitis typically reflects viral invasion of the brain parenchyma. Encephalitis patients usually have alterations in their state of consciousness. Some viruses produce "diffuse" encephalitis in which the predominant features are impaired consciousness, signs of generalized central nervous system (CNS) dysfunction such as generalized seizures, and a cerebral spinal fluid (CSF) pleocytosis. Conversely, other viruses produce "focal encephalitis," in which altered consciousness and CSF abnormalities are accompanied by prominent focal abnormalities on neuroimaging tests or clinical examination including hemiparesis, aphasia, hemisensory loss, ataxia, focal as well as generalized seizures, and, less often, involuntary movements, visual field defects, and cranial nerve deficits. Personality changes, language, and memory disturbances and psychotic features are frequent. Viral encephalitis must be distinguished from nonviral conditions that can present a similar clinical picture, including Lyme disease, tuberculosis, syphilis, Listeria, Mycoplasma, fungal and parasitic infections, brain abscess, subdural hematoma or abscess, brain tumors, CNS vasculitis, and toxic/metabolic encephalopathies.

Viral encephalitis may be epidemic or sporadic (see also Chapter 17). Causes of epidemic viral encephalitis include the togaviruses, enteroviruses (see Chapter 1), mumps and lymphocytic choriomeningitis (LCM) virus (see Chapter 5). The togaviruses are RNA viruses transmitted by mosquitoes or ticks (arthropod-born) (see Chapters 6, 7, and 20), with a peak incidence in the Northern Hemisphere in the warm summer months. This group includes West Nile virus, St. Louis, Eastern and Western equine viruses, Japanese and California encephalitides (see Chapters 6 and 7). Of these, Eastern equine encephalitis is associated with high mortality and morbidity, whereas the rest of the arboviruses have a milder course. The enteroviruses include Coxsackie and ECHO viruses and cause encephalitis during the summer and early fall. Serious sequelae are uncommon. Mumps encephalitis is more common in the winter and spring. LCM is more common in the fall and winter. Sporadic cases of encephalitis are caused by six of the human herpesviruses, primarily type 1 herpes simplex virus (HSV-1) (Chapter 11) and rarely HSV-2, Epstein-Barr virus (EBV), cytomegalovirus (CMV), and human herpesvirus 6 (HHV-6), while varicella-zoster virus (VZV) (Chapter 12) usually produces a uni- or multifocal vasculopathy. The CNS infections most amenable to antiviral treatment are those caused by some of the human herpesviruses. While the neurological symptoms and signs of encephalitis caused by the human herpesviruses are similar, the brain imaging abnormalities seen in HSV-1 encephalitis, VZV vasculopathy, and CMV encephalitis differ markedly and provide important information that assists in identifying the etiologic agent (Figure 19.1). Although there is no specific treatment for most

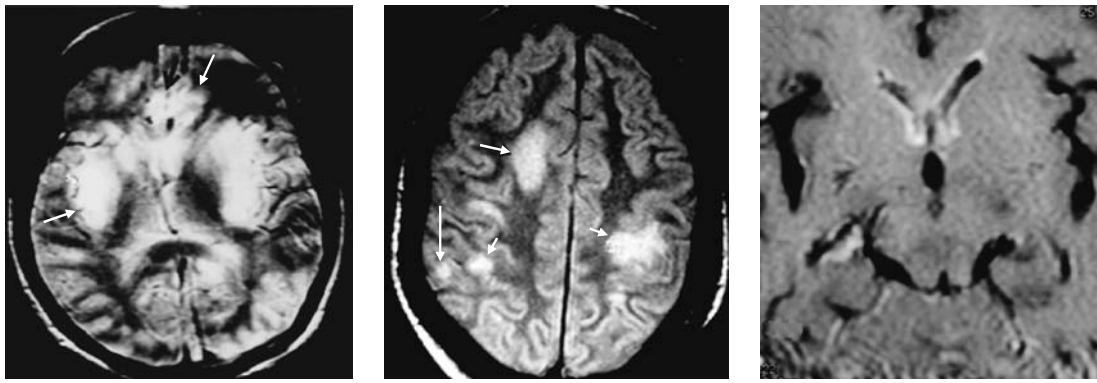

Figure 19.1. Brain imaging abnormalities in CNS virus infections that can be treated with antiviral agents.
(A) Herpes simplex virus-1 encephalitis. Temporal lobe (bottom arrow) and cingulate gyrus (top arrow) involvement.
(B) Varicella-zoster virus vasculopathy. Infarction typically more common in white matter (top arrow), particularly at gray-white matter junctions (short arrows), less frequently in gray matter (long arrow) and may enhance.
(C) Cytomegalovirus encephalitis. Characteristic enhancement in ependyma around lateral ventricles.

viral encephalitides, a few have definitive treatment. Herein, we focus on both the supportive care required in all viral encephalitis as well as specific treatment for encephalitis caused by some herpesviruses, a few RNA viruses, and post-infectious encephalomyelitis.

Viral encephalitis

Treatment

CNS infections by most viruses have no specific treatment. General supportive measures are described below. Nevertheless, there is specific treatment for encephalitis caused by some of the human herpesviruses and post-infectious encephalomyelitis. Prophylactic treatments are available for rabies infection before CNS invasion occurs. There are also possible options to treat CNS infection by enteroviruses and togaviruses.

General supportive measures

The patient should be at bed rest. Isolation procedures should be followed until the cause of the illness is established. The patient's airway should be protected by repeated suctioning and, in some cases, endotracheal intubation or tracheostomy. Nutritional support is important, but only conscious patients with intact brain stem function should be fed orally. All others should be fed by parenteral or nasogastric techniques. Patients should be watched closely for secondary infections, especially of the urinary tract and lungs. Passive range-of-motion exercises and use of foot boards or Styrofoam boots will minimize contractures. Frequent turning decreases the risk of bed sores. Appropriate prophylactic measures should be utilized to prevent development of deep venous thrombosis. Patients with encephalitis may develop an inappropriate secretion of antidiuretic hormone and become water-intoxicated, which can lead to deepening coma and sometimes seizures. Serum and urine electrolytes and urine output should be monitored. Treatment is with fluid restriction and rarely with hypertonic saline. Headache, a frequent problem, is treated with acetaminophen, ibuprofen, codeine, and occasionally, meperidine. Fever is treated with acetaminophen.

Seizures

Seizures may occur in patients with viral encephalitis. Initial seizures are treated with diazepam given 2 mg per minute intravenously for a maximum total dose of 15–20 mg, or with lorazepam given 2 mg per minute intravenously for a maximum total dose of 5–10 mg, followed by a loading dose of phenytoin at 18–20 mg per kg given intravenously no faster than 50 mg per minute. Maintenance doses of phenytoin range from 300 to 400 mg per day in divided doses. Therapy is guided by blood levels, side effects, and absence of seizures. Anticonvulsants should be continued for several months after the acute illness.

Cerebral edema

Most cerebral edema that occurs in patients with viral encephalitis is not life-threatening but can be a serious problem in patients with HSV encephalitis. Patients with increased intracranial pressure (ICP) are best treated in an intensive care unit with ICP monitored using an intracranial pressure bolt. Cerebral edema is treated emergently by intubation and hyperventilation to bring CO_2 partial pressure to 25 mmol per liter. This reduces increased intracranial pressure (ICP) by causing constriction of the intracranial vasculature. After 24–48 hours, hyperventilation is less effective. Dexamethasone should be administered intravenously at an initial dose of 10 mg, then 4–8 mg every 4–5 hours for the next 3 days. The benefit in management of increased ICP is greater than the risk of steroids potentiating virus infection. Total fluid intake should be reduced to one-half to two-thirds of maintenance, and the patient's head should be elevated to 30°. Incipient herniation may be managed with osmotic diuretics. Mannitol is given in repeated doses of 0.25–2 grams per kg. The serum osmolality should be monitored closely and kept below 310 mOsm per liter. Mannitol's effectiveness decreases with repeated use, and rebound increases in ICP may occur. Rare patients may require ventriculostomy or cranial decompression surgery to reduce ICP refractory to other forms of management.

Herpes simplex virus (HSV)

HSV encephalitis in adults

HSV encephalitis (see also Chapter 11) was the first viral CNS infection for which specific therapy with antiviral drugs was attempted [1]. Unfortunately, the agent initially tested, 5-iodo-2′-deoxyuridine, had unacceptably high toxicity [2]. In 1977, the Collaborative Antiviral Study Group (CASG) conducted a placebo-controlled, double-blind randomized multicenter clinical trial of intravenous vidarabine (adenine arabinoside, 15 mg/kg/day for 10 days) in patients with biopsy-verified HSV encephalitis [3]. Vidarabine reduced mortality at 1 month from 70% (7/10) to 28% (5/18) ($p = 0.03$). Of the 13 vidarabine-treated survivors, 7 had only minor or moderate sequelae and 4 patients ultimately recovered completely. Of the remaining 6 treated patients who were alive at 1 month but had severe sequelae, 3 subsequently died, bringing the overall mortality in the vidarabine-treated group to 44% at 1 year. The beneficial results of vidarabine were supported in a subsequent uncontrolled, open-label study including 75 vidarabine-treated patients, for whom mortality was 33% at 1 month and 39% at 1 year; among survivors of the therapy, 54% were reportedly normal at 1 year, 29% moderately debilitated, and 18% severely impaired [4].

In 1986, the CASG conducted a second double-blind, randomized controlled trial to compare the effect of vidarabine (15 mg/kg/day for 10 days) with that of acyclovir (30 mg/kg/day for 10 days) in patients with biopsy-verified HSV encephalitis [5,6]. Mortality at 18 months in the acyclovir-treated patients was 28% (9/32) compared to 54% (19/37) in the vidarabine-treated group. The vidarabine mortality rate was identical to that in a larger cohort pooled from several different trials (54%, 70/129) [5,6]. Six months after infection, 38% of patients who received acyclovir, but only 14% of those who received vidarabine were functioning normally [5]. In acyclovir-treated patients, mortality was influenced by both the patient's level of consciousness (Glasgow coma score, GCS) at the time of institution of

therapy and by the age of the patient. For example, 100% (8/8) of acyclovir-treated patients with a GCS >10 survived, compared to only 75% of those with a GCS of 7–10. Only 6% (1/18) of acyclovir-treated patients younger than 30 years died compared to 36% (5/14) of older patients [5,6]. Nearly identical results were obtained in a Swedish multicenter study comparing acyclovir and vidarabine [7]. Mortality in that study was 19% (5/27) in acyclovir-treated subjects compared to 50% (12/24) in those receiving vidarabine. At 12 months, 64% (14/22) of acyclovir-treated survivors had no or only minor sequelae compared to 25% (3/12) of vidarabine-treated survivors. Level of consciousness at start of treatment was again identified as a prognostic factor. At 12 months, 59% (10/17) of acyclovir-treated patients who were lethargic when therapy was instituted were alive with no or minor sequelae compared to only 40% (4/10) of those who were comatose or semicomatose when treatment was started.

The CASG and Swedish multicenter trials established intravenous acyclovir as the standard of care in the treatment of HSV encephalitis in adults. Although both these trials evaluated 10-day treatment regimens, the use of longer courses (14–21 days) was recommended in later consensus guidelines [8,9,10]. It has been suggested that longer courses of therapy reduce the likelihood of disease relapse or recurrence. But relapse in HSV encephalitis patients after 10 days of acyclovir therapy is unusual in adults [11], occurring in only 4% (2/53) of cases in the Swedish multicenter trials [7] and 7% (4/56) of cases in the CASG study [5]. In contrast, recurrence after HSV encephalitis in children may be as high as 26% (7/26) [12].

Despite the recommended acyclovir treatment for HSV encephalitis patients, treatment is unfortunately often delayed. In a retrospective review of patients presenting to the emergency room with symptoms and signs consistent with a "high suspicion of viral encephalitis or acute viral encephalitis or acute meningoencephalitis" (defined as fever >38°C, abnormal neurological status), and a CSF pleocytosis with a negative gram stain [13], only 29% (7/24) received empiric acyclovir in the emergency department (mean delay, 1.5 hours; range 0–3.1 hours, 95% CI), while the remaining 71% (17/24) did not receive acyclovir until after admission (mean delay, 16 hours; range 7.5–44 hours, 95% CI). Five of the 24 patients were ultimately found to have HSV encephalitis, but only 2 of them had received treatment in the emergency department. This study indicates that empiric treatment of encephalitis with acyclovir remains suboptimal, since a subset of these patients will have HSV encephalitis.

Patients surviving HSV encephalitis even after treatment with 14–21 days of acyclovir (10 mg/kg 3x/day) often have significant neurological sequelae, mainly cognitive impairment and seizures [14]. CASG is conducting a phase III placebo-controlled, randomized multicenter trial (CASG 204) to determine whether oral valacyclovir (2 gm 3x/day) for an additional 90 days after completion of a 14–21 day course of intravenous acyclovir increases survival and reduces the frequency of neurological sequelae including cognitive impairment in HSV encephalitis. To date, the study has recruited about one-third of the expected total enrollment of 132 patients (http://www.clinicaltrials.gov/ct/show/NCT00031486?order = 1).

Valacyclovir and famciclovir are orally available antiviral drugs with efficacy against HSV. After oral administration, valacyclovir is hydrolyzed to acyclovir and L-valine. At an oral dose of 2000 mg 4x/day, valacyclovir produces "area under the curve" (AUC) serum levels similar to those achieved by intravenous administration of acyclovir 10 mg/kg 3x/day, and a valacyclovir dose of 1000 mg 4x/day achieves AUC serum levels similar to those achieved with 5 mg/kg 3x/day of intravenous acyclovir [15,16]. Despite this excellent oral bioavailability profile, it is important to emphasize that oral valacyclovir has never been tested for efficacy in initial primary treatment of HSV encephalitis. There is one report of a 12-year-old Chinese boy with HSV encephalitis who recovered uneventfully after receiving 11 days of intravenous acyclovir followed by 10 days of oral valacyclovir (1000 mg 3x/day) when acyclovir had

to be discontinued due to severe skin reaction at the infusion site [17].

In addition to antiviral therapy, patients with HSV encephalitis are often treated with corticosteroids to reduce cerebral edema. In a mouse model of HSV encephalitis, the combination of acyclovir and methylprednisolone reduced the severity of magnetic resonance imaging (MRI) abnormalities and did not increase HSV load compared to acyclovir alone [18]. Dexamethasone in combination with acyclovir also did not increase HSV replication or dissemination compared to treatment with acyclovir alone in a rat model of HSV encephalitis [19]. Although those studies suggest that steroids combined with acyclovir are not likely to reduce the efficacy of antiviral therapy and may reduce injury from HSV encephalitis, their safety and efficacy in human HSV encephalitis remain unknown. A small uncontrolled study of the effects of pulse steroid therapy (methylprednisolone 1000 mg/day for 3 days, repeated every 2 weeks if no improvement) in five patients with acute viral encephalitis included one patient with definite and one with presumed HSV encephalitis [20]. The patient with presumed HSV encephalitis started his steroid pulse on day 4 of illness and within 24 hours showed a dramatic and sustained improvement in GCS from 9 to 15. The patient with definite HSV encephalitis did not receive steroids until day 22 of illness and showed no improvement until 1 week after initiation of therapy, when GCS score rose progressively from 6 to 15.

A second nonrandomized retrospective study of factors predicting outcome in 45 patients with HSV encephalitis [21] found that absence of corticosteroid therapy predicted a poor prognosis (moderate or severe sequelae or death) in both single (odds ratio 3.5, 95% CI 1–12) and multiple logistic regression analysis (odds ratio 9, 95% CI 1–71). Of 22 patients who received corticosteroids, 82% received dexamethasone with a median dose equivalent to 64 mg/day of prednisolone (mean duration ~14 days, median 6 days). In the same study, other predictors of poor outcome were patients' increasing age and depressed GCS score at time of initiation of acyclovir treatment. Detection of a lesion on initial computed tomography examination was a predictor of poor outcome in single but not multiple logistic regression analysis. Together, these studies suggest that corticosteroid therapy is unlikely to be harmful and may be of benefit when combined with acyclovir to treat HSV encephalitis. However, definitive recommendations about the role of steroids in therapy await randomized controlled trials.

There has been one prospective double-blind trial of interferon-β in combination with acyclovir in the treatment of focal encephalitis in children [22]. In 14 patients with proven encephalitis, there was no difference in outcome between patients receiving interferon-β plus acyclovir compared to those receiving acyclovir alone; in each regimen, 71% (5/7) had a good outcome.

Rare patients with HSV encephalitis have undergone dramatic neurosurgical decompressive procedures to reduce the potential effects of brain swelling or herniation [23,24,25]. Although these treatments appear to have been lifesaving in individual cases, there are no controlled trials establishing efficacy, and the indications for their use and the nature of the procedures used has varied with the individual cases.

Neonatal HSV infection

Neonatal HSV infection differs in many respects from HSV encephalitis encountered in older children and adults. Neonatal infection is typically acquired during delivery, but in some cases also occurs in utero or post-natally [26]. The risk of neonatal HSV infection is generally low in children born to women with only a history of genital herpes (about 3%), but may be as high as 50% in infants born to mothers who develop primary genital herpes in the third trimester [26]. Infection generally falls into one of three patterns: (1) disease localized to the skin, eyes, and mouth (SEM) (18%), (2) CNS disease with or without SEM involvement (34%), and (3) disseminated infection involving visceral organs and the CNS (48%) [26]. Vidarabine was the first drug

shown to be effective in reducing the morbidity and mortality of neonatal HSV infection [27,28]. A later controlled clinical trial established that intravenous acyclovir (10 mg/kg 3x/day for 10 days) was as efficacious as vidarabine in reducing morbidity and mortality in neonatal HSV infections [29], and replaced vidarabine as the treatment of choice. A subsequent clinical trial to investigate the safety and efficacy of higher doses of acyclovir given for 21 days in comparison with the original 10-day course of 10 mg/kg 3x/day [30] revealed a 31% mortality rate for neonates with disseminated HSV disease treated with "high-dose" acyclovir (20 mg/kg 3x/daily for 21 days) as compared to 57% mortality in those treated with "intermediate dose" acyclovir (15 mg/kg 3x/daily for 21 days), and a 61% mortality in historical controls treated with standard-dose acyclovir (10 mg/kg 3x/day for 10 days) ($p = 0.006$ for high-dose vs. standard-dose). In patients with CNS disease, mortality was 6% in the high-dose acyclovir group compared with 20% in the intermediate-dose group and 19% in historical controls, but the differences were not statistically significant. The percentage of children developing normally at 12 months after illness was higher in those treated with high-dose acyclovir compared to standard therapy in neonates with disseminated disease (83% vs. 60%) and CNS disease (31% vs. 29%), but the differences were not statistically significant and the sample numbers in the groups were relatively small. Patients treated with high-dose acyclovir had a higher frequency of neutropenia (absolute neutrophil counts $\leq 1000/mm^3$), but this did not result in any adverse sequelae and resolved either during continuation of acyclovir therapy or after its cessation. Because high-dose acyclovir showed a significant effect in reducing mortality in disseminated disease, a 21-day course of high-dose acyclovir (20 mg/kg 3x/day) has now become the generally recommended standard of care for patients with either disseminated disease or CNS disease. A 14-day course of acyclovir may be adequate for patients with only SEM disease [26].

A phase I/II trial examined the effect of suppressive oral acyclovir therapy (300 mg/m^2 either 2 or 3x/daily) after intravenous therapy for cutaneous recurrences in neonates with SEM disease [31]. Unfortunately, the number of subjects available for efficacy analysis was small (16 in the 3x daily and only 2 in the 2x daily treatment groups). In patients who received oral acyclovir 3x/day, 81% (13/16) were recurrence-free compared to 54% of historical controls who did not receive suppressive therapy. Patients receiving acyclovir 2x/daily had recurrences. However, 46% of the infants receiving acyclovir developed neutropenia (absolute neutrophil count $\leq 1000/mm^3$). Given the frequency of associated neutropenia and the lack of unequivocal data supporting efficacy, oral suppressive therapy for neonatal HSV infection is not currently recommended [26].

HSV meningitis

There are no randomized placebo-controlled trials of the treatment of either single or recurrent episodes of HSV meningitis with antiviral drugs. Case reports and non-controlled case series describe the use of acyclovir for treatment of HSV meningitis [32,33,34]. There has been great variability in the regimens used, with therapy ranging in intensity from oral drugs alone (e.g., acyclovir 200 mg 3–4 times/day for 7 days) to intravenous acyclovir (5–10 mg/kg 3x/day for 7–10 days) [32,35]. Nonetheless, it has been suggested that prophylactic acyclovir may reduce the frequency of attacks of recurrent HSV meningitis in both immunocompetent and immunocompromised adults [32,33,36].

HSV-2 causes meningitis not only in immunocompetent adults but also in immunocompromised adults, including patients with advanced HIV infection and patients receiving chemotherapy [36]. These patients are often treated more intensively than immunocompetent patients with HSV meningitis. For example, in a recent study of 13 severely immunocompromised patients, 85% (11/13) received antiviral therapy (10 with acyclovir and 1 with foscarnet). Although the treatment regimen varied among patients, a typical regimen was intravenous acyclovir 10–15 mg/kg 3x/day for a

median of approximately 14 days (range 3–35 days). Since the study was not blinded and uncontrolled, its efficacy could not be evaluated.

HSV myelitis and brain stem encephalitis

HSV can cause monophasic or recurrent brain stem encephalitis [37,38,39] and myelitis [40,41,42]. Almost all descriptions of these syndromes involve single case reports, so it is not possible to evaluate the efficacy of antiviral therapy in these noncontrolled studies. Because of the severity of these infections, treatment has generally paralleled that utilized for HSV encephalitis. In patients with recurrent disease, valacyclovir or acyclovir has been used as prophylactic therapy with the goal of reducing recurrences [37,39].

Varicella-zoster virus (VZV)

VZV vasculopathy and myelitis

Treatment is intravenous acyclovir (10–15 mg/kg every 8 hours for 10–14 days for adults (Table 19.1), and 500 mg/m^2 body surface area for children for 7 days (see also Chapter 12). Such therapy has led to improvement of patients with VZV myelitis, one of whom had AIDS [43], and was effective in one patient who developed protracted vasculopathy 6 months after zoster [44]. In another subacute case of VZV vasculopathy, serial polymerase chain reaction (PCR) for VZV DNA not only confirmed the diagnosis but also guided the duration of therapy [45]. The role of antiviral therapy in individuals presenting with rarer complications of varicella, such as cerebellar ataxia, has not been studied in a prospective or controlled fashion; however, administration of intravenous acyclovir to such patients is likely to be appropriate. Immunocompromised patients may require longer treatment. Treatment should be discontinued if both VZV DNA and anti-VZV antibody are absent in CSF collected at the time of initiation of treatment. Steroid therapy (prednisone 60–80 mg daily for 3–5 days) should be considered in VZV vasculopathy to reduce inflammation in the CNS.

Cytomegalovirus (CMV)

CMV encephalitis and myelitis in adults

There have been no randomized controlled studies of antiviral therapy in adults with CMV infection of the CNS. Although there are reports of beneficial effects in uncontrolled trials in individual patients or in retrospective case reviews [46,47,48], there is no strong evidence that either foscarnet, ganciclovir, or a combination of the two agents prolongs survival in CMV encephalitis. One nonblinded, noncontrolled prospective study in patients with advanced HIV infection in the era before highly active antiretroviral therapy (HAART) examined the efficacy of combined ganciclovir and foscarnet treatment in 17 patients with acute CMV encephalitis and 14 with acute CMV myelitis [49]. Patients were treated with a combination of intravenous foscarnet (90 mg/kg 2x/day) and ganciclovir (5 mg/kg 2x/day) for 3 weeks (induction therapy) followed by once daily maintenance therapy with both drugs. A response to induction therapy was seen in 71% (12/17) of encephalitis patients and 79% (11/14) of the myelitis patients. Partial, rather than complete, responses predominated and accounted for 100% of responses in encephalitis cases and 82% in the myelitis cases. Of the initial 23 responders, 43% (10/23) subsequently had disease progression (relapse) after a median of 126 days. Relapses occurred earlier in encephalitis (median 101 days) compared to myelitis (median 225 days) cases. Despite treatment, survival was poor in all patients, with a mean and median of 165 and 124 days, respectively, for encephalitis cases and 262 and 114 days, respectively, for myelitis cases. The generally poor results of treatment in CMV encephalitis and myelitis/polyradiculopathy in this series are mirrored in isolated case reports, although there have been exceptions [50,51].

There are isolated case reports of the successful use of cidofovir in patients with AIDS-related CMV

Table 19.1. Antiviral treatment of human herpesvirus infection of the nervous system

Viral infection	Clinical diagnosis	Drug	Dose	Comment
Herpes simplex	Encephalitis	Acyclovir	10 mg/kg IV q8 hr for 14–21 days	Monitor renal function
	Meningitis	Acyclovir	10 mg/kg IV q8 hr for 5–7 days	HSV-2, not HSV-1, usually causes meningitis; no controlled trials have established treatment of single or recurrent episodes of HSV meningitis
	Myelitis	Acyclovir	10 mg/kg IV q8 hr for 14–21 days	
Varicella zoster	Zoster (shingles); herpes zoster ophthalmicus	Valacyclovir	1000 mg PO tid for 1 week; prednisone 1 mg/kg qd for 5 days	
	VZV vasculopathy or myelitis orzoster sine herpete (dermatomal pain without rash)	Acyclovir	10–15 mg/kg IV q8 hr for 14 days; prednisone 1 mg/kg qd for 5 days	Test CSF for anti-VZV antibody and VZV DNA
Epstein-Barr virus			No antiviral treatment	Consider corticosteroids if brain swelling occurs
Cytomegalovirus (immunocompromised)	CNS infection, polyradiculopathy	*Induction* Ganciclovir	5 mg/kg iv q12 hr for 14–21 days	Polyradiculitis most likely in AIDS patients
		Maintenance Valganciclovir or ganciclovir	900 mg PO qd 5 mg/kg IV qd or 6 mg/kg IV qd 5 days/week	Consider combination ganciclovir and foscarnet if neurological disease develops, despite prior anti-CMV therapy
		Induction Ganciclovir and/or foscarnet	5 mg/kg IV qd 12 hr for 14–21 days 60 mg/kg IV q8 hr for 14–21 days	Consider serial CSF PCR for CMV DNA to monitor response to anti-CMV therapy long-term maintenance therapy may be necessary in patients who remain profoundly immunosuppressed
		Maintenance Ganciclovir or foscarnet	5 mg/kg IV qd or 6 mg/kg IV qd 5 days/week 90–120 mg/kg IV qd	Consider combined therapy in HIV-infected patients with $CD4^+$ counts <100 cells/mm^3 and/or in immunocompromised patients with poor response to monotherapy
Human herpesvirus-6 (B variant)	Encephalitis	*Induction* Foscarnet	60 mg/kg IV q8 hr for 14 days	Usually, HHV-6 encephalitis occurs in the setting of severe immunosuppression
		Maintenance Foscarnet	90 mg/kg IV qd for 1–4 weeks	Several reports of successful treatment, but no clinical trial data available
		Induction Valganciclovir	5 mg/kg IV q12 hr for 14–21 days	
		Maintenance Valganciclovir or ganciclovir	900 mg PO qd 5 mg/kg IV qd	

encephalitis [52,53]. In one instance [53], ventriculoencephalitis developed in a bone marrow stem cell transplant recipient, despite combined treatment with ganciclovir, foscarnet, and CMV hyperimmune globulin. The patient initially improved after addition of cidofovir (5 mg/kg intravenously weekly for two doses, then biweekly) with oral probenecid but ultimately died from multiorgan dysfunction and circulatory collapse unrelated to CMV infection.

CMV polyradiculomyelopathy

It has been suggested that CMV polyradiculomyelopathy may be more responsive to antiviral therapy than CMV encephalitis [48]. A retrospective review of 39 cases of AIDS-related CMV myelitis/polyradiculopathy [54] suggested that patients receiving antiviral therapy fared better than untreated patients. Mortality was 100% within 8 weeks in 7 patients who had not received treatment with either ganciclovir or foscarnet, whereas disease in treated patients reportedly stabilized or improved in 78%, although most patients ultimately died from AIDS-related causes. Of the 32 treated patients, 22 received ganciclovir alone, 6 foscarnet alone (5 of whom had previously received prophylactic ganciclovir), and 4 received both drugs. However, the data was insufficient to evaluate the relative efficacy of these different treatment regimens. Ganciclovir-resistant strains have been isolated in some patients who were treated prophylactically [55,56]. Some patients who failed to respond to ganciclovir did stabilize after institution of foscarnet treatment. Although it is difficult to devise treatment recommendations based on retrospective data analysis from noncontrolled studies, Anders *et al.* [54] suggested that patients who develop CMV polyradiculopathy while on ganciclovir or foscarnet therapy should receive a combination of both drugs, whereas patients not on treatment at the time of disease onset can be treated with either drug as monotherapy and switched to combination therapy if they progress clinically and/or have persisting CSF pleocytosis, which may be a marker of drug failure.

Reports of antiviral therapy for CMV-associated mononeuritis multiplex or painful symmetrical neuropathy are too limited to permit conclusions about the efficacy of antiviral therapy [48].

Consensus guidelines for treatment of CMV CNS disease

An international panel has developed guidelines for treatment of CMV diseases in adults with AIDS receiving HAART [47]. The recommendations include treatment with intravenous ganciclovir, intravenous foscarnet, or a combination of the two drugs (category B3, discussed later), with two drugs preferred in patients who had received prior anti-CMV therapy (category A3, discussed later). The "A" level recommendation in favor of combined therapy in patients who had received prior antiviral therapy reflected the panel's opinion that this treatment should "always be offered" and that there was "strong evidence of efficacy and substantial clinical benefit," while the "B" level recommendation for initiating monotherapy indicated the panel's opinion that such therapy "should generally be offered" based on moderate or strong evidence of clinical efficacy but only "limited clinical benefit." The numerical designation ("3") indicated advice representing the opinion of the panel rather than a recommendation based on evidence from clinical trials. A joint guideline for treating opportunistic infections among HIV-infected adults issued by the Centers for Disease Control, National Institutes of Health, HIV Medicine Association, and Infectious Disease Society of America [57] noted that prompt initiation of therapy is critical for an optimal clinical response in CMV-associated neurologic disease and that combination therapy with ganciclovir and foscarnet is preferred as initial therapy to stabilize disease and maximize response (category B2), although this approach is associated with substantial rates of adverse effects, and optimal therapy in patients with optimized antiretroviral therapy is unknown. The category B2 rating indicated that the therapy should generally be offered but that there was either only moderate evidence for

efficacy or only limited clinical benefit based on nonrandomized trials.

The International Herpesvirus Management Forum was considerably less positive about the benefits of antiviral therapy for CMV neurological disease and concluded that although there was a lack of data from randomized clinical trials, "available data pointed to a clear lack of benefit from either ganciclovir or foscarnet therapy of CMV CNS disease" (category 3 recommendation: "opinions of respected authorities based on clinical experience, descriptive studies, or reports of expert committees") [58]. Similar conclusions were reached by the European Union Concerted Action on Meningitis and Encephalitis Expert Group, which found that "monotherapy with ganciclovir or foscarnet is of limited efficacy in the treatment of the complications of CMV infection of the CNS." This group noted that the best available approach was aggressive combination therapy with ganciclovir and foscarnet, although it emphasized that life expectancy remained poor [48]. The recent guidelines for management of viral encephalitis produced by a European Federation of Neurological Societies Task Force also advocated combined ganciclovir and foscarnet therapy for CMV encephalitis (Class IV: evidence from uncontrolled studies, case series, case reports, or expert opinion) [10].

Use of PCR in monitoring response to therapy in CMV CNS disease

The role of quantitative PCR measurement of CSF CMV load in monitoring response to therapy of CMV CNS infections is unclear, since it has been useful in some series [51] but not others [49]. It is important to recognize that CMV replication can be compartmentalized and that patients may have low viral loads in peripheral blood with high viral loads in CSF [59]. Ganciclovir-resistant isolates can cause encephalitis [60], and virus isolates from blood and CSF may also show differences when genotyped for the presence of mutations conferring resistance to antiviral therapy [59]. Patients who respond to therapy typically show a decline in CSF pleocytosis, whereas a persistent pleocytosis is a marker of treatment failure [61].

Prevention of CMV disease in immunocompromised adults and secondary prophylaxis

There have been several randomized controlled trials using antiviral drugs to prevent the development of symptomatic CMV disease in immunocompromised patients, including those with advanced HIV infection (typically with $CD4^+$ cell counts $\leq 100/mm^3$). Oral ganciclovir prophylaxis (1000 mg 3x/day) reduces the risk of developing symptomatic CMV infection by nearly 50% in patients with advanced AIDS [62]. Patients can develop CMV neurological disease despite prophylactic administration of ganciclovir [63], and ganciclovir-resistant CMV emerges in 15–22% of transplant recipients or HIV-infected individuals receiving oral ganciclovir prophylaxis [64,65]. High-dose oral acyclovir prophylaxis (800 mg 4x/day) did not decrease the incidence of either CNS or non-CNS CMV disease in patients with advanced HIV infection [66]. However, compared to acyclovir, high-dose oral valaciclovir (2000 mg 4x/day) reduced the incidence of symptomatic CMV infection by one-third (from 18% to 12%) in patients with advanced HIV disease [67]. In that study, most CMV infection that occurred was nonneurological (retinitis, gastrointestinal disease); however the incidence of CNS disease was also reduced in the valaciclovir arm (1/523, 0.2%) compared to acyclovir (7/704, 1%). These beneficial results were partially offset by the increased risk of thrombotic microangiopathy in 2.6% of the valaciclovir-treated patients compared with 0.6% of those treated with acyclovir.

After induction therapy, immunocompromised patients treated acutely for CMV infection will often require chronic maintenance therapy ("secondary prophylaxis"). Recent recommendations for chronic maintenance therapy indicate this should be lifelong in HIV-infected individuals unless immune reconstitution occurs as a result of antiretroviral therapy (i.e., CD4 T cell count >100–150 cells/μL for

>6 months) [57]. Drugs shown to be effective for secondary prophylaxis in randomized, controlled clinical trials include oral ganciclovir, parenteral foscarnet, and combined parenteral foscarnet and ganciclovir [57]. Oral ganciclovir has largely been replaced in practice with oral valganciclovir, and this drug has been FDA-approved for this use, although data on the use of secondary prophylaxis is limited. Additional regimens and medications are available for secondary prophylaxis of patients with CMV retinitis only [57].

Neonatal CMV infection

There have been two prospective randomized trials of ganciclovir therapy for symptomatic neonatal CMV infection [68,69]. In an initial phase II trial of treatment with 8 or 12 mg/kg/day, hearing improvement or stabilization was found in 16% of treated babies [68]. In a subsequent trial, dose of 6 mg/kg intravenously given twice daily for 6 weeks reduced the number of children with worsening hearing at 6 months compared to baseline of 41% in the no-treatment arm to 0% in the ganciclovir arm ($p<0.01$) [69]. At 1-year follow-up, 21% of ganciclovir-treated children had worsened hearing compared to 68% of the no-treatment group ($p<0.01$) [69]. The proportion of children with worsened brain stem auditory-evoked responses at 6 months was 21% in the ganciclovir arm compared to 68% in the no-treatment arm ($p<0.01$) [69].

Human herpesvirus 6 (HHV-6)

HHV-6 meningoencephalitis

There are no randomized controlled trials of antiviral therapy of HHV-6 CNS disease in either immunocompetent or immunocompromised patients. Although ganciclovir or foscarnet can be used to treat HHV-6-associated neurological disease, their efficacy remains unknown. Improvement after ganciclovir treatment was first reported in a bone marrow transplant recipient [70]. A cardiac transplant patient who failed to respond to ganciclovir (5 mg/kg twice daily) recovered after subsequent treatment with foscarnet (60 mg/kg three times daily for 3 weeks) [71]. Foscarnet (60 mg/kg three times daily for 3 weeks) was also successful in initial treatment of three bone marrow transplant patients [72,73]. However, there are reports of failed treatment with both foscarnet and ganciclovir [74,75]. In one review [76], overall mortality in 12 cases (almost all bone marrow transplant recipients) was 58%, of which 42% was directly attributable to HHV-6 infection. However, 87.5% (7/8) of patients receiving foscarnet or ganciclovir for at least a week were cured of HHV-6 infection compared to 0% (0/4) who were treated for less than a week. There is one recent report of successful treatment of a stem cell transplant recipient with donor lymphocyte infusion (DLI) after failure of both ganciclovir and foscarnet [77]. Initial therapy included ganciclovir (5 mg/kg/d) and 2 days of foscarnet, but the patient continued to deteriorate clinically and subsequently received DLI of 10^6 $CD3^+$ cells/kg followed by a second infusion of 10^7 $CD3^+$ cells/kg 43 days later. After DLI, his clinical status, CSF, and MRI improved dramatically.

Outcome in immunocompetent patients does not appear to be substantially better than that in immunocompromised individuals. There are at least three reports of fatal HHV-6 meningoencephalitis despite antiviral therapy in immunocompetent adults [78,79,80]. Birnbaum and colleagues [81] describe successful treatment of a 21-year-old immunocompetent woman with HHV-6 encephalitis given ganciclovir (5 mg/kg 2x/day for 13 days) after initial treatment with acyclovir (10 mg/kg 3x/day for 13 days) initially instituted for presumed HSV encephalitis. Denes *et al.* [82] describe successful therapy of HHV-6 encephalomyelitis in an immunocompetent 20-year-old woman. The patient initially received a single intravenous infusion of cidofovir (5 mg/kg) with probenecid and began to recover, with reduction in CSF pleocytosis and conversion of HHV-6 CSF PCR to negative status. However, a reaction to probenecid precluded further cidofovir therapy and symptoms recurred, including a positive CSF HHV-6

PCR. She then received ganciclovir (5 mg/kg 2x/day for 15 days) with full recovery.

Real-time PCR was used to follow the effects of antiviral therapy on HHV-6 DNA levels in CSF of 11 hematopoietic stem cell recipients with HHV-6 infection, 8 of whom had encephalitis [83]. All patients received therapy with ganciclovir or foscarnet or both. Within 3 weeks of antiviral therapy, the CSF viral load had decreased from a median of $10^{4.4}$ copies/ml to 10^{2} copies/ml although the difference was not statistically significant ($p = 0.13$). In 4 of the 8 patients with HHV-6 encephalitis, the viral load declined and clinical status improved concurrent with antiviral therapy.

Table 19.1 provides an overview for treatment of CNS infection caused by the human herpesviruses.

Rabies

Rabies is caused by a rhabdovirus that is maintained in an animal reservoir (see also Chapter 3). In the United States, the virus is most common in wildlife (raccoons, skunks, foxes, coyotes, and bats). Transmission to humans is nearly always by animal bite, with rare cases transmitted by inhalation and cornea transplant. Virus in animal saliva is inoculated into the wound and reaches the CNS by retrograde axonal transport in peripheral nerves. The incubation period depends on the location of the bite and severity of exposure and is usually between 2 weeks and 3 months.

Prodromal symptoms of fever, headache, malaise, abdominal pain, sore throat, and pain and paresthesias at the inoculation site lead to one of two clinical syndromes. Between 85–90% of patients will develop "furious" rabies, with agitation, confusion, aggressiveness, and psychosis. Focal and generalized seizures are common. Severe pharyngeal muscle spasms occur with attempts to swallow, producing hydrophobia. Ten to 15 percent of patients develop "dumb" or paralytic rabies, with a progressive flaccid paralysis similar to the Guillain-Barre syndrome. Cranial nerves are predominantly affected, producing ptosis, ophthalmoparesis, hearing loss, dysarthria, and dysphagia. With either form, the acute phase may last several days to 2 weeks. Patients who survive the acute phase progress to coma. There are only isolated reports of recovery.

People who are likely to have been exposed should be vaccinated. Treatment after exposure begins with washing the wound with soap and water. Human rabies immune globulin (HRIG), 20 IU per kg, is given, with half the dose administered intramuscularly in the deltoid muscle and the other half infiltrated around the wound site. Human diploid cell vaccine is started with the HRIG, and 1 ml is given intramuscularly on days 0, 3, 7, 14, and 28. If the biting animal is not captured and examined for rabies infection, the decision to start treatment is based on the nature and circumstances of the bite, the species of the biting animal, and the prevalence of rabies in the region. Treatment started after the onset of symptoms is ineffective.

Togavirus infection

Ribavirin has been used empirically to treat West Nile virus (WNV) encephalitis, but no clinical trials have been conducted. Treatment of patients in Israel with WNV encephalitis was ineffective in an uncontrolled, nonblinded study. Interferon-α inhibits the cytotoxicity of various togaviruses (WNV, St. Louis encephalitis virus, Eastern equine encephalitis virus) *in vitro* but requires further evaluation in animal models (see also Chapters 6 and 7). An open-label trial in humans has not revealed any efficacy of interferon-α, and a blinded, placebo-controlled trial to treat Japanese encephalitis has not demonstrated efficacy. Based on Israeli data in mice that high-titer WNV-specific IV immunoglobulin (IVIG) is efficacious, as well as anecdotal reports of therapeutic efficacy in immunocompromised patients, double-blinded, placebo-controlled trials to evaluate the efficacy and safety of IVIG were initiated in 2003 by the Collaborative Antiviral Study Group and the National Institutes of Health. These trials are ongoing.

Enterovirus infections

There is no specific treatment for enterovirus (Coxsackie, echovirus, poliovirus) infections of the CNS, although patients who have persistent enterovirus infection due to agammaglobulinemia have decreased rates of infection when treated with IVIG (see also Chapter 1). However, once infection is established, eradication with IVIG is difficult. In one study of 16 patients treated with pleconaril, 12 improved and 3 stabilized [84].

Postinfectious encephalomyelitis

Postinfectious encephalomyelitis is associated with the exanthematous childhood diseases, such as measles (see also Chapter 2) and chickenpox (see also Chapter 12). The neurologic features are identical to those that may follow vaccination with live or inactivated virus. Pertinent symptoms and signs include headache, fever, stiff neck, mental status changes, and focal neurologic deficit, which usually occur 7–14 days after infection but might also occur at the time of rash. Although there are no randomized controlled clinical trials, because post-infectious encephalomyelitis is highly inflammatory and associated with brain edema, patients are usually treated with corticosteroids (i.e., intravenous 1434methylprednisolone 1 g per day for 3–5 days, followed by oral prednisone 1 mg/kg/day). Because the natural history of untreated post-infectious encephalomyelitis is often 4–6 weeks, steroid should be continued for weeks before tapering to avoid frequent "relapses" of disseminated encephalomyelitis that occur when steroids are discontinued too soon. Anecdotal reports of improvement with plasmapheresis or intravenous immunoglobulin in patients who fail steroid treatment require further study.

Chronic progressive encephalitis

Some viral meningoencephalitides are chronic forms of proven virus-induced encephalitis. Examples are subacute sclerosing panencephalitis (SSPE) caused by measles (see also Chapter 2) or rubella virus, and progressive multifocal leukoencephalopathy caused by JC virus, a human papovavirus (Chapter 10). These disorders are characterized by slowly progressive neurologic deterioration that occurs over many months or years. Mental changes occur early. Focal neurologic deficits are common, and myoclonic seizures are frequent in the later stages of SSPE. There is no specific treatment.

Today, the most common viral infection of the nervous system is caused by HIV, which can affect any level of the neuraxis. A recent detailed description of treatment with antiretroviral therapy (one or two protease inhibitors or a non-nucleoside reverse transcriptase inhibitor, combined with two nucleoside analogs) is detailed by Nath [85].

Conclusion

Viral encephalitis can be caused by a diverse variety of agents, each of which has distinctive epidemiological, clinical, and diagnostic features. Randomized prospective clinical trials of treatment with antiviral drugs are available for only a few viruses. These studies have provided definitive evidence for the efficacy of intravenous acyclovir in the treatment of HSV encephalitis, which is now the standard of care. Controlled clinical trials are lacking for treatment of other HSV CNS infections, although available data suggests antiviral treatment is likely of at least some benefit in reducing the duration and severity of symptoms in patients with meningitis, recurrent meningitis, and myelitis. Antiviral therapy with intravenous acyclovir is also likely to be of benefit in treatment of VZV CNS infections, although controlled clinical trials are lacking. Although antiviral drugs with activity against CMV are available, their effects on CMV CNS disease remain unknown, despite current treatment guidelines generally advocate instituting antiviral therapy. There is no proven therapy for rabies infection once CNS disease has developed, although rabies vaccination and treatment with rabies immune globulin as

postexposure prophylaxis is considered standard of care. No proven treatments currently exist for togavirus infections, although several controlled clinical trials are currently underway to evaluate potential treatments for WNV CNS disease. No antiviral therapy is currently available for acute enteroviral infections. Despite the absence of proven specific treatments for many types of viral CNS infection, patients almost certainly benefit from appropriate symptomatic and supportive treatments designed to reduce or control complications resulting from their illness.

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20

Influences of arthropod vectors on encephalitic arboviruses

Stephen Higgs

Introduction

Arthropod-borne viruses (arboviruses) are found in several virus families including *Bunyaviridae, Togaviridae, Flaviviridae, Orthomyxoviridae, Rhabdoviridae*, and *Reoviridae*. Many are not pathogenic for their natural vertebrate hosts, but those that do cause disease are often associated with a broad range of clinical manifestations. For example, most human West Nile virus (WNV) infections are asymptomatic, but some progress rapidly to a fatal encephalitic outcome [1]. Although this variability can be partly correlated with host characteristics (e.g., age and immune status) interactions between the virus and the vertebrate host that lead to disease are often complex and poorly understood. For a particular arbovirus, different species of vertebrate may play different roles in the viral maintenance and transmission cycle. It is not well understood why some species are apparently genetically resistant to infection (i.e., not a host for replication) whereas others are highly susceptible (i.e., readily infected following exposure to a low viral dose). Maintenance vertebrate hosts that play a critical role in virus amplification and the infection of vectors must produce a sufficiently high titer of virus in the blood (viremia) to ensure that the recipient vector becomes infected. This is usually referred to as the infection threshold and may be accompanied by clinical symptoms such as fever, but it does not necessarily correlate with disease severity. Conversely, hosts that do not produce threshold viremic levels sufficient to infect vectors (so-called "dead-end" hosts) may succumb to infection. For example, although many fatal equine infections have occurred in the United States due to WNV, viremic levels in horses are relatively low and of short duration and are thus below the threshold level required to infect mosquitoes [2]. Often, susceptible vertebrate species that are not a typical component of the life cycle (i.e., incidental hosts) may develop more severe disease. Thus, domesticated animals encountering an endemic virus may develop fatal infections, whereas related native species that have evolved in the presence of the pathogen may develop no clinical symptoms, despite playing an essential role in the transmission cycle as amplifying hosts.

Arboviruses that cause disease in humans and other vertebrate hosts can be broadly categorized into (1) those that cause hemorrhagic disease, for example Crimean Congo hemorrhagic fever virus (CCHFV), Kyasanur Forest disease virus (KFDV), Rift Valley fever virus (RVFV), and Dengue virus (DENV), and (2) those that cause encephalitic disease, for example, WNV, tick-borne encephalitis virus (TBEV), Venezuelan equine encephalitis virus (VEEV), and others. Table 20.1 summarizes the typical vertebrate hosts and vectors associated with arboviruses. These two different disease categories (i.e., hemorrhagic or encephalitic) do not correlate absolutely with the virus genera. For example, some flaviviruses cause hemorrhagic disease, whereas others cause encephalitic disease. In the genus *Flavivirus*, a phylogenetic analysis revealed a correlation between a) mosquito-borne flaviviruses that cause encephalitis and are associated primarily with *Culex* spp. (i.e., ornithophilic mosquitoes), and b) mosquito-borne

Table 20.1. Typical vertebrate hosts and vectors associated with arboviruses

Family	Virus	Principal vertebrate hosts	Vector type	Vector species
Flaviviridae Genus *Flavivirus*	Powassan (POWV)	Marmots (*Marmota monax*)	Hard ticks	*Ixodes angustus* *Ix. cookei* *Ix. persulcatus* *Ix. scapularis* *Dermacentor silvarum* *D. variabilis* *Haemaphysalis concinna* *Ha. longicornis* *Ha. neumanni*
			Mosquitoes	*Aedes togo* *Anopheles hyrcanus* *Ae. aegypti* (experimental)
	Deer tick virus (DTV) – perhaps	Deer	Hard tick	*Ixodes scapularis*
	Tick-borne encephalitis (TBEV)	Rodents (*Microtus arvalis* *Clethrionomys rufocanus*)	Hard tick	*Ix. hexagonus* (experimental) *Ix. ovatus* *Ix. persulcatus* *Ix. ricinus* *D. nuttelli?* *D. pictus* *D. silvarum* *Ha. concinna* *H. japonica douglasi?* *Hyalomma asiaticum?* *Hy. dromedarii?* *Hy. turkmeniense?*
	Louping Ill (LIV)	Mountain hare (*Lepus timidus*) Red grouse (*Lagopus lagopus scoticus*)	Hard ticks	*Ix. ricinus*
	Langat (LGTV) – perhaps	Rodents (*Rattus muelleri validus*, *Rattus sabanus vociferans*)	Hard ticks	*Ix. granulatus* *Ha. spinigera.*
	Japanese encephalitis (JEV)	Birds and pigs	Mosquitoes	*Culex tritaeniorhynchus*
	St. Louis encephalitis (SLEV)	Various species of bird	Mosquitoes	*Cx. nigripalpus* *Cx. pipiens pipiens* *Cx. p. quinquefasciatus* *Cx. tarsalis*
	Murray Valley encephalitis (MVEV)	Various species of bird	Mosquitoes	*Cx. annulirostris*

(*Continued*)

Table 20.1. *(Continued)*

Family	Virus	Principal vertebrate hosts	Vector type	Vector species
	West Nile (WNV)	Numerous species of bird	Mosquitoes Ticks?	*Cx. pipiens pipiens* *Cx. p. quinquefasciatus* *Cx. tarsalis* and many other species
Togaviridae Genus *Alphavirus*	Eastern equine encephalitis (EEEV)	Glossy ibis (*Plegadis falcinellus*) and other species of bird	Mosquitoes	*Ae. albopictus?* *Ae. canadensis* *Ae. sollicitans* *Ae. vexans* *Coquillettidia perturbans* *Culex* *Culiseta melanura*
	Western equine encephalitis (WEEV)	House finches and sparrows (*Carpodacus mexicanus, Passer domesticus*) and numerous species of bird. Jackrabbits (*Lepus californicus*), horses	Mosquitoes	*Ae. campestris?* *Ae. dorsalis?* *Cx. erythrothorax?* *Cx. tarsalis* *Ae. melanimon*
	Venezuelan equine encephalitis (VEEV)	Horses	Mosquitoes	*Cx. (Melanoconion)* *Cx. taeniorhynchus*
Bunyaviridae Genus	California encephalitis (CEV)	Rabbits and rodents	Mosquitoes	*Ae. malanimon*
Bunyavirus	Jamestown Canyon (JCV)	White-tailed deer (*Odocoileus virginianus*)	Mosquitoes	*Cs. inornata,* *Ae. stimulans,* *Ae. communis, other Aedes, Anopheles,* Tabanid flies?
	LaCrosse (LACV)	Eastern chipmunk (Tamius striatus) Gray squirrel (Sciurus carolinensis)	Mosquitoes	*Ae. triseriatus*
	Snowshoe hare (SSHV)	Red fox (Vulpes fulva)	Mosquitoes	*Ae. communis and other Aedes*
	Trivittatus (TVTV)	Hares (*Lepus americanus*) and other mammals. Rabbits?	Mosquitoes	*Ae. trivittatus* *Ae. infirmatus*
Reoviridae Genus *Coltivirus*	Colorado tick fever (CTFV)	Numerous rodents and other mammals	Hard tick	*D. andersoni*
	Eyach (EYAV)–perhaps	Probably rodents	Hard tick	*Ix. ricinus* *Ix. ventalloi*

flaviviruses that are more likely to cause hemorrhagic disease and are associated with *Aedes* spp. mosquitoes and mammalian species [3]. Currently, there is no similar type of analysis for other virus families, although in the context of arboviruses it is possible that similar relationships might exist. Although there are accumulating sequence data, for complete virus genomes of many arboviruses, the genetic basis for the two phenotypes (i.e., hemorrhagic vs. encephalitic) has not yet been identified [4].

Earlier chapters by Griffin (Chapter 6) and Johnson (Chapter 7) specifically discuss alphaviruses and flaviviruses, respectively, that can cause encephalitic diseases in humans and other vertebrates. This chapter also deals with arboviruses, but it focuses on the role that arthropod vectors play in the evolution, life cycle, and pathogenicity of these viruses. Since general transmission cycles have been discussed in several comprehensive reviews [5,6,7,8,9], this chapter examines some intriguing aspects of vector-virus-vertebrate relationships that have been described relatively recently.

Encephalitic arboviruses: Types of arthropod vector and evolution

By definition, arboviruses have the capacity to infect and replicate efficiently in both invertebrate and vertebrate hosts. While arthropods reproduce most efficiently under warm ambient conditions, development, reproduction, and activity may be arrested under adverse conditions. Arthropods have developed stages in their life cycles that enable them to survive seasonal environmental fluctuations, although behavioral patterns may prevent exposure to extremes. For example, ixodid ticks that feed three times during their lives may be attached to a host for only a few days each year and spend much of their life when not actively feeding in relatively protected cool and humid locations. In contrast, vectors such as mosquitoes that feed frequently and live in temperate-tropical regions may experience a broad range of temperatures. As a consequence of these differences, the reproductive dynamics of tick-borne and mosquito-borne viruses may be very different. As discussed below, arboviruses have evolved various survival strategies in response to the challenges associated with components of the vectors' biology and the environmental fluctuations that they encounter.

Interestingly, although arboviral infections of invertebrate hosts may typically be sustained for the life of the individual arthropod and, for some arboviruses sustained from one generation to the next, these infections typically have no detectable impact on vector longevity, feeding, migration, or reproductive patterns. This is in contrast to the often brief but potentially fatal infections observed in vertebrates. The basis of the disparity between viral pathogenicity in the vector and in the vertebrate is uncertain, but it emphasizes how different the virus-vector and virus-vertebrate relationships are.

The challenges presented for arboviruses to survive in vertebrate hosts are different from those associated with invertebrates. The normal body temperature of vertebrates is typically maintained within a narrow range. It can vary between different vertebrate species/groups, for example that of birds is typically higher than that of mammals, and there may be short-term fluctuations, for example during fever. A challenge that is better developed in the vertebrates than in the invertebrates is the highly adaptive and specific antiviral immune response. The vertebrate immune responses may effectively limit the duration and magnitude of viremia and the severity of a viral infection. These effects may directly determine success/failure of the arboviral life cycle. However, provided that the virus generates an adequate viremia, vectors may be infected while feeding on the viremic vertebrate, and the transmission cycle will be maintained.

The dependence on different types of arthropod vectors to sustain the arbovirus transmission cycle has impacted arboviruses in many ways. Arboviruses that cause encephalitis may be divided into (1) those transmitted by species in the order Acari (ticks) and (2) those transmitted by Diptera (two-winged flies), primarily mosquitoes. The incidence of human tick-borne encephalitis cases, for example in Far East

Russia, has been correlated with the distribution and abundance of *Ix. persulcatus* and *H. concinna* [10]. Interestingly disease severity correlates with the relative abundance of these two species of tick, with more severe disease apparently associating with years when *Ix. persulcatus* was the predominant species [10]. The basis for this relationship has not been fully explained. However, as discussed below, as a result of the differences between ticks and mosquitoes, the viruses they transmit may be under different selection pressures [11,12].

As with the invertebrate, the type of vertebrate host (mammal vs. bird) may also have important consequences for arboviruses, and, for example, has been shown to be related to alphavirus epidemiology [13] and flavivirus pathogenicity. The tick-borne flaviviruses can broadly be divided into those that have a seabird cycle with their associated ticks and those that cycle in other vertebrate hosts such as rodents in the forests and a variety of introduced species, such as sheep, deer, horses, and grouse on the moorlands. The seabird viruses are not associated with encephalitic diseases. With the exception of Powassan virus (POWV) and the closely related deer tick virus (DTV), all tick-borne flaviviruses occur exclusively in the Old World. DTV is generally not thought to cause human disease, although there is one case in which DTV may have caused a fatal encephalitis [14,15]. Langat virus (LGTV) is regarded as relatively nonpathogenic for humans, but in clinical trials of candidate vaccines in humans in Russia, a relatively high proportion of volunteers developed meningoencephalitis with permanent sequelae [4].

An interesting consequence of the differences between ticks and mosquitoes, upon the viruses that they transmit, is that the evolutionary rate of mosquito-borne flaviviruses seems to be approximately 2.5 times more rapid than that observed in the tick-borne viruses [16]. The differences in the rate of evolution are probably determined by the transmission dynamics, and the proposed explanation for this correlation relates to the basic difference in the biology and metabolic rate of the vectors. While ticks may live for long periods – perhaps, several years in a relatively quiescent state, and feed only three times (as a larva, nymph, and adult), mosquitoes are relatively short lived and must feed frequently in order to complete as many reproductive cycles as possible. These simple but fundamental differences in vector biology mean that the mosquito-borne viruses have a relatively brief opportunity for transmission. For maximum transmission efficiency of mosquito-borne arboviruses, the salivary glands must be infected as quickly as possible after the mosquito has fed upon a viremic host (i.e., a short "extrinsic incubation period"). Mosquito-borne viruses have therefore evolved to infect and replicate multiple times in a short time period to produce high titers of virus and to disseminate quickly from the midgut to the salivary glands. As a consequence of the short generation time with multiple replication cycles in the vector, mutations may accumulate relatively quickly in the mosquito-borne viruses, resulting in the generation of many different genetic lineages. If the mutations are advantageous then the lineage will survive, but others will be deleterious and the lineage will be lost. In contrast, the opportunity for transmission of the tick-borne flaviviruses is relatively long, but because each instar feeds only once, the tick-borne flaviviruses must survive in the vector through the process of metamorphosis. If infected at an immature stage, a tick will be infected for the rest of its life. The tick-borne viruses are therefore transmitted between vectors and vertebrates less frequently than the mosquito-borne viruses and also disseminate to secondary tissues more slowly. This lower virus turnover, combined with nonviremic transmission that can play a critical role in tick-to-tick transmission (discussed later) means that tick-borne viruses evolve and disperse more gradually than a typical mosquito-borne virus. Viral lineages may therefore survive unchanged for relatively long periods. These biological differences between the vector-virus relationships may explain why the phylogenetic tree of tick-borne flaviviruses is characterized by gradual branching that correlates with geographical distance between the higher levels of divergence between the viruses (indicative of a gradual "clinal" mode of dispersal and evolution). In contrast, mosquito-borne flavivirus trees show

"discontinuous" branching with frequent extinctions and little correlation with geography [17,18].

Interestingly, the taxonomic classification of flaviviruses based on both serology [19] and sequence analysis [3,16,20,21,22], discriminates the viruses according to the vectors by which they are transmitted (tick, mosquito, or no known vector – NKV). Phylogenetic analysis of the family *Flaviviridae* suggests that the entire genus evolved less than 10 000 years ago from nonarthropod-borne viruses [23,24] and that the tick-borne lineage diverged from the mosquito-borne lineage less than 5000 years ago [17,25]. This is consistent with the impact of the most recent ice age that ended approximately 12 000 years ago after having existed for up to 150 000 years. One difference between tick- and mosquito-borne viruses is that the mosquito-borne flaviviruses have aspartic acid at position 336 in the envelope protein that is absent from the E protein of tick-borne viruses. Interestingly, the tick-borne Powassan virus (POW) has been isolated from mosquitoes and will replicate in *Aedes aegypti* mosquitoes and cell cultures derived from them [26]. The relevance of this epidemiologically has not been investigated, but a possible explanation for this unusual ability to replicate in mosquitoes is that E protein of POWV is unique among the tick-borne viruses and contains aspartic acid at position 336, as is characteristic of mosquito-borne E proteins [27]. The ability of tick-borne flaviviruses in the seabird-associated Tyuleniy group to infect mosquito cells *in vitro* may also be attributed to their possession of some properties that are typical of the mosquito-borne flaviviruses, including the presence of a common glycosylation site in the envelope protein and lack of a hexapeptide insertion (24). Based on some phylogenetic analysis, POWV appears to represent the most ancestral lineage for the tick-borne viruses infecting mammalian hosts [3,25,28], but recent studies by Cook and Holmes [24] and Grard *et al.* [29] support the conclusion of Kuno *et al.* [20] that POWV does not represent an outgroup. TBE viruses have occasionally been isolated from flies, fleas, lice, and mosquitoes, but whether or not these arthropods play a role in transmission is uncertain [30,31].

Phylogenetic analysis by Gaunt *et al.* [3] has also revealed that the mosquito-borne flaviviruses divide into two distinct epidemiological groups. Although the analysis separates them based on the mosquito genera by which they are transmitted, interestingly, from the perspectives of this chapter, the analysis also cosegregates them with respect to the type of disease that they typically cause. The two groups are: (1) the neurotropic viruses, often associated with encephalitic diseases in humans, correlated with *Culex* species and avian reservoirs, and (2) the nonneurotropic viruses, associated with hemorrhagic disease in humans, correlated with the *Aedes* species and primate hosts. This division is supported by more recent phylogenies based either on multigenic or whole genome analyses [24]. To date, the molecular basis underlying the correlation between the type of disease produced and the mosquito clade (*Aedes* vs. *Culex*) in which the viruses are grouped remains unidentified. However, in biological terms, these correlations do demonstrate the dependence of these viruses on their particular vertebrate host and/or invertebrate vector associations.

The encephalitic *Culex* clade contains 23 viruses, 12 of which (e.g., St. Louis encephalitis virus (SLEV) and WNV) are known to be transmitted in a mosquito-bird cycle. The evolution of the *Culex* clade seems to have occurred after the separation of the mosquito-borne viruses from the tick-borne and NKV viruses. Essentially, the *Culex* species-associated viruses diverged after the *Aedes* species-associated viruses [24,32]. The mosquito-borne flaviviruses are presumed to have originated in the Old World, and since many of the most divergent mosquito-borne flaviviruses are closely related to indigenous African viruses, it is believed that they probably originated in Africa [18,24,32,33].

The biology and life cycles of ticks and mosquitoes

As described above, tick-borne and mosquito-borne viruses have different characteristics and are

apparently evolving at different rates. These differences probably reflect adaptations to their respective vectors, and so the next section presents descriptions of the differences between ticks and mosquitoes that may contribute to this viral variability. Key common attributes of ticks and mosquitoes that enable some of them to be efficient vectors of arboviruses are as follows.

Hematophagy

With respect to being vectors of viruses, the most important characteristic of ticks and mosquitoes is their dependence on blood as an essential nutrient source. In general, eggs are only produced after the adult female has taken a blood meal. Both male and female ticks feed on blood during all mobile life stages (larva, nymph, adult). In mosquitoes only the adult female feeds on blood. Feeding on blood (hematophagy) has evolved multiple times in the arthropods [34], presumably a reflection of the richness of blood with respect to its nutritional value. To obtain blood, an arthropod must overcome a number of potentially high-risk challenges. First, the arthropod must locate a host. To find the host, hematophagous arthropods have developed behaviors and responses that enhance the probability of contact with a vertebrate. A common component is the presence of sensory organs that can detect host-specific signals. The most frequently used host cue is carbon dioxide. Hard ticks locate their hosts by a behavior known as "questing" during which they climb from the ground to the top of the vegetation and actively survey the habitat by holding out the forelegs on which are located the CO_2-sensitive Haller's organs. Mosquitoes fly with frequent turns until they locate an elevated CO_2 level. The turning rate is then modified to keep them not only within the higher CO_2 zone, but also to promote flight up an increasing concentration gradient to the source – a respiring vertebrate. Different arthropod species have different sensitivities and behavioral patterns (for example, crepuscular flight activities vs. diurnal) that bring them into contact with preferred host species. The basis of host specificity is not well-understood. Upon contact with the host, both hard ticks and mosquitoes use specialized mouthparts to penetrate the skin and obtain blood. It is while feeding on blood from an infected vertebrate that the arthropod may become infected with an encephalitic arbovirus. As described below the efficiency of this transmission process may be influenced by several factors.

Vector saliva

A critical factor in successful blood feeding is the production of highly specialized saliva by the arthropod. The saliva of all hematophagous arthropods share common activities that facilitate blood feeding by preventing clotting and promoting blood flow to the feeding site. The specific molecules responsible for a particular activity/function can be different in different species. As a result of their long duration feeding process, ticks may be exposed to potentially harmful blood components, for example antibodies against salivary gland components. Ticks have a more complex repertoire of substances that are secreted in their saliva than that seen in mosquitoes [35], and this has probably arisen as a mechanism of immune evasion, either through saliva plasticity or through the secretion of immunomodulatory substances [36,37,38,39,40,41,42,43,44,45]. Antivector vaccines based on immunization of the vertebrate against proteins in saliva or in vector organs have recently been developed and may directly or indirectly protect vertebrates from infection [46,47,48].

As reported with ticks, mosquito saliva also contains salivary components that may be pharmacologically active [49,50,51,52,53,54,55,56,57,58,59,60,61,62,63,64,65,66,67,68,69,70,71,72,73,74,75,76,77,78,79,80,81,82,83,84,85,86,87,88,89,90,91]. Although mosquito feeding may induce hypersensitivity reactions, because of the rapid feeding, one might not expect immunomodulatory activities to have evolved. However, recent research has revealed that mosquito saliva does have immunosuppressive components [58,89,90,91,92] and as discussed below this activity can influence pathogen establishment and disease development [59,64,65,68,81,82,84,172,173].

Tick life cycles, tick biology, and their influences on encephalitic arboviruses

Although the suborder Ixodida includes the soft ticks (family Argasidae) and hard ticks (family Ixodidae), only hard ticks are discussed here, since encephalitic virus has not been isolated from soft ticks, which typically transmit the seabird viruses. All ticks have a holometabolous life cycle in which all stages have a similar general appearance (although larvae have six legs while nymphs and adults have eight) and growth occurs by molting. There are several characteristics of ticks that contribute to their capacity to be efficient arboviral vectors:

1. Males and females of all active stages are persistent blood feeders.
2. They are slow feeders.
3. They often have a broad host range.
4. They are relatively long lived.
5. They may transmit pathogens transstadially, transovarially, and nonviremically.
6. They have relatively few predators.
7. They are heavily sclerotized.
8. They have a large reproductive capacity.

The time spent feeding on the host may be long (from a few days by the larva to perhaps 2 weeks for adults). This means that the period during which the tick can become exposed to a virus is relatively long. Many vertebrates develop a transient viremia that may last for just a few days. By feeding for an extended period, the probability that viremic blood is imbibed by a tick is therefore increased, compared for example to a mosquito that feeds for just a few minutes. Similarly, because ticks salivate to varying extents [93] throughout the period of attachment, there is a prolonged opportunity for pathogen transmission to the vertebrate.

Although some ticks may have preferred host species, many will feed on a wide variety of hosts. Unlike a mosquito, which may feed on many different individuals, a tick will feed on three or fewer different individual hosts during its lifetime. Hard ticks (Ixodidae) may be classified based on the number of different individual hosts upon which they feed during their life span. The most typical life cycle involves three hosts. The larva feeds on one host and when fully engorged falls to the ground where it molts to the nymphal stage. The nymph subsequently attaches to a second host, feeds, drops off, and molts into an adult. The adult feeds on a third host, mates, and then falls to the ground where, after laying eggs, it dies. Although this limited number of individual host contacts may restrict the opportunity of an individual tick to feed on a viremic host if the prevalence of infection in the vertebrate population is low, the long feeding period, long life span that allows a virus to survive through periods when vectors are not feeding, and the intensity of infestation, means that ticks can be very efficient vectors. Since ticks lay very large numbers of eggs as a batch, vertebrates can become infested with very large numbers so that a single viremic vertebrate can infect many individual ticks. As detailed below, the combined effects of high-density infestations and phenomenon of nonviremic transmission (NVT) of virus between cofeeding nymphs and larvae can be critical for virus survival.

Transmission of tick-borne encephalitic flaviviruses

The tick-borne viruses that can cause encephalitic disease are: Powassan virus (POWV), tick-borne encephalitis virus (TBEV), louping ill virus (LIV), and perhaps langat virus (LGTV). These viruses are typically transmitted to ticks as they are feeding on a viremic vertebrate, and because all active stages are hematophagous, ticks may be exposed to virus as larvae, nymphs, and adults. Interestingly, not only can the distribution of TBEV foci be predicted based on seasonal environmental/climate data [94], but also it has been discovered that synchronous cofeeding of tick larvae and nymphs is important [95] because of the phenomenon of nonviremic transmission (discussed later). Alternating transmission between the vertebrate and the vector is known as *horizontal transmission* and is the basis of most arboviral cycles. For an arthropod to be a competent

vector, the concept of an *infection threshold* is recognized. This assumes that there is a certain minimum viral titer to which the arthropod must be exposed in order for it to become infected. This is well-documented for mosquito-borne viruses [96], but because ticks salivate and feed intermittently during the period of attachment it is very difficult to determine exactly how much virus is taken into the gut [97]. It has been estimated that to be infected with RSSEV (i.e., TBEV-Siberian), *Ixodes pursulcatus* must engorge 10^3mouse LD_{50} [98] and for *D. andersoni* to be infected with Colorado tick fever virus (CTFV), a viremia of at least 10^2 mouse LD_{50} was required [99]. Since each stage of ixodid ticks feeds only once, for transmission to occur the virus must disseminate from the gut, survive through the process of molting, and infect the salivary gland of the subsequent stage so that when this feeds, the virus can be secreted with the saliva. This type of vertical transmission from one tick stage to the next is called *transstadial transmission*. This is relatively rare for mosquito-borne viruses; however, for transmission of tick-borne viruses to vertebrates, this mode of transmission is an absolute requirement. Because of their importance, this and several other phenomena, including transovarial transmission (TOT), so-called "nonsystemic" or "nonviremic" transmission (NVT), and "salivary activated transmission" (SAT) that are associated with tick-borne pathogens but are rare or have not been documented for mosquito-borne viruses are discussed in detail below.

Transstadial transmission is the means by which virus is passed directly from one life stage of an individual tick to the next – in the case of ticks, for example, from the larva to the nymph or from the nymph to the adult. In this way, ticks may be infected for most of their life span. Not only does this enable the virus to persist in the environment, perhaps for years, but also since all of these tick stages are hematophagous, it provides up to two opportunities for the virus to be transmitted to different vertebrate hosts. The mechanism by which virus survives the process of metamorphosis is unclear. Most tissues, including the salivary glands of immature ticks, are lysed and replaced; however, this regenerative process is phased [97], suggesting that virus may survive in different tissues at different points in the regenerative cycle. Titers of virus present in different stages and the pattern of change vary according to virus [100,101,102].

Transovarial transmission (TOT) is a mechanism by which some female vectors transfer virus via the reproductive tract to the eggs and hence to the offspring. High proportions of the offspring may be infected in this way with TBE viruses [103]. TOT has been reported for several viruses including TBEV-RSSEV [100,104,105,106], LIV [107], and CCHFV [108]. Not only does TOT represent a long-term survival mechanism, but it increases the number of opportunities for viral transmission to the vertebrate to three, since larvae can transmit when feeding. This is also important since larvae represent the most abundant cohort of the tick population. It is suggested that TOT may compromise the efficiency of horizontal transmission and reduce viral pathogenicity for the vertebrate but may increase the pathogenicity for the tick [109]. There are reports of transovarial transmission of mosquito-borne viruses by ticks (e.g., WEEV by *D. andersoni* [110] and of SLEV by *D. variabalis* [111] and by the chicken mite *Dermanyssus galinae* [112]), but this is probably unimportant with respect to the normal cycle of these viruses.

Nonsystemic transmission (NST) is a phenomenon in which a pathogen is transmitted from an infected to an uninfected vector, without a detectable viremia/parasitemia in the vertebrate host. Nonviremic transmission (NVT) is the term specifically applied to viruses and occurs when infected and uninfected vectors feed together on a nonviremic or apparently uninfected host. Although the process is well-documented, NVT challenges the paradigm that to become infected a vector must be exposed to a threshold viral titer. NVT can play a very important role in maintaining the life cycle of tick-borne viruses because of aggregated cofeeding by different tick instars, allowing, for example, virus transmission between nymphs and larvae [95]. NVT of mosquito-borne viruses has only recently been described [113,114,115,116], and although its

Table 20.2. Viruses transmitted nonviremically between arthropod vectors in laboratory studies

Family	Virus	Vector	Reference
Orthomyxoviridae	THOV	*Rhipicephalus appendiculatus*	119, 123, 124
Flaviviridae	TBEV	*Ixodes ricinus*	125, 126, 127, 128
		Dermacentor reticulatus	129
	LIV	*Ix. ricinus*	130
	WNV	*Ornithodorus moubata*	131
		Cx. p. quinquefasciatus	113, 114
		Cx. p. quinquefasciatus	115
		Cx. p. quinquefasciatus	116
		Cx. tarsalis	116
	KFDV	*Argas persicus*	132
Bunyaviridae	BHAV	*D. marginatus, D. reticulatus*	133
	Palma	*D. marginatus, D. reticulatus,*	133
		R. appendiculatus, R. sanguineus	133
	CCHFV	*Hyalomma spp.*	134
Rhabdoviridae	VSV	*Simulium vittatum*	135

THOV, Thogoto; TBEV, tick-borne encephalitis; LIV, Louping Ill; KFDV, Kyasanur Forest; BHAV, Bhanja; CCHFV, Congo-Crimean hemorrhagic fever; VSV, vesicular stomatitis (New Jersey serotype).

potential importance has been highlighted [117, 118], to date this has not been widely discussed. The NVT rate (the percentage of uninfected cofeeders that become infected) for tick-borne pathogens, for example Thogoto virus (THOV), may be 100% [119]. Transmission of viruses during feeding, from an infected arthropod to an uninfected arthropod, in the absence of a detectable viremia in the vertebrate host, has been reported for a broad range of arboviruses [120,121; Table 20.2) and also for *Borrelia* [122].

Lord and Tabachnick [136] reviewed systemic and nonsystemic transmission of arboviruses and formulated a general model of arbovirus transmission. They identified four critical factors: (1) latent period in the host, (2) duration of infectivity of the host, (3) virus transmission rates from vector to host and host to vector, and (4) vector:host ratio. The relative impact of these factors varies between conventional systemic transmission and nonsystemic (nonviremic) transmission. A fundamental assumption in this model is that "nonsystemic transmission will depend on an infected and uninfected vector feeding sufficiently close together in time and space" [117,136]. Although the assumption is logical, more experiments are needed to determine the precise relationship between these factors. For example, due to its dependence on two different categories of vector (infected and uninfected), NVT is sensitive to additional factors such as host availability, host preferences, feeding behavior, preferred feeding sites on a host, and circadian rhythms of insect activity; all of these factors influence the proximity and abundance of vectors as they feed together on a host. NVT may, however, not be sensitive to the host's antiviral immune status.

Spatial aspects of NVT

Although it is assumed that NVT efficiency is influenced by the spatial and temporal proximity of the infected and uninfected vectors [117,136], there is relatively few data to support this. From the demonstration of NVT of THOV when donor and recipient ticks were 160 mm apart, Jones and Nuttall [137] concluded that the spatial relationship was less

important than the temporal effects. Labuda *et al.* [133] investigated NVT of TBEV by *Ixodes ricinus* ticks in which two chambers were placed next to each other on rodent hosts. The first contained both donor and recipient ticks; the second chamber contained only recipients. Under all experimental conditions, infection of recipients was consistently higher when housed in the same container with the donors, compared to when they were housed separately. The percentage of recipients infected was 72% vs. 38% in nonimmune rodents, 57% vs. 15% in needle-immunized rodents, and 24% vs. 3% in rodents that had previously been fed upon by infected ticks.

Temporal aspects of NVT

In experiments with ticks, donor and recipients have invariably been fed on the host at the same time. As noted by Lord and Tabachnick [136] and Lord *et al.* [128], NVT is assumed to be most efficient if the arthropods feed simultaneously. The rationale is that with increasing time, infectious virus delivered to the vertebrate by the donor becomes less accessible for recipient infection due to dilution or diffusion from the feeding site, sequestration by immune cells, neutralization by antibody, or some other mechanism resulting in reduced availability/infectivity of the virus. Jones and Nuttall [137] manipulated the duration of cofeeding ticks and reported that while 80% of recipients became infected with THOV if they cofed with donors for 5 days, none became infected if cofeeding was limited to 3 days.

Potential mechanisms underlying NVT of tick-borne viruses

Despite years of research, the underlying mechanism of NVT is not entirely clear.

Viremia below the level of detection

A possible explanation for NVT is that there is a viremia, but that it is below a detectable level, or that it is so brief that the probability of detection is low. In this case, NVT could be a misnomer and simply reflects the use of techniques that are not sensitive enough to detect very low level or transient viremias. This is plausible, since NVT was discovered prior to the development of highly sensitive molecular techniques for viral detection. Jones *et al.* [119] collected blood by cardiac puncture throughout the period of tick engorgement but found no THOV by plaque assay. Inoculated guinea pigs produce only low-level THOV viremias ($\sim$2.0 $\log_{10}$ LD_{50}/mL), but interestingly, ticks feeding on these viremic animals were infected at a lower rate than those that cofed in the presence of infected ticks on an uninfected animal. This suggested that a novel mechanism of transmission was operating that was independent of viremia.

Transmission via skin

Another possible mechanism for NVT is that virus is transmitted via the skin. Labuda *et al.* [127] re-evaluated studies by Jones *et al.* [119] and proposed that viremia is a product of NVT rather than a prerequisite for it. Labuda *et al.* [127] demonstrated that virus transmission from donor to recipient was correlated with infection in the skin at the site of tick feeding. Viral antigen was detected in migratory Langerhans cells (LCs) and neutrophils, derived from skin explants taken from tick feeding sites, by immunocytochemistry, and infectious TBEV was recovered from migratory monocytes/macrophages. Since ticks often feed for several days and have numerous pharmacologically active substances in their saliva [41,42,138], one can envision that the feeding site is a highly active environment, with migration and emigration of multiple cell types. Interestingly, TBEV was detected in the skin of natural rodent hosts where uninfected recipients were feeding, but it was absent from similar sites where no ticks were present. The observation suggests that virus may be preferentially recruited to the site of the feeding ticks. Given the balance between the inflammatory responses caused by tick feeding and immunomodulatory effects of tick saliva, one can imagine that infected cells may migrate from the feeding site of infected ticks to sites where

uninfected ticks are feeding rather than migrating randomly. In response to ticks, cellular migration varies in the degree of prior exposure of the host to tick bites. In sensitized hosts, more cells are attracted to the bite site and do so more rapidly.

Salivary-activated transmission

Nuttall and Labuda [139] concluded that so-called "salivary-activated transmission" (SAT) plays a critical role in the life cycle of TBEV [140,141,142]. Supporting observations are: a) successful transmission of TBE virus between cofeeding infected and uninfected ticks does not depend on a detectable viremia in the host [125,129]; b) NVT can be mimicked by syringe inoculation of TBEV if it is mixed with tick saliva/salivary gland extract [126,143]; c) NVT occurs in nature in wild vertebrate hosts [144]; d) NVT can occur in ticks feeding on immune hosts [128]; and e) based on the calculated R_0, survival of TBEV in nature, NVT is necessary to maintain the virus [145,146]. Nuttall and Labuda [139] conclude that the basic mechanism of NVT is TBEV's exploitation of the pharmacological activities of tick saliva at the feeding site (the basis of SAT). When ticks were fed on guinea pigs inoculated with TBEV a significant increase in infection rate was observed if virus was inoculated with salivary gland extract (SGE) [144]. The effect was not tick species-specific, SGE from *Rhipicephalus appendiculatus, Ixodes ricinus,* and *Dermacentor reticulatus* being equally effective. SGE from partially fed ticks resulted in a four-fold increase in the infection rate when compared with SGE from unfed ticks. SGE also increased the level of viremia. In NVT experiments with THOV [140], no effect was seen using mosquito SGE. The components of tick saliva involved in SAT are uncertain [120], although a protein/peptide seems to be involved, since SAT activity is destroyed if SGE is treated with pronase or proteinase-k [123].

Epidemiological significance of NVT

The epidemiological significance of NVT between cofeeding ticks was reviewed by Randolph *et al.* [121], with a comparison of the relative contributions of systemic and nonsystemic transmission for TBEV and *Borrelia burgdorferi*. In a model to evaluate the quantitative aspects of the two modes of transmission, several parameters were particularly influenced by nonsystemic transmission. First, the latent period between infection and infectivity in the vertebrate (intrinsic incubation period) is shortened with NVT. Coefficients of transmission, based on specific interactions of the vector and host, determine transmission from vector to host, from host to vector, and from vector to vector, all of which may be species-specific. An important consideration of NVT is that transmission does not require a susceptible host [128]. In the tick model, transmission rates are influenced by the vector infestation level, and the efficiency of NVT depends on close association between infected and uninfected ticks. The duration of host infectivity for biting arthropods in viremic transmission is often relatively short, but for NVT it may be for the life of the host. From this model, the duration of one episode of transmission for TBEV virus was doubled for NVT when compared with conventional systemic transmission. NVT of TBEV results in 50% more virus transmission than does systemic transmission.

Host immunity and NVT

With regards to vector-borne pathogens, the vertebrate host may display immune responses to the pathogen and to vector saliva, and both may influence the potential for a vertebrate to infect vectors. In the conventional transmission model, immune vertebrates are regarded as "dead-end" hosts with no role in perpetuating the transmission cycle; however, because of the rapidity of the process, virus-neutralizing antibodies may not prevent NVT. Labuda *et al.* [128] immunized rodents against TBEV either by needle inoculation or via the bite of TBEV-infected *Ixodes ricinus* ticks, and then at 4 weeks postinfection, when the presence of neutralizing antibodies was confirmed, they placed infected donor and uninfected recipient ticks on immune animals or nonimmunized controls.

Recipients were consistently infected when cofeeding on both immune and nonimmune rodents; however, the proportion of animals permissive to NVT was lower in those immunized by tick bite (75%) than in those immunized by needle inoculation (89%). To explain this he suggested that either tick feeding elicits a stronger immune response, or alternatively virus transmission is inhibited due to previous exposure to tick feeding. Interestingly, Labuda *et al*. [128] reported that NVT of TBEV is more efficient in field mice (*Apodemus flavicollis*) than in bank voles (*Clethrionomys glareollus*), a possible explanation being that the bank vole mounts an immunologically based resistance to ticks, whereas field mice do not [147]. Jones and Nuttall [148] also showed that transmission of THOV was reduced in guinea pigs pre-exposed to ticks, but not in hamsters.

Tick-borne coltiviruses

The coltivirus Colorado tick fever virus (CTFV) occurs in the Western United States, throughout the geographic range of the tick vector, *Dermacentor andersoni* [149]. There are approximately 200 cases reported per year. Severe disease is relatively uncommon; however, fatalities in children have resulted due to central nervous system (CNS) damage [150,151,152]. The related Eyach virus (EYAV), which has been isolated from *Ix. ricinus* and *Ix. ventalloi* in Europe, has been associated with meningoencephalitis [153,154].

Mosquito life cycles, mosquito biology, and their influences on encephalitic arboviruses

In contrast to ticks, mosquitoes have a hemimetabolous life cycle in which distinct morphological and physiological characteristics are displayed by different life stages. Eggs may or may not be resistant to desiccation but are typically laid in or close to water and hatch to produce an aquatic filter-feeding larvae. Larvae molt to produce progressive larger but similar larvae until the final larval stage, which molts to produce a nonfeeding pupal stage. This metamorphoses to produce the winged adult. In the genera that are associated with arboviral transmission, only the female feeds on blood, the male feeding on sugars.

Transmission of mosquito-borne encephalitic arboviruses

All of the mosquito-borne arboviruses follow basically the same relationship with their vectors. Mosquitoes are typically infected by feeding on a viremic vertebrate.

There are several reviews on arbovirus-vector interactions [5,6,7,8,9]. Hardy *et al*. [6] provided an excellent flow chart describing the pathway of arboviral infection within a mosquito including hypothesized and conceptual infection and escape barriers. All mosquito-borne arboviruses that cause encephalitis are enveloped, and for these to be transmitted by a mosquito following an infectious blood meal, a number of criteria must be satisfied. The virus-vector relationship can be regarded as consisting of a number of stages.

1. The mosquito must feed on a viremic host.
2. An infectious dose of virus (greater than the minimum threshold) must enter the mosquito midgut lumen.
3. Virions must bind to the membrane of midgut epithelial cells.
4. Following endocytosis or fusion of the viral envelope and cellular membranes, the virus genome must enter the cell cytoplasm and replicate to produce infectious virions.
5. Virions must disseminate from the midgut epithelial cells and enter the haemocoel.
6. Virions must infect salivary glands.
7. Virions must be secreted in saliva when the mosquito feeds upon a host.

The time between feeding on a viremic vertebrate and the ability to transmit the virus in saliva is known as the *extrinsic incubation period*. The successful completion of the transmission process is influenced by various factors, but the overriding consideration is that the virus and species of mosquito must be

"compatible." The "competence" of a mosquito to transmit a virus is determined by factors that allow progress from each of the stages to the next. There are a number of potential "barriers" to this progression that may affect the success/failure and rate of the dissemination and transmission process. While some of these are undoubtedly mosquito-related, others may be virus-dependent. Although we do not know the identity of the hypothesized midgut receptor, infectious clone technology is providing insight into the role of different viral genes in the determining vector specificity [155,156] and in the infection and dissemination processes [157,158,159]. Stages one and two are influenced by host factors and by mosquito behavior (host preference, time and frequency of feeding, etc.).

Flaviviruses

All of the encephalitic mosquito-borne flaviviruses are members of the Japanese encephalitis virus serocomplex: Saint Louis encephalitis virus (SLEV), West Nile virus (WNV), Murray Valley encephalitis virus (MVEV), and Japanese encephalitis virus (JEV). Until 1999, SLEV was the only member of the serocomplex present in the Americas; however, the introduction of WNV into New York has demonstrated the capacity of arboviruses to utilize new vectors and vertebrates and rapidly expand their geographic range. The biology of WNV, its introduction and spread in the United States has been described in Chapter 8, Flaviviruses. An important component of this spread has been the extraordinary ability of WNV to infect a very broad range of arthropods [1,160]. As shown in Table 20.1, most of the encephalitic arboviruses infect very few species of vector, and indeed although they are listed, many of the species named in Table 20.1 probably contribute relatively little to the natural transmission cycle. Like the other viruses, WNV is transmitted by certain species (e.g., *Cx. p. pipiens*, *Cx. p. quinquefasciatus,* and *Cx. tarsalis*) far more frequently than by other species; however, it has already been identified in some 60 North American species of mosquito, can infect ticks [128], and be experimentally transmitted nonviremically [131] and transstadially [161]. The basis of WNV's ability to infect so many species of vector has yet to be determined. In their molecular epidemiological studies, Weaver *et al.* [13] discussed why encephalitic arboviruses, such as WEEV, EEEV, and presumably WNV, that utilize highly mobile avian enzootic hosts have evolved as relatively few, highly conserved genotypes that extend over wide geographic locations. This is in contrast to viruses (e.g., JEV) that utilize mammalian hosts, which have evolved as multiple genotypes with a more restricted range.

Alphaviruses

As described in Chapter 6, Neurotropic alphaviruses, there is a closely related group of alphaviruses: Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), and Venezuelan equine encephalitis virus (VEEV), that typically circulate in cycles involving mosquitoes, birds/rodents, and horses. Because of their infectivity and pathogenicity, these New World alphaviruses are appropriately designated as National Institutes of Allergy and Infectious Diseases (NIAID) priority pathogens (http://www3.niaid.nih.gov/topics/BiodefenseR/Biodefense/research/CatA.htm), and due to their potential development and use as aerosolized bioterrorist agents, EEEV and VEEV are also categorized as "select agents" (http://www.cdc.gov/od/sap/docs/salist.pdf). Molecular epidemiological studies [13] have contributed much to our understanding of these and other encephalitic arboviruses. However, several phenomenon remain to be addressed. For example, in contrast to the New World alphaviruses, the Old World alphaviruses characteristically produce arthritic/polyarthritic diseases. We do not know the basis of this difference, and it is unclear why alphaviruses are transmitted only by Diptera, but both Acari and Diptera are involved as vectors for flaviviruses.

Bunyaviruses

Several bunyaviruses in the California serogroup may cause encephalitis (Table 20.1) as reviewed by

Calisher [162] and Tsai [163]. However, almost all of the infections in the United States with this group of viruses are with La Crosse virus (LACV). Approximately 99% of infections are inapparent, and fatal encephalitis is extremely rare (0.5% of clinical cases). In view of the actual number of severe infections with LACV, the often repeated description of LACV as "the most important arboviral cause of pediatric encephalitis in the United States" [162] seems to perhaps exaggerate the actual importance of this particular virus when placed in the context of other causes of pediatric fatalities. Although of undeniable public health importance, LACV is not a credible agent for development as a bioterrorist agent, and considering our extensive knowledge of its biology, its wide geographic distribution in the southern half of the United States, the ratio of apparent:inapparent infections, and its relatively low pathogenicity in humans, its inclusion, and that of CEV, as NIAID priority pathogens seems to be misguided.

Transovarial and transstadial transmission

Transovarial transmission has been reported for several mosquito-borne viruses [164,165,166]. For most viruses, TOT is probably unimportant with respect to maintaining the life cycle; however, for bunyaviruses and particularly those in the California serogroup, such as LACV, TOT is very important, especially as an overwintering mechanism [167]. Due to its geographic distribution in a temperate climate with characteristically cold winters and dependence upon a principal vector – *Ae. triseriatus* – that survives through the winter as a diapausing egg, LAC has evolved to be efficiently transmitted by the female mosquito to the egg [166]. These embryonate prior to the winter with the virus present in the embryo. These eggs hatch in the spring, the virus replicates in the larva, survives through each molt (i.e., is transstadially transmitted), and then the adult ecloses with a disseminated infection – fully capable of immediate LACV transmission. Since both male and female adults may be infected by TOT, venereal transmission has also been documented for LACV. Thanks to an experimental ability to generate reassortant bunyaviruses, the molecular basis of vector infections is relatively well-understood for these viruses [168]. For the related Rift Valley fever virus (RVFV), transovarial transmission may be the mechanism by which the virus survives the dry hot desert conditions.

Mosquitoes and NVT

As discussed above, NVT of viruses by ticks has been relatively well-studied and represents an important component of the viral life cycle. Mead *et al.* [135] described NVT between black flies infected with VSV (for which there is one reported case of human encephalitis [163]. More recently NVT of WNV has been described [113,114,115,116]. With respect to spatial and temporal aspects of NVT of WNV by mosquitoes, uninfected recipient *Cx. p. quinquefasciatus* became infected when feeding simultaneously up to 40 mm from infected donor mosquitoes, and also became infected when feeding up to 40 minutes after the infected mosquitoes had been removed from the host animal [115]. Given the high mosquito densities, the high minimum field infection rates for WNV at certain times of the year [169] http://www.cdc.gov/ncidod/dvbid/westnile/software.htm), and the high titers of virus present in mosquito saliva [170], the discovery of NVT of WNV is perhaps not surprising. In a mouse model, no viremia was detected [113,114,115], however, low-level viremias were observed in NVT studies performed by Reisen *et al.* [116]. This discrepancy may reflect the use of different vertebrates or detection techniques. The factors involved in facilitating NVT by ticks may be quite different from those involved in NVT by mosquitoes. Due to differences in the biology and physiology of ticks and mosquitoes, caution must be applied before extrapolating between the two systems, but given the potential importance of NVT [117,118,137] further research to determine the mechanism seems warranted.

Lord and Tabachnick [137] developed a model of NVT of VSV, and Lord *et al.* [117] discussed NVT in relation to our WNV data. Their conclusions were similar to those of Randolph *et al.* [121]; although

differences were observed due to the differences in the biology of acarid and dipteran vectors. The Lord and Tabachnick model was also based on the determination of a basic reproductive number R_0 to compare the efficiency of viremic and nonviremic transmission. Their analysis showed that elimination of the latent period would allow a more rapid increase in the VSV infection rate in the arthropods early in the development of a new outbreak. The aggregation of black flies at favored feeding sites on the host was considered to be particularly influential. Another important difference between the tick and dipteran vectors is the relative mobility of latter arthropods, with flies able to move rapidly and frequently between different animals. This greatly accelerates the speed of transmission and may result in large numbers of flies becoming infected in a short time period. Also, for conventional systemic transmission, the progress of an epizootic can be influenced by host mortality and immunity, as the pool of susceptible vertebrates is limited [171]. With NVT this pool is increased, because immunity, either resulting from natural infection or vaccination, does not prevent transmission [128].

Salivary potentiation

The studies by Cross *et al.* [58] were the first to demonstrate that mosquito saliva influenced murine cellular immune responses. The application of modern immunological and molecular techniques has advanced our knowledge of the types of cells and cytokines that are affected [89,91]. Given that the success and severity of arboviral infections in vertebrates are impacted by the specificity and effectiveness of the vertebrates' immune responses, it follows that the immunodulatory activity of vector saliva may influence arboviral infections [171,172]. Not only have studies now revealed enhancement of arboviruses by mosquito feeding/mosquito saliva for several viruses including Cache Valley virus [59], LACV [68], Sindbis virus [81], vesicular stomatitis virus (VSV) [64,65], and WNV [82,172], but they have directly linked salivary-induced effects on specific components of the immune system (e.g., interferons) as a mechanism to explain enhancement of viral infections. Recently, it has been demonstrated that sensitization to mosquito saliva by pre-exposure to mosquito feeding exacerbates disease, leading to increased mortality following WNV infection [172]. The aggravated disease course was associated with enhanced early viral replication, increased interleukin-10 expression, and elevated influx of WNV-susceptible cells to the inoculation site. Interestingly, the exacerbated disease course was mimicked by passive transfer of mosquito-sensitized serum to naïve animals. The machanism of this potentiation is not fully understood but these data have important implications with respect to infections in nature, where host animals, including humans, may be intensely exposed to arthropod feeding.

Conclusion

The long association between encephalitic arboviruses and their arthropod vectors has resulted in the development of complex interspecific relationships. As the vectors evolved to successfully exploit vertebrate hosts, so the arboviruses had to adapt to survive in both the arthropod and the vertebrate. Differences between the types of vector and the types of vertebrates infected by the arboviruses seem to have inevitably shaped the evolution of these viruses. Although many aspects of the virus-vector-vertebrate relationship are poorly understood, the employments of new highly sensitive techniques, for example to accurately quantify multiple components of the immune responses, are giving new insight into viral infections. An understanding of the mechanistic basis of how arboviral infections are established and how arthropod vectors influence the processes may provide data for the optimization of intervention strategies, including those designed to minimize the risk of infection or to reduce the probability of disease progression. In laboratory investigations, the vector is often ignored; however, as described in this chapter the vector plays a critical role in the arboviral transmission cycle and is often

the factor that determines the geographic distribution of the virus, the potential for establishment if introduced into a new region, and whether or not an epidemic occurs. We cannot afford to ignore the fact that most human infections with encephalitic arboviruses begin with a single bite of an infected vector.

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21

The role of bats as reservoir hosts of emerging neurological viruses

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Introduction

It is now well-recognized that more than 75% of emerging diseases over the past 2 decades have been zoonoses. Many of these zoonotic viruses have caused neurological disease, especially those emerging during this period in the South–East Asian and Western Pacific regions [1,2]. Most of the diseases emerging from wildlife have been from bats and rodents. Bats are only second to rodents in terms of mammalian species richness [3] and constitute about 20% of all mammalian species. Thus, with their wide distribution and abundance, it is not surprising that there is growing awareness that bats are the reservoir hosts for a number of these emerging viruses [4,5,6,7] and suspected of being associated with many others on serological grounds. Not only have they been shown to be the reservoir hosts for rabies and related lyssaviruses but also for other human pathogens, or potential pathogens, such as SARS-coronavirus-like viruses [8,9,10], Ebola virus [11,12], Menangle virus [13], and Hendra and Nipah viruses [14,15,16]. This brief review looks at the biological features that make bats good reservoir hosts, and the more important neurological viruses associated with bats that are, or have the potential to be, transmitted to humans.

Bats as reservoir hosts: implications for virus transmission

The Order Chiroptera, their diversity, evolution, abundance, and social behavior

The mammalian Order Chiroptera is divided into two suborders, the Megachiroptera, or Old World fruit- and nectar-feeding bats, including flying foxes, and the Microchiroptera, or echolocating bats [17]. All 188 species of megachiropteran bats are grouped within a single family, *Pteropodidae*, and are geographically confined to tropical regions of Africa, Asia, Australia, and many South Pacific islands [18]. In contrast, the 18 families and 917 species of microchiropteran bats inhabit both temperate and tropical regions throughout the world [18]. Molecular phylogenies suggest an early Tertiary origin of the Chiroptera [19], with three major microchiropteran lineages traced to Laurasia and a fourth to Gondwana [19]. At least 24 genera of bats were extant by the Eocene [50 to 52 million years ago] [17,19], and the earliest known fossil Eocene bats could already fly and use echolocation. The divergence of the Megachiroptera and Microchiroptera had to have occurred well prior to the oldest fossil record. Following the early evolution of flight and echolocation, bats have changed little as a taxonomic group relative to other mammals [20].

Bats are unique with regard to the abundance and density achieved by certain cave-dwelling species. Colonies of Mexican free-tailed bats (*Tadarida brasiliensis*) can achieve numbers in excess of a million individuals, reaching densities of 500 individuals per square foot, and several species of *Myotis* achieve hibernating population densities of >300 per square foot [21,22,23,24]. Tree roosting bats can also be highly gregarious with camps of fruit bats containing thousands of individuals, often representing more than one species, clustered within trees in close proximity to each other. In Australia, little red flying foxes (*Pteropus scapulatus*) hang together with up

to 30 individuals clustered on a single branch [25]. Other species of bats may roost in colonies of several dozen to hundreds of individuals while less gregarious species may roost in small colonies or singly. Roosting bats engage in many maintenance activities, such as grooming, and frequently move about the roost site. The close proximity of numerous individuals packed into dense concentrations can obviously facilitate virus transmission by direct contact, such as biting or licking and other means, such as through respiratory transmission or contact transmission by transfer of infectious secreta and excreta. It is in caves harboring millions of closely packed free-tailed bats that airborne rabies virus transmission was documented [26,27].

Bat flight and movements

Bats are the only mammals able to fly, and they may fly considerable distances from roost sites to feeding locations. Although most tropical bats will travel distances <200 km during a season when shifting roosts in response to the availability of fruit production by tropical trees [28,29], a few species, such as *Eidolon helvum*, will seasonally travel approximately 1500 km in one-way migrations from forest habitats to savannahs in Africa [29]. Pteropus species have been recorded traveling across open sea between peninsular Malaysia and Sumatra and between Australia and New Guinea [30]. Migratory behavior among temperate bat species has been categorized as sedentary, regional, and long distance [29]. Regional migration (typically <500 km) is common among European and North American species of *Myotis* while the long distance, one-way migrations of the subtropical/tropical Mexican free-tailed bats, *Tadarida brasiliensis*, exceed 1800 km [31,32]. Locally abundant but widely distributed fruit resources may serve to aggregate species of bats and other terrestrial fruit-eating mammals, such as great apes and ungulates, at feeding sites, thus potentially enhancing the risk of intra- and interspecific transmission of viruses. An example is the potential enhancement of interspecific Ebola virus transmission from a putative fruit bat reservoir host [11] during dry seasons when fruiting trees are restricted in number [33].

The long-distance migratory behavior of bats presumably influences the geographic distribution and genetic variability of lyssaviral variants associated with specific bat species (Table 21.1). The LN/PS variant of rabies virus is maintained by the silver-backed bat, *Lasionycterus noctivagans*, and the eastern pipistrelle, *Pipistrellus subflavus*, and this variant has been the most commonly recognized cause of indigenously acquired human rabies in North America over the last few decades [34,35,36]. The summer and winter range of *L. noctivagans* in North America extends from Central Canada to the Southern United States [37], overlapping the distribution of human rabies cases associated with the LN/PS variant (Figure 21.1). In Europe, phylogenetic studies of European bat lyssavirus (EBLV) subgroup 1a (EBLV-1a) suggest that virus trafficking between migratory bat species and the sedentary *Eptesicus serotinus*, a principal host species, has contributed to the genetic homogeneity observed among EBLV-1a isolates [38]. Within a bat species, such as *T. brasiliensis*, both sedentary and migratory populations may exist and intermingle seasonally [31,39], providing a mechanism for virus exchange and introduction.

Long-distance movements of bats may also lead to regular, but not constant, contact between individual bats from different subpopulations allowing partial connectivity between colonies of bats (e.g., *Pteropus* spp. in Australia). A metapopulation may exist where a spatial mosaic involves a constellation of subpopulations of which, at any given time, some are susceptible, some infected, and some immune to a particular disease. This may permit viruses to persist in a species with a total population that would otherwise be too small to maintain the pathogen [40].

Bat echolocation

Microchiropteran bats are the only land mammals to use "sophisticated echolocation" [17], although *Rousettus aegyptiacus*, a megachiropteran bat, uses a brief, low amplitude clicking that may aid in orientation [41].

Table 21.1. Recognized or proposed members of the genus *Lyssavirus*, family *Rhabdoviridae*

Name (Serotype/ genotype) ICTV abbreviation*	Species implicated in maintenance	Distribution	Annual human deaths	Reference
Rabies (ST 1/GT 1) RABV	Dogs, wild carnivores, bats > 50 spp.	Worldwide among dogs (with exception of Australia, Antarctica, and designated rabies-free countries): Restricted to New World bats	~55,000 (dog related)	[74,84,87, 88,221]
Lagos bat (ST 2/GT 2) LBV	Bats-Megachiroptera; *Eidolon helvum*, *Micropterus pusillus*, *Nycteris gambiensis*, *Epomophourus wahlbergi, Rousettus aegyptiacus*,	Africa: Central African Republic, Ethiopia, Egypt, Nigeria, Senegal, South Africa	Not reported	[102,108]
Mokola (ST 3/GT 3) MOKV	Shrew-Insectivora; *Crocidura* spp.; Rodentia; *Lopyhromys sikapusi*	Africa: Cameroon, Central African Republic, Ethiopia, Nigeria, South Africa, Zimbabwe	Occasional	[97]
Duvenhage (ST 4/G 4) DUVV	Bats-Microchiroptera; *Miniopterus schreibersii*, *Nycteris gambiensis*, *N. thebaica*	Africa: South Africa, Guinea, Zimbabwe	Occasional	[59]
European bat Lyssavirus 1 (GT 5) EBLV-1	Bats-Microchiroptera; *Eptesicus serotinus, Tadarida teniotis, Myotis myotis, Myotis nattererii, Miniopterus schreibersii, Rhinolophus ferrumequinum*,	Mainland Europe	Occasional	[38,54,222]
European bat Lyssavirus 2 (GT 6) EBLV-2	Bats-Microchiroptera; *Eptesicus serotinus, Myotis dasycneme M. daubentonii*	Europe, The United Kingdom	Occasional	[222,223]
Australian bat Lyssavirus (GT 7) ABLV	Bats-Megachiroptera; *Pteropus alecto*, *P. scapulatus, P. poliocephalus, P. conspicullatus*. Bats-Microchiroptera; *Saccolaimus flaviventris*	Australia, 1996; possibly SE Asia mainland	Occasional	[128]
Aravan virus** ARAV	Bats-Microchiroptera; *Myotis blythi*	Kyrgyzstan, 1991	Not reported	[224]
Khujand virus ** KHUV	Bats-Microchiroptera; *Myotis mystacinus*	Tajikistan, 2001	Not reported	[225]
Irkut virus** IRKV	Bats-Microchiroptera; *Murina leucogaster*	Eastern Siberia, 2002	Not reported	[225]
Wet Caucasian bat virus** WCBV	Bats-Microchiroptera; *Miniopterus schreibersi*	Caucasus Mountains, 2003	Not reported	[226]

* ICTV = International Committee on Taxonomy of Viruses.

** As yet unclassified new lyssaviruses [227].

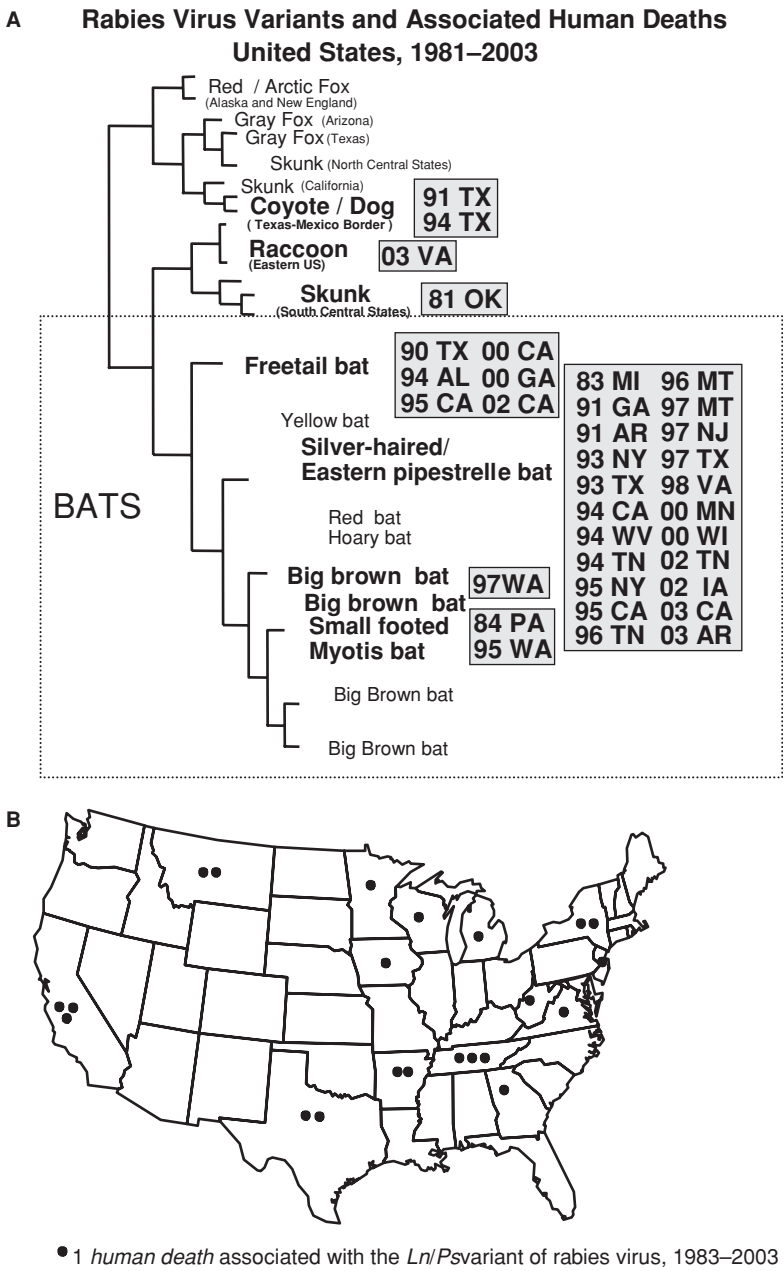

Figure 21.1. The phylogeny of rabies virus variants and associated human deaths in the United States, 1981–2003. (A) shows the phylogenetic relationships of various rabies variants, based on partial sequences of 320 bps of the N gene, associated with terrestrial mammals and bats in the United States. Note the large number of human deaths associated with *Lasionycterus noctivagans*/*Pipistrellus subflavus* [silver-haired/eastern pipistrelle (LN/PS) variant]. (B) shows the location, by state, of human deaths caused by the LN/PS variant of rabies virus. Human rabies deaths due to domestic dog exposures and "imported" into the United States do not appear on this figure.

The intense energy required to produce echolocation emissions [42] may promote virus transmission by aerosols or droplets when bats are aggregated and in close proximity. This transmission route has been hypothesized to occur with rabies virus as virus can be recovered from the mucous or respiratory fluids of infected bats [43]. The evidence supporting possible rabies virus transmission by an airborne route under natural conditions in caves harboring large colonies of Mexican free-tailed bats is discussed below in the section on transmission of lyssaviruses.

Bat hibernation and torpor

Most, if not all, temperate bat species are capable of entering into regulated torpor whereby their body temperature (T_b) is allowed to fall below ambient temperature (T_a) [44], and many species enter hibernation during winter [45]. Additionally, some tropical microchiropteran and megachiropteran species reduce T_b, but whether this is regulated torpor or caused by extreme peripheral vasoconstriction remains to be determined [44].

The impact of torpor and hibernation on the immune response and the persistence of viral infections among experimentally infected bats has been investigated for Japanese encephalitis virus (JEV) and rabies virus [46]. Decreased rabies virus activity has been inferred by subjecting experimentally infected bats (*Myotis lucifugus*,*T. brasiliensis*, and *Anthrozous pallidus*) to low temperatures (4°–10°C) and then observing the onset of rabies after transferring animals to temperatures of 22°–29°C [46,47,48]. The thermogenic organ, or brown fat, of bats has been suggested as a storage depot as rabies virus has been isolated from this tissue in experimentally infected bats kept at low temperatures [48] and from naturally infected bats [49]. JEV studies with persistence are described below in the section "Flaviviruses."

There is data suggesting that on rare occasions bats may experience abortive infection by rabies virus or unusually long incubation or latency periods [50]. The presence of neutralizing antibody among apparently healthy bats and the delayed development of rabies among captured bats has been interpreted as suggesting recovery from or prolonged incubation following infection [51,52]. Apparently healthy common vampires, *Desmodus rotundus*, surviving experimental rabies virus challenge can excrete virus in their saliva [53]. Similarly, apparently healthy bats have been shown to harbor low levels of EBLV RNA suggesting that there is a nonreproductive infection stage or subclinical persistence of viral RNA [54,55].

Bat longevity

Bats mature slowly and live long lives [56]. Several species of microchiropteran bat, *M. lucifugus*, *Plecotus auritus*, and *Rhinolophus ferrumequinum*, have been shown to have life spans exceeding 30 years in the wild (reviewed in [56]). This extreme longevity in a small mammal places bats well outside the traditional regression line scaling life expectancy to mammalian size [57]. The impact of extreme longevity on the potential for bats to maintain and transmit viruses could be enormous when coupled with the possibility of bats developing persistent infection following infection by certain viruses.

The role of bats as reservoirs of specific viruses

Lyssaviruses

Members of the genus Lyssavirus, and their association with specific Chiropteran hosts.

The single-stranded, negative-sense RNA viruses of the family *Rhabdoviridae*, Order Mononegavirales, exhibit an extraordinary host range, infecting plants, invertebrates, fish, and mammals [58]. However, viruses within individual genera of this family can exhibit exquisite host specificity as exemplified by the 11 viruses currently classified within or proposed as members of the genus *Lyssavirus* (Table 21.1) (see also Chapter 3). There are currently six serotypes (STs) and seven genotypes (GTs) recognized: classical rabies virus (RABV) is ST1/GT1; Lagos bat virus (LBV) is ST2/GT2; Mokola virus (MOKV) is ST3/GT3;

Duvenhage virus (DUVV) is ST4/GT4; European bat lyssavirus 1 (EBLV-1) is ST5/GT5; European bat lyssavirus (EBLV-2) is ST6/GT6; and Australian bat lyssavirus (ABLV) is ST1/GT7. In addition, four other viruses have been proposed as members of the genus: Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV), and West Caucasus bat virus (WCBV). With the single exception of MOKV, each of the representative genotypes of lyssaviruses has been isolated from microchiropteran or megachiropteran bats, which are also believed to serve as their reservoir hosts. Six bat-associated GTs (GTs 1,3,4,5,6,7) have been transmitted from bats to species of other mammalian orders, a process termed "spillover," causing fatal neurological disease among humans and other animals (Table 21.1). The term rabies was once strictly reserved for the acute fatal encephalomyelitis caused by rabies virus, ST1/GT1. However, the clinical disease of rabies is now widely used to include the clinically and pathologically indistinguishable diseases caused by any lyssavirus [59,60,61,62,63].

Lyssaviruses and subgroups among different GTs can be broadly differentiated by antigenic and immunologic characteristics and more finely by characteristic patterns of nucleotide substitutions in their genome. The genus *Lyssavirus* has been divided into two phylogroups of different virulence: phylogroup I includes RABV, GT 1; DUVV, GT 4; EBLV-1, GT5; EBLV-2, GT6; and ABLV, GT7; and phylogroup II includes LBV, GT2, and MOKV, GT3 [64]. Specific vaccines and immunoglobulins only exist for the pre- or postexposure treatment (PET) of phylogroup I rabies virus [65]; however, these vaccines elicit high levels of neutralizing antibodies to other phylogroup I lyssaviruses [66]. Immunization of laboratory animals with rabies vaccine with subsequent challenge with other lyssaviruses indicates that diminishing efficacy is a function of increasing phylogenetic distance from rabies virus [67].

Characteristic differences in sequence variation of lyssaviral isolates have permitted identification of the primary reservoir host species. Characterization and typing of distinct virus variants within a GT has provided insights into the evolution, host species range, and geographic distribution of specific genetic lineages of viruses circulating among bats and led to the identification of bat-associated variants, which through spillover, have caused rabies in humans and animals [38,68,69,70,71,72,73].

Rabies virus (GT1) and bats

The reservoir hosts for rabies virus are mammalian species in the Orders Chiroptera and Carnivora, although virtually all of the approximately 55 000 human deaths occurring globally each year are due to virus variants maintained by domestic dogs [74]. Clinical features of rabies are described in detail in Chapter 3. The first observation linking bats to rabies was made in 1911 when the common vampire bat (*D. rotundus*) was identified as the source of an epidemic of rabies among cattle in Brazil [75]. Human deaths attributed to bites received from vampire bats were first recorded from the island of Trinidad [76,77]. Outbreaks of human rabies due to vampire bats continue to be reported from Brazil [78,79], Costa Rica [80], Peru [81], Venezuela [82], and Mexico [83,84].

The recognition of insectivorous microchiropteran bats as reservoirs of rabies virus dates from 1953, when rabies was first described in a bat that attacked a 7-year-old boy in Florida [85]. Since that time, the number of rabid bats reported to CDC has increased, reaching 1408 in 2005 [86]. The majority of indigenously acquired human rabies cases in North America over the past 3 decades have been caused by bat-associated variants [35,50]. More than 30 species of North American bats have been identified as naturally infected by rabies virus [87,88], and the number of bat species infected in Mexico and in South America is rapidly growing [72,84,89,90,91]. Distinct virus variants of rabies virus may be associated with one or more bat species, and research into species specificity and the evolution of bat-associated rabies virus is rapidly changing our knowledge base on this subject [92,93]. Rabies virus (GT1) has not been isolated from bats outside North, Central, and South America.

Rabies virus variants circulating among bats are currently divided into four major groups and

several additional subgroups [93,94]. Group I (four subgroups) contains virus variants primarily originating from highly colonial, migratory bats of the genera *Myotis* and *Eptesicus*, and are endemic to Eastern Canada and the United States as far west as Colorado [93]. Group II (two subgroups) contains variants primarily originating from solitary or moderately colonial, migratory species of the genera *Lasiurus* and *Pipistrellus* and *Lasionycteris noctivagans* and are endemic to Canada and most of the United States; included here is a virus variant isolated from *L. noctivagans* and *P. subflavus* (LN/PS), which is the most common variant recovered from indigenously acquired human rabies in North America [35] (Figure 21.1). Group III contains variants from *Eptesicus fuscus*, genetically distinct from group I, and is restricted to Western Canada and the Western United States [93,94]. Group IV (three subgroups) contains variants originating from colonial species of hematophagous bats and insectivorous bats from Central and South America. Various subgroups and paraphyletic clades within groups preclude making any unequivocal statements concerning the endemic range and bat species infected by a particular group of viruses at this time [93]. As additional samples become available, finer resolution of phylogenetic relationships between virus variants and individual species can be achieved, as exemplified by recent studies of rabies virus from the genus *Pipistrellus* and *L. noctivagans:* the LN/PS variant may be two independently maintained rabies virus variants with distinct hosts [71].

African lyssaviruses (GT 2, 3, and 4)

In addition to rabies virus, three other lyssavirus GTs are endemic to Africa; DUVV (GT 2), LBV (ST 3/GT 3), and MOKV (GT 4) (Table 21.1) [95]. Bats are believed to be the reservoir hosts for DUVV and LBV [96]; the only wildlife species MOKV has been isolated from are shrews (genus *Crocidura*, order Insectivora) and rodents (*Lopyhromys sikapusi*, order Rodentia) [97,98]. Little is known about the epidemiology of African lyssaviruses other than rabies. Dogs are the major reservoir for GT 1 viruses in Africa, with indigenous species of wild carnivores serving as reservoir hosts within several regions [99]; African bats have never been implicated in rabies virus maintenance or transmission.

DUVV and MOKV have been linked to sporadic cases of fatal encephalitis among humans [59,100,101]; LBV has only been isolated from megachiropteran bats and domestic animals dying of rabies [96], with a single exception of one isolate from a water mongoose, *Atilax paludinosus*, order Carnivora [102]. DUVV, which is closely related to EBLV-1 [38], has been reported from Guinea, South Africa, and Zimbabwe; the only human deaths were from South Africa, northwest of Johannesburg, in 1971 and 2006 [59,101,103]. Although MOKV has never been recovered from a bat, humans and domestic cats and dogs have been diagnosed with rabies caused by MOKV over an extensive geographic range including Ethiopia, Cameroon, Central African Republic, Nigeria, South Africa, and Zimbabwe [95,100,104,105,106,107]. The first isolate of LBV was obtained from a fruit bat in Nigeria in 1956 [108], and since that time additional isolates have been obtained from fruit bats from Central African Republic, Egypt, Senegal, South Africa, and Zimbabwe [96,109,110,111], from single cats in South Africa and Zimbabwe [105,111], and a dog in Ethiopia [106].

European bat lyssaviruses (GT 5 and 6)

EBLV-1 (GT5) has been isolated from bats throughout Europe, mostly from the Serotine bat (*Eptesicus serotinus*), although the host range may be relatively broad, whereas EBLV-2 (GT6) has been associated exclusively with Myotis bats (*Myotis daubentonii* and *M. dasycneme*) with cases observed most recently in Netherlands, United Kingdom, and Switzerland [63,112]. From molecular studies, EBLV-1 and EBLV-2 have been further subdivided into two subgroups [38,63,112]; EBLV-1a has been primarily isolated from the non-migratory, colonial species *E. serotinus* in Northern Europe; EBLV-1b has been isolated from *E. serotinus* obtained from Northern Europe, France, and Spain; EBLV-2a has been primarily isolated from *M. dasycneme* from the Netherlands; EBLV-2b has

been isolated from *M. daubentonii* from Switzerland [38,54].

Since 1977, four human deaths have been attributed to EBLV: two from EBLV-1 and two from EBLV-2 [60,62,113,114,115,116]; the recent case of fatal EBLV-2 infection in a Scottish bat conservationist was the first indigenously acquired case of rabies in the United Kingdom in 100 years [62]. EBLV-1 has been recovered from terrestrial mammals, five sheep in Denmark [117,118] and a stone marten in Germany [119], and in captive zoo Egyptian fruit bats (*R. aegyptiacus*) in Denmark [120], but spillover is either rare or goes undetected. It is possible that the virulence of EBLVs is lower than that of GT 1 viruses; experimental inoculation of EBLV-1 into red foxes and sheep has resulted in death in only one of fourteen sheep [118,121,122]. Furthermore, EBLV-1 was detected in a range of tissues from apparently healthy bats (Schreiber's bent-winged bats, *Miniopterus schreibersii*, and greater horseshoe bats, *Rhinolophus ferrumequinum*) in Spain [54] and in healthy zoo fruit bats (*R. aegyptiacus*) [55], showing that bats may survive infection with possible long-term maintenance of the virus in infected healthy animals. There have been no reported spillover cases of EBLV-2 into either wild or domestic animals.

Human cell culture-derived rabies (GT1) vaccine prevented infection of mice from challenge with an EBLV-1 isolate from *E. serotinus* [123], as also demonstrated for EBLV-2 and ABLV [66]. As in Australia, humans exposed to potentially rabid bats in Europe are treated with traditional rabies biologics [124].

Australian bat lyssavirus (GT 7)

In 1996, the GT 7 *Lyssavirus* group was established when Australian bat lyssavirus (ABLV) was unexpectedly isolated from brain sample of a black flying fox (*Pteropus alecto*) collected in New South Wales, Australia [125]. ABLV is genetically most similar to rabies virus [126] and is classified as a serotype 1 virus. In Australia to date, ABLV has been found in four species of flying fox (genus *Pteropus*), as well as in the yellow-bellied sheath-tailed bat, *Saccolaimus flaviventris* [127]. Serological evidence of infection has been found in a number of other genera, indicating that the ecology and diversity of this virus is yet to be fully understood. Phylogenetic analyses have indicated that ABLV forms a monophyletic group which differentiates into two distinct clades, one associated with the four *Pteropus* species, and one with the insectivorous bat species, and that the two clades have a nucleotide divergence of up to 18.7% [128].

ABLV has caused two human deaths in Australia [129]. The first case involved a bat rehabilitator who had been scratched by a yellow-bellied sheath-tailed bat 5 weeks previously [130,131] and the second death occurred in a woman 2 years after she had received a bite from a flying fox while removing it from a child on whom it had landed [132]. In both cases, the disease was similar to that caused by classical rabies (GT1), namely a severe non-suppurative encephalitis associated with abnormal clinical signs, including hypersalivation, aggression, and agitation [130,132]. As ABLV is classified as a ST1 virus along with rabies virus, standard preparations of cell-culture vaccine and human immunoglobulin against rabies virus have been used to treat persons exposed to ABLV; this regimen protects mice in experimental challenges [66,127]. Bat rehabilitators and others likely to be exposed to infected bats are offered cell-culture vaccine. Additional research is currently underway to investigate the effectiveness of rabies vaccination in protecting against ABLV infections. No spillover cases of ABLV infection have been reported from other Australian mammals [133]. In limited studies to date, experimental exposure of domestic cats and dogs to ABLV appeared to cause occasional mild clinical signs, but no evidence of virus persistence could be found. Most of the exposed animals seroconverted; some had anti-ABLV antibodies in the cerebral spinal fluid [134]. This raises a number of questions about the susceptibility of terrestrial animals to bat lyssaviruses.

Other lyssaviruses isolated from bats

Other proposed members of the genus *Lyssavirus* have been isolated from insectivorous bats sampled

from Eurasia (Table 21.1). Based on genetic phylogenies and the diminished ability of rabies virus vaccines and immunoglobulins to protect immunized animals from challenge with the viruses, these newly identified viruses are quite distant from GT 1 lyssaviruses [67].

Transmission of lyssaviruses from and between bats

Transmission of bat-associated lyssaviruses occurs primarily by bite, when virus present in the saliva of an infected individual is directly inoculated into a susceptible individual. The potential for nonbite transmission of rabies virus among bats through saliva exchanged during mutual grooming has been suggested; transmission by such a mechanism may have precipitated an epidemic of rabies among kudu, an African ungulate [135]. Mexican free-tailed bats may transmit rabies virus *in utero*, as virus isolates have been obtained from cell lines established with fetal tissue [136]. Airborne transmission of rabies virus was suggested as the possible event leading to two cases of rabies in humans visiting a cave harboring millions of Mexican free-tailed bats [137,138]. In subsequent experiments, a number of caged animals placed within caves developed rabies, and rabies virus has been isolated from air sampled from these same caves [26,27,139]. Experimental aerosol infection of mice with RABV and EBLV-2 found that mice were highly susceptible to RABV infection by inhalation whereas EBLV-2 required direct intranasal inoculation [140]. Most recently, laboratory mice and wild-caught big brown bats (*E. fuscus*) and Mexican free-tailed bats were exposed to aerosolized rabies virus. All the bats and some of the mice survived exposure and produced rabies-neutralizing antibody, but this antibody provided poor protection for the bats to a subsequent challenge with rabies virus 6 months later [141]. Corneal transplants have been the source of human-to-human transmission of rabies virus on several occasions [142,143,144,145], and tissues transplanted from an individual infected by a bat-associated rabies virus variant caused multiple deaths among recipients in the United States [146]. Most human rabies cases caused by bat-associated variants of rabies virus have involved "cryptic" exposures, as patients or family members often cannot provide a positive history of bat bite [34,35,71,147].

Although spillover of bat-associated lyssaviruses to terrestrial mammals is rarely found in systematic surveys [69], clusters of bat-associated rabies have been documented in gray foxes (*Urocyon cinereoargenteus*) [148], red foxes (*Vulpes vulpes*) [149], and skunks (*Mephitis mephitis*) [73] in North America. Such data suggests that rabies epidemics among terrestrial mammals may in rare instances be seeded by spillover from bats.

Henipaviruses

The genus *Henipavirus* consists of two novel viruses – Hendra virus and Nipah virus – which have recently emerged from fruit bats of the genus *Pteropus* (*Pteropodidae*). Both have been associated with severe neurologic disease in animals and humans, and both are classified as biosafety level 4 (BSL4) agents because they pose a high risk of laboratory transmission and life-threatening disease. As a consequence, laboratory work involving live virus has to be done under BSL4 conditions. Hendra virus was first described in 1994 in Australia after a fatal disease outbreak in horses and humans in a horse-racing stable. Nipah virus was first described in 1999 in the investigation of a major outbreak of disease in pigs and humans in peninsular Malaysia.

Hendra virus

In September 1994, an outbreak of acute respiratory disease of unknown aetiology occurred in thoroughbred horses in a training complex in Brisbane (Queensland, Australia) [150]. The syndrome was characterized by severe respiratory signs and high mortality, with thirteen horses dying from acute disease. The trainer and the stablehand suffered a concurrent severe febrile illness, with a fatal outcome for the trainer. Quarantine procedures and movement restrictions were applied, including a complete

Table 21.2. Details of identified Hendra virus incidents in Australia, 1994–2006

Location[a]	Cases	Time
Mackay, Queensland	2 horses & 1 human	1994 (August)
Brisbane, Queensland	20 horses & 2 humans	1994 (September)
Cairns, Queensland	1 horse	1999 (January)
Cairns, Queensland	1 horse & 1 human	2004 (October)
Townsville, Queensland	1 horse	2004 (December)
Peachester, Queensland	1 horse	2006 (June)
Murwillumbah, New South Wales	1 horse	2006 (November)

[a] See Figure 2 map also.

shutdown of the racing industry, and epidemiological investigations commenced. The outbreak was contained, and within days exotic diseases were excluded, and a causal agent was identified [151,152]. The virus, a previously undescribed member of the family *Paramyxoviridae*, was initially named equine morbillivirus (EMV) but was renamed Hendra virus (after the Brisbane suburb where the outbreak occurred) when further characterization identified features inconsistent with morbilliviruses [153]. As of December 2006, seven Hendra virus incidents have been identified, with a total of 27 equine cases (20 fatal) and 4 human cases (2 fatal). All cases have been in Australia (Table 21.2, Figure 21.2).

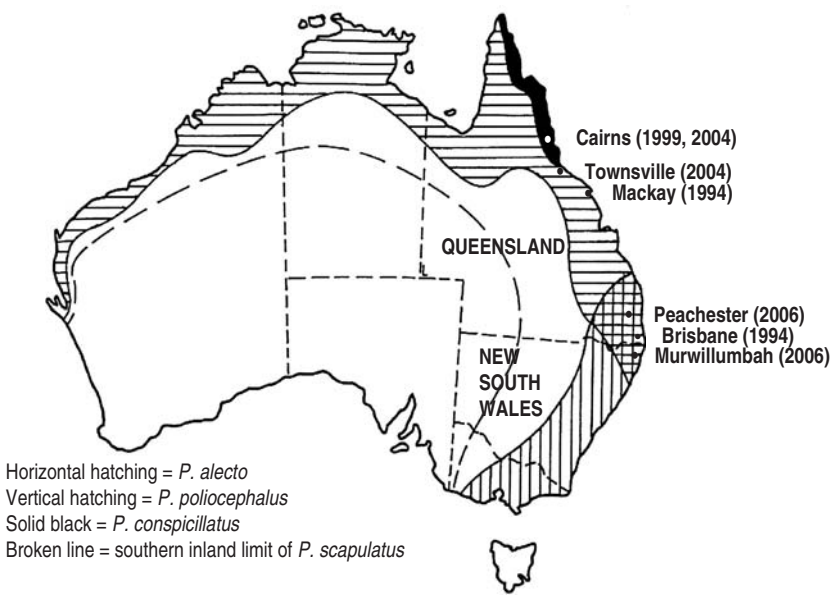

Figure 21.2. Location of Hendra virus incidents, and distribution of Australian flying fox (*Pteropus* spp.). Horizontal hatching = *P. alecto*; Vertical hatching = *P. poliocephalus*; Solid black = *P. conspicillatus*; Broken line = southern inland limit of *P. scapulatus*. Adapted from [25].

Nipah virus

A major outbreak of disease in pigs and humans occurred in peninsular Malaysia between September 1998 and April 1999, resulting in the death of 106 of 265 reported human cases and the culling of over 1 million pigs [154,155]. The outbreak spread to Singapore where a cluster of 11 cases with one death occurred at an abattoir [156]. Initially attributed to Japanese encephalitis virus, the aetiological agent was eventually shown to be another previously undescribed virus of the family *Paramyxoviridae*. Preliminary characterization of an isolate at the Centers for Disease Control and Prevention (CDC) in Fort Collins and Atlanta, United States, showed the new virus had ultrastructural, antigenic, serologic, and molecular similarities to Hendra virus [157]. The Malaysia outbreak primarily impacted pig and human populations, although horses, dogs, and cats were also infected. No cases of Nipah virus have been recorded in Malaysia since 1999. The disease reappeared, however, in Bangladesh and West Bengal, India, in 2001. Regular seasonal clusters of human Nipah virus infections have been recorded in Bangladesh since 2001, and in contrast to Malaysia, the Bangladesh cases appear not to involve a domestic animal cycle, and human-to-human transmission is evident [158,159,160,161,162,163,164] Outbreaks in West Bengal have occurred in 2001 and 2007 [165,166]. The number of human cases and geographic location are shown in Table 21.3.

Phylogeny

Elucidation of the complete nucleotide sequence of the matrix (M) and fusion (F) proteins and partial sequence information from the PV proteins established that Hendra virus had a greater homology with known morbilliviruses than with other genera of the family *Paramyxoviridae* [167]. However, the sequence comparisons also revealed substantial divergence with other morbilliviruses. Subsequent sequencing of the entire genome confirmed Hendra virus as a member of the subfamily *Paramyxovirinae* but identified differences that supported the creation of a new genus. Hendra virus had a larger genome size, the replacement of a highly conserved sequence in the L protein gene, different genome end sequences, and other sequence and molecular features [153]. "Henipavirus" was proposed as the new genus, with Hendra virus the type species and Nipah virus the second member. The International Committee on Taxonomy of Viruses (ICTV) has formally recognized the genus *Henipavirus*, and the virus names Hendra virus and Nipah virus [168] (Figure 21.3).

Species of fruit bats (sub-order *Megachiroptera*), commonly known as flying foxes, were eventually identified as the likely reservoir host of both Hendra and Nipah viruses [14,15,169,170]. This finding was a major breakthrough in understanding the ecology of these "new" viruses, and not only informed management strategies but precipitated further

Table 21.3. Nipah virus cases, 1999–2007

Dates	Location	No. cases	No. deaths	CFR(%)
Sep 1998–Apr 1999	Malaysia;	265	105	40
	Singapore	11	1	9
Feb 2001	Siliguri, W. Bengal, India	66	45	68
Apr–May 2001	Meherpur, Bangladesh	13	9	69
Jan 2003	Naogaon, Bangladesh	12	8	67
Jan–Apr 2004	Goalando, Bangladesh	29	22	76
	Faridpur, Bangladesh	36	27	75
Jan–Mar 2005	Tangail, Bangladesh	12	11	92
Mar–Apr 2007	Kushtia, Bangladesh	19	5	26
	Nadia, W. Bengal, India	5	5	100

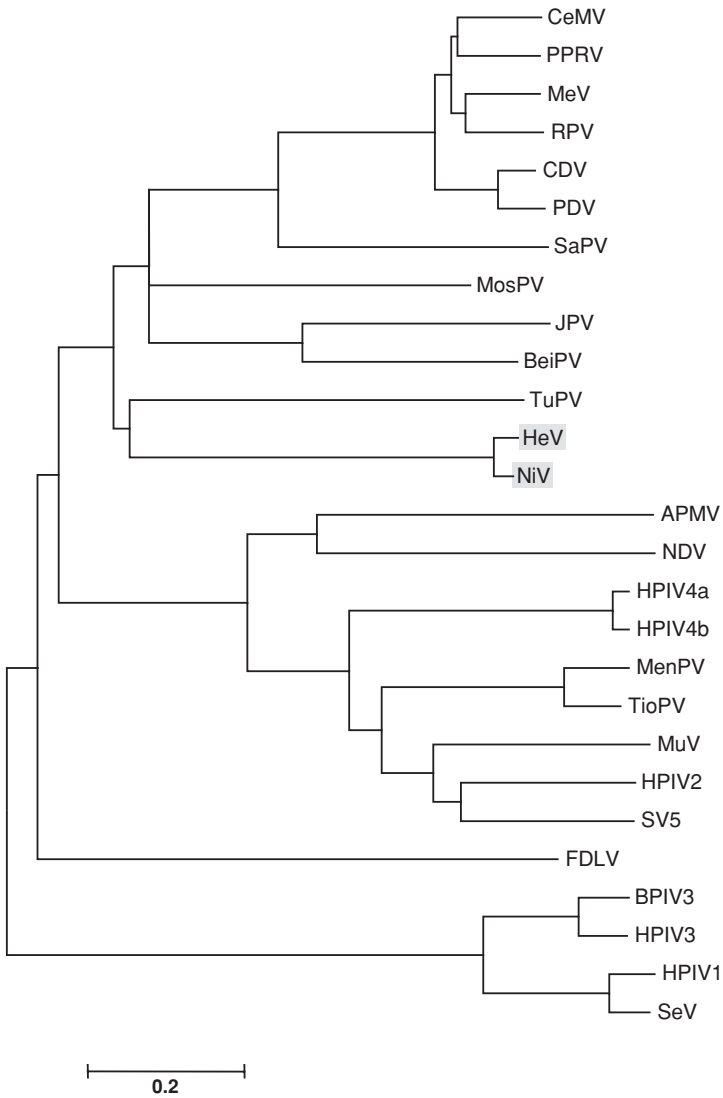

Figure 21.3. A phylogenetic tree based on the deduced amino acid sequences of the nucleocapsid protein (N) of members of the subfamily *Paramyxovirinae*. Branch lengths represent relative evolutionary distances. (Note: The new abbreviation system adopted in the 8th ICTV report is used.) APMV = Avian paramyxovirus, BeiPV = Beilong virus, BPIV3 = bovine parainfluenza virus 3, CDV = canine distemper virus, CeMV = Cetacean morbillivirus, FDLV = Fer-de-Lance virus, HeV = Hendra virus, HPIV1 = human parainfluenza virus 1, HPIV2 = human parainfluenza virus 2, HPIV3 = human parainfluenza virus 3, HPIV4a = human parainfluenza virus 4a, HPIV4b = human parainfluenza virus 4b, JPV = J-virus, MeV = measles virus, MenPV = Menangle virus, MosPV = Mossman virus, MuV = Mumps virus, NDV = Newcastle disease, NiV = Nipah virus, PDV = phocine distemper virus, PPRV = Peste-des-petits-ruminants virus, PoRV = porcine rubulavirus, RPV = Rinderpest virus, SaPV = Salem virus, SeV = Sendai virus, SV5 = Simian virus 5, TioPV = Tioman virus, TuPV = Tupaia paramyxovirus.

investigation of the ecology of henipaviruses and factors associated with their emergence.

The role of bats

The emergence of Hendra virus caused consternation for Australian animal and public health authorities. Zoonotic infections of horses were previously unknown, yet it became evident that the severe febrile illness suffered by the trainer and the stablehand in the Brisbane outbreak was attributable to infection with the new discovered virus through their close contact with the index horse case. When the novel nature of the virus was established [171], the search for its origin began. Surveillance of wildlife species was undertaken to evaluate the hypothesis that the virus existed in a wildlife reservoir. Serological screening of ubiquitous native and introduced fauna in the Brisbane index case paddock found no evidence of Hendra virus infection. Subsequent to retrospective identification of a Hendra virus outbreak in horses near the city of Mackay (1000 kilometers north of Brisbane), the focus of the wildlife surveillance shifted to species that were common to both locations and capable of moving between locations. Mammal species were given a higher priority than avian species. Of 27 flying foxes from two species tested in an initial survey, 40% had anti-Hendra virus antibodies by virus neutralization test [172]. The finding of neutralizing antibodies to Hendra virus in flying foxes was a major breakthrough in the search for the origin of Hendra virus. In September 1996, 2 years after the first reported outbreak, virus was isolated from a grey-headed flying fox (*P. poliocephalus*) [15]. A concurrent survey of over 1000 flying foxes from the four mainland species identified an estimated crude seroprevalence of 47% in Australian flying foxes. In a retrospective serological survey, antibodies neutralizing Hendra virus were identified in the sera of flying foxes collected in 1982 [172].

Investigation of the origin of Nipah virus was an integral part of the Malaysian outbreak investigation. When nucleotide sequence analysis showed that Nipah and Hendra viruses were closely related, Malaysian bat species were targeted, based on the established bat-Hendra virus link in Australia. Of 324 bats from 14 species surveyed in peninsular Malaysia, neutralizing antibodies to Nipah virus were found in 21 bats from 5 species, but predominantly in 2*Pteropus* species, *P. vampyrus* and *P. hypomelanus* [169]. Subsequently, Nipah virus was isolated from the urine of a colony of *P. hyomelanus* on Tioman Island in Malaysia [16] and from bat saliva on partially eaten fruit [170]. Nipah virus was also isolated from Lyle's flying foxes (*P. lylei*) in Cambodia [173].

Most strategies for managing Nipah virus infection have been directed at farm-gate security and reducing the potential for exposure of the spillover host (pigs) to flying foxes [174,175].

From virus isolation and various serological surveys in Southern and Southeastern Asia, and Australasia, there is little doubt that bats of the genus *Pteropus* are the major reservoir hosts of Hendra- and Nipah-like henipaviruses [176], and it should not be surprising for other related viruses to emerge throughout their range. The world distribution of the genus *Pteropus* extends from the west Indian Ocean islands of Mauritius, Madagascar, and Comoro, along the sub-Himalayan region of Pakistan and India, through Southeast Asia, the Philippines, Indonesia, New Guinea, to the southwest Pacific islands, and Australia. There are about 60 species in total. Flying foxes range in body weight from 300 g to over 1 kg, and in wingspan from 600 mm to 1.7 m. They are the largest bats in the world, do not echolocate and navigate at night by eyesight and their keen sense of smell. Females usually have only one young a year after a 6-month pregnancy. The young are independent after about 3 months. All species eat fruits, flowers or pollen, and roost communally in trees. Flying foxes are nomadic species, capable of traveling distances of hundreds of kilometers. Where the distributions of different species overlap, roosts are shared [25,177,178,179]. Thus the potential exists for interaction between flying fox populations across much of their global distribution.

Calisher and colleagues [4] review the apparent association between bats and emerging infectious diseases. They contend that information about the

natural history of most viruses in bats is limited, and specifically in relation to the family *Pteropodidae*, that only half of the 64 genera in this family (which includes flying foxes) have been adequately studied. Thus we know relatively little about the bats from which the henipaviruses have emerged. Calisher and colleagues [4] pose a number of questions in relation to the role of bats and emerging zoonoses. Do bats possess special attributes that equip them to host highly pathogenic zoonoses? Are emergences such as Hendra and Nipah viruses infrequent and incidental events, or are we detecting only the tip of the iceberg? They conclude by calling for pre-emptive potential pathogen screening in wildlife, rather than the outbreak-response surveillance that typically occurs currently.

Clinical presentation

Hendra virus in animals

The putative index case in Brisbane (September 1994) was a heavily pregnant thoroughbred mare at pasture. She was moved to a training stable for nursing and died within 48 hours. A further 12 horses in the stable and an adjoining training stable died in the following 14 days. Clinical signs included fever, facial swelling, severe respiratory distress, ataxia, and terminally, copious frothy (sometimes blood-tinged) nasal discharge. The incubation period based on clinical observations was 8–16 days. There were four nonfatal cases, two of which were left with mild neurological signs. A further three horses were subsequently found to have seroconverted in the absence of obvious clinical signs [151,152]. A small number of horses in the stable remained unaffected. A second Hendra virus outbreak in horses was retrospectively diagnosed in October 1995 after the Hendra virus-attributed death of a farmer who suffered a relapsing encephalitic disease. This second incident (1000 km north of Brisbane) chronologically *preceded* the Brisbane outbreak by several weeks and resulted in the death of two horses. The first horse, a 10-year-old heavily pregnant thoroughbred mare, died after exhibiting simlar symptoms to those in Brisbane over a 24-hour period. The second horse, a 2-year-old colt in an adjoining paddock, died 11 days later, again after a 24-hour clinical course [180,181]. Numerous other horses on the property remained unaffected.

Extensive investigations were undertaken in relation to these two outbreaks. No antibodies to Hendra virus were found in over 5000 domestic animals surveyed (including 4000 horses) [180,182], and no epidemiological link was identified between the two outbreaks. Retrospective investigations found no evidence of previous infection in horses in Queensland (P.J. Ketterer, personal communication; P.T. Hooper, personal communication).

A single case near Cairns in 1999 exhibited inappetance, depression, and swelling of the face, lips, and neck. Despite symptomatic treatment, the mare deteriorated and was recumbent the next morning with copious quantities of yellow frothy nasal discharge, and was euthanased. A companion horse was unaffected on clinical and serological examination [183]. Another case near Cairns in October 2004 had a clinical presentation suggestive of colic. Over a 24-hour clinical course, the horse exhibited restlessness, inappetance, and profuse sweating. Rectal temperature, heart rate, and respiratory rate were elevated, capillary refill time was slowed, and mucous membranes were injected. The horse had severe dyspnoea, a large amount of bloody froth flowing from the nostrils, and was very weak, unable to stand or hold his head off the ground. None of seven companion horses was affected.

In December 2004, a single case near Townsville, 400 kilometers south of Cairns, exhibited an elevated rectal temperature, moist rales, increased heart rate, and injected mucous membranes. The horse reportedly had a nasal discharge and a variable appetite during the previous week but had deteriorated in the final 48 hours, walking abnormally and repeatedly attempting to lie down (signs again suggestive of colic). In 2006, two unrelated cases were reported: the first in June near Peachester in southeast Queensland; and the second in October 2006, near Murwillumbah, 300 kilometers south. These cases had a number of features in common with previous index cases, including breed (thoroughbred),

age (>8 years old), housing (paddocked), and close proximity to flying fox feeding or roosting trees [184].

With the exception of the Brisbane and Mackay outbreaks (20 and 2 cases, respectively), all spillovers have involved single cases only, notwithstanding the presence of in-contact horses on each occasion. While overinterpretation of a sample size of seven spillover events is unwise, this observation suggests that the scale of the Brisbane outbreak is anomalous, and it is speculated that inadvertent human-assisted transmission (e.g., via shared saliva-contaminated bridles or blood-contaminated hypodermic needles) may have facilitated transmission. The typical absence of transmission to in-contact horses suggests that Hendra virus is not normally highly contagious in horses and that direct contact or mechanical transmission of infectious material is necessary for transmission to occur.

Hendra virus in humans

There are four known human cases of Hendra virus infection. In Brisbane in 1994, the horse trainer and a stablehand, both closely involved with the nursing of the index case, became ill and presented with myalgia, lethargy, headaches, and vertigo within a week of the death of the index case. The stablehand recovered, but the trainer developed pneumonitis, respiratory failure, renal failure, and arterial thrombosis and died a week later from cardiac arrest. At autopsy, both lungs were congested, hemorrhagic, and filled with serous fluid. Histology revealed focal necrotizing alveolitis with many giant cells, some syncytia formation, and viral inclusions. Infection with Hendra virus was demonstrated in both cases [185]. The third case was that of a farmer who presented first with meningitis and a 12-day history of sore throat, headache, vomiting, drowsiness, and neck stiffness. He appeared to fully recover but then developed a fatal encephalitis 13 months later and was admitted to hospital with a generalized tonic-clonic seizure after 2 weeks of irritable mood and lower back pain. Recurrent focal motor seizures occurred over the next week as did secondarily generalized seizures and low-grade fever. This was followed by dense right hemiplegia, signs of brain stem involvement, and depressed consciousness requiring intubation. The patient remained comitose and died 25 days after admission. Distinctive cortical changes were observed on magnetic resonance neuroimaging and histopathological examination of the brain at autopsy [186]. The equine cases in Mackay in 1994 were only identified after the death of the farmer 14 months after assisting his veterinarian wife's necropsy of two horses [187].

In November 2004, a Cairns veterinarian reported the onset of influenza-like symptoms a week after performing a necropsy on a horse. No antibodies to Hendra virus were detected by immunofluorescent antibody test (IFT) and enzyme-linked immunosorbent assay (ELISA) at that time, but a second blood sample taken 14 days later showed anti-Hendra virus antibodies by IFT and ELISA, and neutralizing antibodies were detected by virus neutralization test [184,188]. The patient remained well, and no relapse was observed over the next 12 months.

Human-to-human transmission was not observed, and a serological survey of bat rehabilitators found no evidence of bat-to-human transmission [189]. Thus all human cases arose from contact with sick horses.

Nipah virus in animals

Pigs on commercial pig farms were the predominant infected species in the Malaysian outbreak. Herd-level infection was typically subclinical, with estimated morbidity and mortality rates of 30% and 5%, respectively [155]. The incubation period was estimated to be 7 to 14 days. Observations of clinical cases suggested a varying presentation in different classes of animals. Affected weaners and porkers (2–6 months) typically showed acute febrile illness with respiratory signs ranging from rapid and labored breathing to harsh nonproductive cough. Attributed neurological signs included trembling, twitching, muscular spasms, rear leg weakness, and variable lameness or spastic paresis. Adult

sows and boars typically suffered a peracute or acute febrile illness with labored breathing (panting), increased salivation, and serous, mucopurulent or blood-tinged nasal discharge. Neurological signs including agitation and head pressing, tetanus-like spasms and seizures, nystagmus, champing of mouth, and apparent pharyngeal muscle paralysis were observed. The primary means of spread between farms and between regions was the movement of pigs. The primary mode of transmission on-farm was likely oronasal. Secondary modes of transmission between farms within local farming communities may have included roaming infected dogs and cats. Transmission studies in pigs in Australia at the CSIRO Australian Animal Health Laboratory established that pigs could be infected orally and by parenteral inoculation. It was observed that infection could spread quickly to the in-contact pigs. Neutralizing antibodies were detectable 10–14 days postinfection. Evidence of infection (virus isolation, IHC, serology) was also found in dogs and horses with neurological disease in the outbreak area [155]. Experimental infection in cats caused neurological disease [190].

The early epidemiology of the outbreak in the northern state of Perak, and the spillover mechanism that first introduced the infection to pigs, remains uncertain; however, retrospective investigations indicated that Nipah virus was responsible for sporadic disease in pigs in Perak since late 1996 [191]. Mathematical modeling supports the hypothesis that at least one spillover event occurred before the 1998–1999 outbreak, and that a level of residual immunity in sows provided the right herd immunological conditions for infection to become endemic in the pig index case farm in 1998, thus providing a sustained reservoir of virus from which to infect other farms [192] (J.R.C. Pulliam, J.H. Epstein, J. Dushoff1, S.A. Rahman, G. Meehan, M. Bunning, A.A. Jamaluddin, A.D. Hyatt, H.E. Field, A.P. Dobson1, and P. Daszak, unpublished observations).

The outbreak caused a drastic change in the direction of the future of pig industry in Malaysia. Pig farming is now allowed only in identified pig farming areas, with farmers in other areas encouraged to undertake other agricultural and livestock activities.

Nipah virus in humans

At least 105 people died during the course of the Malaysian outbreak. The majority of human cases had a history of direct contact with live pigs. Most were adult male Chinese pig farmers [154,193]. Identified risk factors for human infection in Malaysia were activities requiring direct contact with pigs, with handling sick pigs and assisting with birthing posing the highest risks. Most patients presented with acute encephalitis characterized by fever, headache, myalgia, disorientation, dizziness, vomiting, and more than 50% had a reduced level of consciousness [154,194]. The major clinical signs included areflexia, segmental myoclonus, tachycardia, hypertension, pinpoint pupils, and an abnormal doll's eye reflex. These clinical features suggested involvement of the brain stem and upper cervical spinal cord and were observed most commonly in those patients who had a reduced level of consciousness, generally a symptom indicative of a poorer prognosis [194]. Most patients who survived acute encephalitis made a full recovery, but about 20% had residual neurological deficits [194,195,196]. Neurological sequelae included cognitive difficulties, tetraparesis, cerebellar signs, nerve palsies, and clinical depression. A number of patients developed relapse encephalitis or late onset encephalitis. About 7.5% of patients who recovered from acute encephalitis and 3.4% of those who experienced nonencephalitic or asymptomatic infection developed late neurological disease, presenting several months to 4 years after the initial infection [194,197]. The clinical features associated with relapse and late onset encephalitis resembled those found with acute encephalitis, but there was a decreased incidence of fever, coma, and segmental monoclonus, and an increased incidence of seizures and focal cortical signs [197]. The mortality rate associated with relapse and late onset encephalitis was 18%, which was lower than the 40% associated with acute encephalitis. However, 61% of patients with relapse or late onset had further

neurological sequelae compared with 22% after acute encephalitis. The involvement of the cortex in relapse and late onset encephalitis suggests a different pathological mechanism compared with acute encephalitis. The occurrence and frequency of clinically undetected Nipah virus infections was also notable: 6% of persons from farms without reported encephalitis cases and 11% of persons from farms with reported encephalitis cases. In addition, 8% of cases reported no contact with pigs [193]. There was no evidence of human-to-human transmission during the initial outbreak in Malaysia.

Clinical presentation of human cases in Bangladesh and West Bengal, India, has typically been similar to that in Malaysia: fever, central nervous system signs, and a high case fatality rate. However, in 2004, a cluster of cases in the Faridpur district exhibited an acute respiratory distress syndrome. Human infection in Bangladesh has not been typically associated with disease in pigs (or other domestic species), and the pattern of transmission has suggested multiple-generation human-to-human transmission in some clusters. Evidence of horizontal transmission is particularly strong in the Faridpur respiratory cluster [160,163,198]. The number of reported cases and the case fatality rates of Nipah virus infection in Malaysia, Singapore, Bangladesh, and India are shown in Table 21.3.

Diagnosis

Six diagnostic methods have been described for the detection of henipavirus infection: virus isolation, electron microscopy, immunohistochemistry, PCR sequencing (see also Chapter 18), virus neutralization tests, and ELISA [199]. The first four techniques detect virus, virus antigen, or virus nucleotide sequence; the latter two detect antibody. Because Hendra and Nipah viruses are classified as BSL4 agents, tests necessarily involving live virus (virus isolation beyond primary diagnosis, serum neutralization tests) should only be carried out under BSL4 conditions after appropriate training.

The recent development of multiplexed microsphere assays presents an additional option for henipavirus serology, and one that promises simultaneous detection and differentiation of Hendra virus and Nipah virus-neutralizing antibodies without the constraints of working with live BSL4 agents [200].

Flaviviruses

There are many reports demonstrating serological evidence of flaviviral infections of bats, especially in microchiropteran bats, but there has been relatively little direct evidence to substantiate a role for bats in virus transmission cycles [4,6]. Various flaviviruses have also been isolated from bats, but it is not known whether they could be transmitted from bats to other species, including humans. Nevertheless, there is good circumstantial and experimental evidence to suggest that some bat species could serve as hosts for Japanese encephalitis virus (JEV), but the evidence for other members of the *Flavivirus* family is very tenuous at best. The ecology and clinical disease caused by encephalogenic flaviviruses has been described in more detail in Chapter 7.

Japanese encephalitis virus (JEV)

JEV has been isolated from a number of bats of the families *Pteropodidae*, *Rhinolophidae*, *Hipposideridae*, and *Vestpertilionidae*. Early studies found that high titers of virus could be detected in the brains of microchiropteran bats infected by intracerebral inoculation, and although the titers were as high as found in fatal murine infection, the bats appeared free from disease [201]. Early studies also demonstrated that mosquito-bat-mosquito transmission was successful at room temperature and at 10°C in simulated cave situations [202], thus making bats potential maintenance hosts of JEV and participants in wildlife transmission cycles. These very early findings led to some extensive investigations by Sulkin, Allen, and their colleagues (reviewed in [46]). Their studies together with those of others showed the following:

1. Nearly 100% of bats inoculated subcutaneously with small doses of virus developed viremia within 24–72 hours, and some animals circulated

virus for as long as 25–30 days at titers high enough to infect mosquitoes.
2. Bats did not develop encephalitis despite significant virus titers in the brain.
3. Following subcutaneous inoculation, replication was demonstrated in the brown adipose tissue, and this tissue was able to sequester the virus in an inactive state during hibernation, and then seed virus to provide further viremia once hibernation ended.
4. Transplacental transmission could readily be demonstrated, particularly in the latter stages of pregnancy, providing a mechanism for virus perpetuation in nature.
5. Anti-JEV antibody could not be reliably detected or measured by hemagglutination-inhibition, but only by neutralization.
6. Bats maintained at room temperature developed a viremia in 2–3 days postinoculation in most animals, which usually persisted for 10–15 days, and neutralizing antibodies developed in 3–7 weeks.
7. Bats maintained at 37°C developed a viremia more rapidly than those at room temperature and reached higher titers, but the duration of viremia was shorter and there was little evidence of replication in brown fat, brain, or kidney, and neutralizing antibodies responses were faster.
8. About 25% of bats at both temperatures failed to develop neutralizing antibodies despite being shown to be viremic.
9. Studies of field-caught bats collected in different seasons yielded 24 JEV isolates (16 from 1139 *M. schreibersii* and 8 from 267 *Rhinolophus cornutus*), with a significant number of isolates coming from those collected in the fall.
10. Neutralizing antibodies to JEV were found in sera from 5% of *M. schreibersii* and 9% of *R. cornutus*.
11. Isolations of JEV from bats were also extended to China (Taiwan), and one isolate was obtained from *Hipposideros armiger* and two from *M. schreibersii* (it is interesting to note that nine JEV isolates were also obtained from *Culex annulus* mosquitoes at the same time and cave as the latter *M. schreibersii* isolates).
12. Neutralizing antibodies to JEV were found in a number of other species of bats in Japan, including 21/79 *R. ferrum-equinum*, 9/72 *Myotis macrodactylus*, 4/25 *Myotis mystacinus*, 1/31 *Pipistrellus abramus*, 10/110 *Vespertilio superans*, and 1/22 *Plecotus auritus*.

More recently, neutralizing antibodies were found to JEV in 46 of 626 sera collected from insectivorous bats in Karnataka, India. The positive sera were from five species: *Hipposideros pomona*, *H. speoris*, *H. bicolor*, *H. cineraceus* and *Rhinolophus rouxi*. The incidence of antibodies in bats was reasonably well-correlated with the incidence of JE in humans in Kolar district during 1983 and 1985 [203], and it was suggested that bats may be involved in virus amplification.

The involvement of Megachiroptera, family *Pteropodidae*, or fruit bats, in the ecology of JEV was first indicated from studies in Thailand in which neutralizing antibodies to JEV were observed in 22 of 245 *Cynopterus brachyotis* (P.K. Russell, 1968, personal communication to [46]), a species widely distributed in Southeastern Asia from Thailand through peninsular Malaysia to Singapore, Sumatra, Borneo, Sulawesi, Lombok, and the Philippines. Experimental infection has been studied in two species of fruit bat in India, *Rousettus leschenaulti* [204] and *C. sphinx* [205]. The former study demonstrated a low level of viremia after subcutaneous inoculation of JEV lasting up to 9 days. In the latter study, bats were infected intramuscularly with JEV and, during the subsequent viremic phase, *Cx. bitaeniorhynchus* and *Cx. tritaeniorhynchus* mosquitoes were allowed to feed on them. Transmission was observed between bats, from bats to chickens, and from chickens to bats. Thus frugivorous bats are potential candidates for virus maintenance and may assist in virus movement. Most recently, van den Hurk and colleagues have shown that the black flying fox, *P. alecto*, could be infected with JEV after being bitten by infected *Culex annulirostris* mosquitoes. No viremia could be detected by either virus isolation or real-time polymerase chain reaction (PCR), but a small, significant number of recipient mosquitoes allowed to feed

on the infected flying foxes 4–5 days postinfection became infected. In addition, 60% of the flying foxes seroconverted (A.F. van den Hurk, C.R. Smith, H.E. Field, I.L. Smith, J.A. Northill, C.T. Taylor, C.C. Jansen, J.S. Mackenzie, G.A. Smith, unpublished observations). These results demonstrate that the black flying fox could potentially participate in natural transmission cycles of JEV.

Other encephalogenic flaviviruses

Two other members of the JEV-serological complex have been associated with bats, St Louis encephalitis (SLEV) and West Nile viruses (WNV). Early studies on natural and experimental infection of bats with SLEV were largely confined to the Mexican free-tailed bats (*T. brasiliensis*) and little brown bats (*M. lucifugus*) (reviewed by [46]). A number of isolates of SLEV were obtained from Mexican free-tailed bats during epizootic activity, and as isolations continued over winter, these investigations indicated that SLEV could persist in this species. Experimental studies in Mexican free-tailed bats and big brown bats (*E. fuscus*) found that SLEV produced an intense and long-lasting viraemia in the Mexican free-tailed bats and was maintained during hibernation in the big brown bats [46,206], providing further evidence of SLEV persistence in bats. Serological studies suggested that up to 9% of big brown bats and little brown bats were seropositive for SLEV in a nonepizootic period, suggesting that these species are involved in the maintenance of SLEV in enzootic foci and could have a role in dissemination of SLEV to epizootic foci [206]. While there is evidence that bats might play a role in the persistence, over-wintering and possibly in the spread of SLEV, the evidence for bats playing a role in the natural transmission of WNV is much less certain. Serological evidence of infection of bats with WNV has been reported from a number of countries (reviewed in [46]), but there has only been a single report of virus isolation from a fruit bat, *R. leschenaultia*, in India [207]. Interest in a possible role for bats in WNV transmission has increased since the emergence of WNV in North America, but although infection was confirmed in a big brown bat and a little brown bat in New York state, both of which were sick [208], only occasional bats have been found to have antibody to the virus, including big brown bats, little brown bats, and Mexican free-tailed bats [209,210,211]. There is no evidence to suggest they are involved in either transmission or persistence of the virus.

SARS-coronavirus

There is recent evidence to suggest that bats may be the ultimate reservoir of severe acute respiratory syndrome-associated coronavirus (SARS-CoV), although bats may not be directly involved in animal-to-human transmission (see also Chapter 4). Neurological symptoms from SARS-CoV infection were generally uncommon [212] and consisted of isolated reports of epileptic fits, mental confusion, and disorientation [213]. No focal neurological deficit or structural abnormality on computed tomography (CT) and magnetic resonance (MR) scans was found [212]. A number of patients developed affective psychosis during the acute phase of their illness associated with high-dose steroid use, personal vulnerability, and psychosocial stress [214].

There has been considerable effort by a number of laboratories and organizations to determine the possible reservoir host(s) of SARS-CoV, including a joint field study group under the auspices of the WHO, FAO, and OIE. The most direct evidence ascertaining the animal origin of SARS-CoV came from the direct isolation of almost identical viruses Himalayan palm civets (*Paguma larvata*) and a raccoon dog (*Nyctereutes procyonoides*) at a live animal market in Guangdong, China [215]. However, subsequent studies showed that palm civets in farms and field were largely free from SARS-CoV infection [216,217]. These results suggested that palm civets played a role as intermediate host rather than as a natural reservoir. Subsequent surveillance studies revealed the presence of a diverse group of coronaviruses closely related to the SARS-CoV in genome

organization and sequence, named SARS-like coronaviruses (SL-CoVs) or SARS-CoV-like viruses, in several horseshoe bat species in the genus *Rhinolophus* [8,9]. These discoveries raised the possibility that bats could be the natural reservoirs of SARS-CoV [218].

One interesting observation was the consistent failure of PCR detection in respiratory specimen for SL-CoVs in bats while high level of viral RNA was detected in anal swabs [9]. This suggested that fecal-oral contact is most likely the main route of transmission among bats and from bats to other wildlife animals. This also implied that direct contact between animals may not be a prerequisite for animal-to-animal transmission of this group of coronaviruses. Considering that bats and a diverse group of wildlife animals cohabitate in their natural environment (e.g., in caves) and that live bats are housed and traded with all sorts of different animals in live animal markets in Southern China and southeast countries, one would expect that there will be ample opportunities for fecal-oral transmission to occur.

The fact that SARS outbreaks have not occurred more frequently than observed so far might be due to the following two reasons. First, although SARS-CoV is able to infect a number of mammalian species, their susceptibility and tissue tropism vary significantly. Civets appear to be extremely susceptible in both natural infection [216,217] and laboratory challenge [219], and they shed viruses in feces. Second, the original bat viruses may not be very effective in infecting humans, and significant transmission in humans will only occur after the viruses have undergone an adaptation process in one or more intermediate hosts [220]. It is therefore unlikely for a direct bat-to-human transmission to occur without intermediate amplification and adaptation. Since it is not realistic or possible to control or eliminate the bat populations, the most effective risk management strategy to prevent human infection will be to identify and control the intermediate hosts (in addition to civets and raccoon dogs), which are highly efficient and competent in transmitting SARS-CoV from bats to humans.

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Viral oncolysis of glioblastoma

William T. Curry, Jr. and Robert L. Martuza

Viral oncolysis

"Viral oncolysis" refers to the ability of some viruses directly to kill cancer cells by infecting them, replicating intracellularly, and then lysing the cells as infectious viral progeny are released and subsequently infect surrounding cancer cells. Ideal anticancer agents specifically target neoplastic cells, effectively kill them, and are nontoxic both systemically and to surrounding tissues. Molecular engineering techniques have permitted design of viral "vectors" that retain the ability to replicate yet are nonpathogenic. Viral oncolysis differs from what is typically thought of as "gene therapy" in that efficacy depends not on the efficient transfer of a gene of interest into a cancer cell but rather on the ability of the virus itself to kill the cell. For instance, "replication-defective" or nonreplicating viruses have been used in preclinical models and cancer clinical trials to transfer genes that correct cancer-associated genetic defects such as p53 mutations or encode for prodrug-activating enzymes, such as for HSV-thymidine kinase (HSV-tk) or cytosine deaminase [1]. Other delivered genes include those that inhibit angiogenesis or stimulate antitumor immunity. Nonreplicating viruses can be further modified to enhance tropism for their intended cancer targets and to more specifically and efficiently transfer genes. On the other hand, viral oncolysis depends fully on the ability of the virus to replicate in tumor tissue. Safe, targeted viral oncolysis is made possible by the ability to engineer DNA viruses in the laboratory for tumor selectivity (HSV and adenovirus) or by use of wild-type or spontaneously arising attenuated RNA viruses with intrinsic tumor selectivity (reovirus, Newcastle disease virus, poliovirus, measles, and vesicular stomatitis virus). The ability to replicate, though a *sine qua non* for oncolysis, is not necessarily sufficient. Replication-competent retroviruses (RCR) have been engineered to allow efficient and tumor-restricted replicative spread in a variety of cancer cell lines, including glioblastoma [2], but they require delivery of transgenes for cytocidal effect.

The fundamental paradigm of viral oncolysis is as follows: virus enters and infects a target cancer cell. Viral replication occurs intracellularly, lytic cell death occurs, and numerous viral progeny are released and then infect surrounding tumor cells. The cycle continues either until tumor cells are eradicated or the immune system eliminates the viral particles. Safety is achieved by targeting the virus specifically to tumor cells or by using a virus that is nonpathogenic in surrounding tissues.

History of viral oncolysis

The concept that viruses might function as anticancer agents extends to the nineteenth century when, in several patients, it was observed that viral infection coincided with regression of malignant disease [3]. Typically, this occurred in young immunosuppressed patients with hematological malignancies (leukemia or lymphoma), the effect was transient, and the causative infection was documented to be varicella or measles virus. Kelly and Russell [3] provide a detailed review of the history of

oncolytic viruses, commenting as well on some of the ethically questionable clinical trial practices of the mid-twentieth century. Four clinical virotherapy trials of historic significance are described. In 1949, 35 patients with Hodgkin's lymphoma were treated with parenteral injection of sera or tissue extracts containing "hepatitis virus" obtained from patients with known hepatitis infection. Several short-lived responses were documented; toxicities, including hepatitis infection, were not well-recorded. Three years later, 34 patients with advanced malignancies of various pathologies underwent inoculation with the "Egypt 101" isolate of West Nile virus. A few tumor responses were accompanied by several cases with severe neurotoxicity; immunosuppressed patients were the most likely to respond but also suffered from encephalitis.

Adenovirus, then known as adenoidal-pharyngeal-conjunctival (APC) virus, gained newfound attention as a potential oncolytic agent because of its relatively well-tolerated toxicity profile. In 1956, 30 patients with epidermoid carcinoma of the cervix were treated with adenovirus, administered intraarterially, intratumorally, or intravenously. Local antitumor effect was seen in more than half of the patients, consisting usually of intratumoral necrosis and the shedding of large amounts of malignant tissue. Survival was not prolonged, however, foreshadowing some of the results of future gene therapy trials wherein the effects of viral treatment and gene delivery were impeded by immune-mediated abrogation of the adenovirus life cycle.

Mumps, a widespread paramyxovirus, gained favor after preclinical studies and clinical trials using enteroviruses, vaccinia, vesicular stomatitis virus (VSV), and picornaviruses were shown to lack meaningful efficacy against a number of cancers. Deviating from use of an inactivated form of mumps known to be an immune adjuvant in melanoma vaccine trials, Asada in Japan [4] inoculated 90 cancer patients with nonattenuated mumps virus preparations via a range of delivery approaches. The results, published in 1974, were very promising for a high degree of antitumor response and limited toxicity but could not be replicated in subsequent trials.

Study of oncolytic viruses waned after this initial era, as the toxicities associated with effective anticancer effect were deemed too severe to warrant further clinical testing. However, the advent of techniques in genetic engineering in the 1980s and 1990s created renewed enthusiasm for the development of targeted and nontoxic conditionally replicating viruses, development and clinical examination of which continues today. In 1991, this new approach was introduced in a model of oncolytic herpes simplex virus (HSV) infection of human glioblastoma, both *in vitro* and in mice [5]. Since then, hundreds of preclinical studies using oncolytic viruses in many different cancer models have been reported, and a large number of clinical trials have demonstrated safety as well as provided some suggestion of effective antitumor therapy.

Oncolytic viruses for glioblastoma

"Astrocytomas" are a subset of glial tumor (or "gliomas") derived from astrocytes, supporting cells in the brain and spinal cord. Dysregulated growth leads to proliferation of cells and invasion of normal central nervous system structures. The World Health Organization (WHO) grades astrocytomas from I to IV, with grades III and IV considered "malignant." Grade IV tumors are also known as "glioblastoma." Adults rarely have grade I tumors, which are largely curable with complete excision or can be indolent. Grade II tumors typically progress over time, despite treatment, and eventually transform into a more malignant type.

Glioblastoma is both the most aggressive and the most common of primary brain tumors. Glioblastoma afflicts 4.5 per 100 000 American adults. Despite advances in diagnosis, surgical technique, radiation methodology, and chemotherapy, survival rates remain poor. Complete surgical excision is essentially impossible because of the infiltrative nature of the tumor; microscopic disease almost always exists centimeters away from the grossly or radiographically distinguishable tumor. With the combination of surgery followed by concomitant

temozolomide chemotherapy and radiation, mean survival has reached 14.6 months, and 2-year survival may reach 26% [6]. Glioblastoma becomes resistant to therapy and recurrent disease, at this time, is relentlessly progressive. Also, astrocytomas typically recur within a 2 cm margin of the primary tumor, making it reasonable that more effective local therapy might prolong survival [7]. Glioblastomas are both pathologically and molecularly heterogenous. Common mutations include, but are not limited to, 1688loss or mutation of p53, epithelial growth factor receptor (EGFR), amplification or mutation, PTEN mutation, loss of heterozygosity at chromosome 10q, and deletion of p16 (INK4a) [8]. Deeper understanding of the molecular characteristics of these tumors have led to development and application of rational and promising targeted therapies, such as small-molecule inhibitors and monoclonal antibodies; however, clinical outcomes, thus far, have been disappointing [9,10]. The molecular heterogeneity both between and within tumors presents a great therapeutic challenge. A great need, therefore, exists for novel approaches, and the development of oncolytic viruses will continue to play a prominent role.

In this chapter, we review the current state of replication-competent or replication-selective oncolytic viral treatment of malignant astrocytomas. This includes studies examining the DNA viruses herpes simplex type I and adenovirus, both of which can be engineered to replicate only in dividing cancer cells, and RNA viruses, including reovirus, measles virus, Newcastle disease virus (NDV), VSV, some of which replicate selectively in brain tumors because of transformed cellular defects in antiviral immunity and others that require modification to render them nonpathogenic in healthy brain tissue while destroying resident tumor cells. Of the oncolytic viruses reviewed, two are truly neurotropic: HSV and polio. Wild-type infection in the central nervous system with either HSV or poliovirus is associated with well-characterized and debilitating neurological syndromes encephalitis and poliomyelitis, respectively. The neurotropism of HSV and polio makes adaptation for use against glioblastoma seem natural and likely contributes to efficacy of infection; however, avoidance of neurotoxicity and, if necessary, treatment of and abrogation of virus-associated neurotoxicity must be foremost when considering design of these agents. The other viruses described in this chapter are not neurotropic, *per se*, but have natural or enhanced tropism for malignant cells.

An oncolytic virus for glioblastoma should have the following qualities:

1. Replication and cell lysis should be limited to tumor cells, and neither surrounding tissues nor distant organs should be impacted.
2. The virus must be able to propagate and disperse progeny across a wide area, allowing for infection of cells in the leading edge of invasion.
3. Viral oncolysis must be able to proceed in the setting of an intact antiviral immune response.

Herpes simplex virus

HSV-1 is particularly attractive as an oncolytic agent for brain tumors for several reasons, not least of which is its neurotropism and known capacity for replication in nervous system tissues (see also Chapter 11). Wild-type HSV-1 causes encephalitis and, therefore, could never be considered a safe option for injection intracerebrally. However, sequencing of the HSV-1 genome allowed identification of the genes associated with neurovirulence and led to study of modifications that make administration into brain tumors safe for the host. Advantages of HSV-1 include the following: (1) Up to 30 kb of viral genome can be replaced with foreign DNA without disabling replicative capacity in the appropriate cellular environment. (2) Multiple genes associated with neurovirulence can be deleted without affecting the virus' capacity to replicate within tumor cells and to destroy them during the normal lytic phases. (3) Effective and widely available anti-HSV medications can be administered in the event of local or systemic infection. (4) HSV-1 does not integrate into the host genome, remains episomal, and, therefore, is incapable of insertional mutagenesis, as opposed to a retrovirus infection, which is potentially carcinogenic [1].

Detailed description of the HSV-1 genome is covered in other chapters in this text, but a summary is important in the context of the specific engineering that has permitted effective oncolysis [11]. The genome consists of unique long and short segments, flanked by inverted repeats. During lytic infection, HSV-1 gene expression occurs in an ordered temporal sequence. Immediate-early α genes (including α47, discussed later in this section) regulate gene transcription. Next, β genes promote viral DNA synthesis, including expression of viral DNA polymerase, the large segment of ribonucleotide reductase (UL39), and HSV-TK (UL23). Finally, the γ genes create an environment permissive for viral protein synthesis, contribute to encapsulation of DNA, and support viral envelope formation. The γ34.5 gene is present in two copies in the terminal repeats and is responsible for neurovirulence, neuroinvasion, prevention of the shutoff of host protein synthesis that occurs in response to infection, viral egress and glycoprotein processing, and inhibition of cellular autophagy. Mutants with deletions in both copies of γ34.5 are, therefore, avirulent in the central nervous system and, in part because of their inability to prevent viral-associated shutoff of host protein synthesis, are also relatively attenuated in their ability to replicate. The first HSV-1-engineered mutants for the treatment of brain tumors included those with deletions or alterations in HSV-TK, the large subunit of RR, or in both copies of γ34.5.

Single-mutated oncolytic HSV-1 for brain tumors

Actively dividing tumor cells upregulate endogenous thymidine kinase (TK), providing enzymatic activity in support of TK-negative mutant HSV-1 such as *dl*sptk [5]. In 1991, it was demonstrated that, at low titer, *dl*sptk could destroy a glioma cell monolayer in culture within several days and that intratumoral inoculation in the brains of athymic mice resulted in a dose-dependent regression of tumors and prolongation of survival, with several cures. Effect was shown to be dependent upon actual viral replication. Efforts with *dl*sptk were abandoned prior to clinical translation because later studies demonstrated that, at high titers, the virus could be neurotoxic. In addition, unlike other mutants where viral TK remained intact, *dl*sptk was insensitive to treatment with acyclovir or ganciclovir, which requires TK phosphorylation for its antiviral effect.

Working essentially in parallel, several groups began study of RR mutants and viruses lacking one or both copies of the γ34.5 gene. RR mutants such as hrR3 replicate conditionally in dividing tumor cells, again, because of upregulation of cellular ribonucleotide reductase, which complements the viral mutation. Two separate studies highlighted the efficacy of treating malignant glioma with RR mutants, and there has been little neurotoxicity [12,13,14]. However, again, because of fear of encephalitis as well as concerns about potential for recombination or acquisition of a conversion mutation, singly mutated RR HSV-1 agents have not been brought to clinical trial in patients with astrocytomas.

γ34.5 mutants, as described above, do not have the capacity to replicate within healthy adult neurons and cannot cause encephalitis [15]. They do retain susceptibility to acyclovir and ganciclovir. R3616 has both copies of γ34.5 deleted. γ34.5 mutants replicate selectively in dividing cells because of mitotic cellular upregulation of the DNA repair enzyme GADD34, whose carboxyl terminus can substitute for the homologous γ34.5 [16]. γ34.5 mutants lack the capacity to shut off the cellular stress response that leads to postinfectious apoptosis in normal, quiescent cells and can therefore replicate in tumors, achieving effective and safe oncolysis in several preclinical intracranial glioma models [17,18]. In short, γ34.5 mutants will not replicate in neurons but will do so in tumor cells that have upregulated GADD34.

The low toxicity profile and the preclinical efficacy of γ34.5 mutants have led to execution of phase I clinical trials in patients with glioblastoma, details of which are discussed below. Concerns exist regarding the use of any singly mutated HSV-1 in the brains of patients. First, it may be possible for intracerebral delivery of an engineered HSV-1 to reactivate latent wild-type HSV that is already present. Also, recombination with latent wild-type HSV-1 is a

theoretical possibility. Wang *et al.* [19] demonstrated that in rats with latent wild-type HSV-1 infection (kos) via the cornea, introduction of hrR3 into the central nervous system (CNS) was not associated with reactivation or with encephalitis. Reactivation has not been demonstrated in any experimental model. Recombination with wild-type HSV-1 has not been fully studied and has not been observed. It has been shown, however, that a γ34.5-deleted HSV-1 can mutate during serial passage and can reacquire the wild-type HSV-1 phenotype of sustained late protein synthesis – diminishing its safety profile in the brain and elsewhere [20]. The subsequent development of a multimutated oncolytic HSV-1 provided insurance against viral reversion to wild-type.

Multimutated oncolytic HSV

G207 is a multimutated oncolytic HSV-1, developed from a Strain F backbone [13]. Both γ34.5 genes are deleted, and a lacZ insertion inactivates UL39, the gene for the large subunit of ribonucleotide reductase. As such, it combines replication selectivity in dividing cells with safety, afforded not only because of the γ34.5 deletions but also by the wide spacing between the mutations. Reversion at one of the loci would still leave a vector that is markedly attenuated for neurovirulence, and it would be highly unlikely for both loci to undergo reversion. G207 effectively kills a wide range of murine and human tumor cell types, and efficacy has been established in multiple preclinical models. For instance, in U87 glioma tumors implanted in the frontal lobes of athymic mice a single intratumoral injection of 2×10^6 pfu G207 resulted in significantly longer survival in mice. All mock-treated animals died by day 38, and the median survival for mice receiving G207 was significantly longer, with 2 of 13 surviving at 6 months post-treatment ($p<0.01$ at day 38 and $p<0.0001$ at 6 months using Wilcoxon and log rank tests [13]).

By adulthood, most humans develop immunity to HSV-1. However, this may not negatively impact the antitumor effects of treatment with G207. In a subcutaneous neuroblastoma tumor model in immunocompetent syngeneic mice, efficacy of treatment with intratumorally injected G207 was not affected by prior HSV immune status [21].

G207 has undergone significant safety testing, both in mice and in *Aotus Nanymae*, a primate species that is hypersensitive to HSV [22,23]. Sixteen Aotus monkeys were inoculated by intracerebral injection with G207, wild-type HSV-1 strain F, or buffer. Thirteen animals received G207 at doses of 1×10^7 or 1×10^9 pfu, one was inoculated with 1×10^3 pfu wild-type HSV-1 strain F, and two were inoculated with buffer. Strain F caused rapid mortality and symptoms consistent with HSV encephalitis, including fever, hemiparesis, meningitis, and hemorrhage in the basal ganglia. In contrast, 1 year after G207 inoculation, seven of the thirteen treated animals were alive and exhibited no evidence of clinical complications. Of the other six animals, three died from non-neurologic causes unrelated to HSV infection, and three animals were sacrificed for histopathological examination. In a further evaluation, two animals were reinoculated with 1×10^7 pfu G207 at the same stereotactic coordinates 1 year after initial inoculation with G207. These animals remained healthy during the 2-year observation period following the second inoculation. Magnetic resonance imaging (MRI) before and after G207 inoculation revealed no evidence of HSV-related sequelae. Histopathological analysis of multiple organ tissues showed no evidence of HSV-induced histopathology or dissemination.

Oncolytic HSV-1 clinical trials in patients with malignant glioma

On the basis of safety analyses, including the above-described *Aotus* model, a clinical trial using G207 was initiated in patients with recurrent malignant glioma [24]. In this phase I dose-escalation trial of stereotactic intratumoral injection of G207, the maximal tolerated dose of virus was not achieved, as no adverse events secondary to administration of virus were recorded. Twenty-one patients were treated with doses beginning at 1×10^6 pfu in 0.1 ml. Patients treated at the highest dose received 3×10^9 pfu. Virus was not identified in patient saliva or conjunctival

secretions. One of five HSV-seronegative patients converted following G207 treatment. Fourteen of 20 patients had posttreatment radiographic diminution of the enhancing portion of their tumor as measured by gadolinium-enhanced brain MRI. Average time to disease progression after injection was 3.5 months. There were four long-term survivors (7–19 months at the time of manuscript submission). No histopathologic specimen demonstrated encephalitis or HSV antigen by immunostaining. This clinical trial demonstrated that oncolytic HSV-1 can be engineered for safe administration into malignant brain tumors in patients without viral shedding and without delayed reactivation of the virus.

HSV-1716 is a γ34.5 null vector that also has efficacy against numerous cell types, including gliomas. Despite some evidence that HSV-1716 may retain pathogenicity in the CNS [25], two clinical trials in malignant glioma patients have proceeded without evidence of therapy-related adverse events or toxicities [26,27]. In one of the HSV-1716 clinical protocols [27] the tumor was inoculated with oncolytic virus through a stereotactically guided needle. Several days later, gliomas were resected by craniotomy, and the tissue was analyzed for viral replication and HSV antigens. In two patients, replication of virus was confirmed by recovery of infectious virus at higher levels than inoculated. Again, no therapy-related toxicity including encephalitis, edema, or viral shedding was identified.

To date, phase I trials utilizing oncolytic HSV-1 in patients with malignant glioma have been marked by feasibility of intratumoral injection as well as safety. Viral particles do not seem to shed into accessible body secretions/fluids. In addition, there has been suggestion of biological activity by way of imaging and studies of intratumoral viral replication.

Immune aspects of oncolytic HSV-1

Much of the initial preclinical work on oncolytic HSV-1 was performed in athymic or SCID mice, making study of the immune response to viral infection impossible. However, more recent studies have emphasized analysis and manipulation of the immune responses generated by oncolytic HSV-1. Chalavi *et al.* demonstrated that prior exposure to HSV-1 and immunoglobulin seropositivity did not negatively impact G207 oncolysis in subcutaneous tumors [21]. Furthermore, multiple inoculations of virus into tumor over time were more likely to eradicate the lesion than a single injection – again suggesting that development of antiviral immunity did not significantly hinder oncolysis. Similarly, dexamethasone, a corticosteroid commonly used to treat brain tumor-associated edema with well-known immunosuppressive effects, did not alter or enhance viral replication in a syngeneic murine tumor model [28]. On the other hand, several studies have demonstrated that inhibiting development of innate immune responses allows for enhancement of oncolytic virotherapy, including for glioma [29,30,31]. Preadministration of cyclophosphamide (CPA), an alkylating agent, allows increased intratumoral replication of HSV by suppressing immune function. In a rat intracranial glioma model, virus was cleared more slowly from tumors in the CPA-treated group [29]. CPA reversed the HSV-mediated increases in mononuclear cells after infection of brain tumors, and tumor infiltration of phagocytic cells was blocked. Similarly, administration of CPA allowed for dose reduction of an oncolytic HSV-1 in treatment of a murine glioma [31]. Reduced dosing could help make clinical translation more feasible, alleviating the need to scale up to high-titer virus production, which is labor intensive and costly.

Although immunosuppression via alkylating agents or other inhibitors of innate immunity may be an attractive means of enhancing viral oncolysis, it may offset the advantageous "vaccine" effect that oncolytic HSV-1 infection of tumors seems to induce. Infection of a subcutaneous flank tumor in a syngeneic mouse with G207 results in an effective, cytocidal antitumor immune response that can be directed against a synchronous contralateral flank tumor or an intracranial tumor [32]. This response is $CD8^+$ T lymphocyte-dependent and can be abrogated with immunosuppressive corticosteroid treatment [28]. Cured animals reject tumor cells injected months later, demonstrating

durable immune memory. Oncolytic HSV-1 can be further engineered to express proinflammatory cytokines such as GM-CSF or IL-12 with immune-enhancing effects [33]. Recently, a "triple combination" of oncolytic HSV vectors bearing transgenes for IL-12, IL-18, and soluble B7–1 (a costimulatory molecule associated with effective antigen presentation) showed enhanced antitumor efficacy when compared to backbone oncolytic HSV without transgenes or to virus bearing any of these genes alone [34].

Particularly in environments where HSV does not replicate well, taking advantage of virus-generated antitumor immunity may be an effective way of treating established tumors. Delivery of some proinflammatory transgenes directly in the central nervous system may not be appropriate as heavy inflammation may be less tolerable than in other sites, but this deserves continued study. Also, although preadministration of CPA permits enhanced viral replication, it is unknown how this affects the antitumor immune response. Does insertion of a proinflammatory transgene impair viral replication while enhancing antitumor immunity? These issues require further study in the laboratory.

Increasing the cell specificity of HSV vectors

Although the deletion of genes such as ribonucleotide reductase, thymidine kinase, and γ34.5, confers replication selectivity in HSV to dividing cancer cells, this comes at some cost to virulence and effective lytic cellular destruction. Other means of targeting viruses to cells have been explored, including transcriptional regulation of viral gene expression. For instance, immediate-early gene transcription can be placed under the control of a tissue or tumor-specific promoter. In oncolytic HSV-1 G92A, the albumin enhancer/promoter sequence drives expression of the gene for ICP4, which is required for replication [35,36,37]. As a result G92A replicates 1000-fold greater in hepatoma cells that are expressing albumin. As another example, oncolytic HSV-1 has been engineered to specifically divide in tumor cells with strong beta-catenin/T cell factor signaling. Beta-catenin/T cell factor is an oncogene that can be upregulated in some cancers due to APC or beta-catenin gene mutations [38]. Once viral replication is initiated in these tumors, there is no attenuation in response, and it may be feasible to use lower titers than with the current replication-conditional vectors.

Combining oncolytic HSV-1 with established therapies for glioblastoma

A successful approach to glioblastoma therapy will likely be multimodal and combine locoregional therapies such as surgery and radiation with systemic treatments such as chemotherapy. Oncolytic HSV-1 has great promise as a component of an antiglioma arsenal and therefore must be studied in the context of other effective therapies. Advani *et al.* examined the impact of sequentially treating glioma cells implanted in murine hind limbs with R3616 and ionizing radiation [39]. In the combined virus and radiation group, average tumor volumes were lower and more cures were effected. Furthermore, more virus was harvested at various posttreatment time points from tumors that had been treated with combination R3616 and radiation. This study was followed by more detailed examination, including reducing the radiation dose to a more clinically relevant level in an intracranial glioma model. Intracranial U87 tumors were treated with injection of 1×10^7 pfu of R3616 followed by administration of 30 gray of radiation in 5-gray fractions [40]. Again, the combination of virus and ionizing radiation was more effective than either treatment alone. Immunohistochemistry demonstrated a higher number of viral particles within the tumors of the combination-treated mice. In the context of ionizing radiation, R3616 appeared capable of more sustained and effective replication and oncolysis.

It has been hypothesized that the synergistic and viral-sensitizing effect of radiation for γ34.5 mutant oncolytic HSV-1 occurs as a result of radiation induced upregulation of GADD34, one of the "growth arrest and DNA damage"-induced genes [41]. As mentioned previously, GADD34 is a cellular homolog

for γ34.5 and can restore viral protein translation. Therefore, ionizing radiation may be able to complement defects in G207 and R3616 and enhance viral replication [42]. A number of studies in different tumor models corroborate these effects, but the efficacy of the combination is not related to upregulation of GADD34 alone. Cellular ribonucleotide reductase is also upregulated by low-dose ionizing radiation [43], also enhancing G207 replication. In addition, p38, part of the MAP kinase cascade, is activated by irradiation, and can, in turn, enhance HSV-1 replication by increasing transcription of late viral genes [44]. Precise mechanisms by which radiation potentiates viral replications are not clearly elucidated and require further study.

The combination of oncolytic HSV-1 and radiation is currently in clinical translation in glioblastoma patients. Preclinical work demonstrated that the combination of G207 and radiation results in significantly longer survival in athymic mice bearing intracranial U87 MG (human glioblastoma) tumors. Mice with tumors that received a single intratumoral injection of G207 that was followed by exposure to 5 Gy of radiation at 5 hours and also at 24 hours lived significantly longer that mice treated with G207 or radiation alone [45]. The majority of animals receiving the combination treatment survived through the end of the study period. Furthermore, it was determined the delivery of radiation 24 hours after viral inoculation was critical in achieving this synergistic effect.

In combination with ionizing radiation (5 Gy), there was no toxicity when G207 (5.6×10^7 pfu) was administered by intracerebral injection into BALB/C or athymic (nu/nu) mice. All injected mice tolerated virus injections and irradiation procedures without any adverse effects, except for modest weight loss in irradiated mice, which gradually resolved over 30–45 days. Currently a phase I clinical trial is examining the safety of this combination in glioblastoma patients (http://clinicaltrials.gov/show/NCT00157703, accessed on May 6, 2007).

Temozolomide is an alkylating agent that is currently FDA-approved for treatment of glioblastoma. Tumor expression of O-6-methylguanine-DNA methyltransferase (MGMT) is associated with temozolomide resistance [6]. Aghi *et al.* [46] have demonstrated the synergistic effect of G207 and temozolomide in killing glioma cell lines and, also, have related response to tumor cell genotype. Following pretreatment with temozolomide, p53-intact/MGMT(−) glioma cells suffered increased cell death after inoculation with G207 – mediated by drug-induced upregulation of GADD34 and subsequent enhancement of viral replication. P53-mutated/MGMT (+) tumors were not affected by pretreatment with temozolomide, unless also treated with the AGT inhibitor 06-benzylguanine. In these p53-mutated cells, enhanced viral replication – seen after MGMT inhibition – was related to upregulation of RR, rather than GADD34. These differences were related to MGMT status and independent of the presence of mutated p53. RNA interference experiments confirmed the specific actions of GADD34 and RR in the synergistic effect, and enhanced survival was achieved with combination treatment of mice bearing intracranial U87 gliomas. Of note, synergy was not observed between G207 and cisplatin, an alkylating agent that upregulates neither GADD34 nor RR. These studies not only document the advantages of sequential treatment of gliomas with temozolomide and G207 but also correlate response to MGMT status, which may be associated with the presence of p53 mutation [47]. None of the mice in these studies suffered any toxic effects. Such studies demonstrate the possibility that specific viral mutations may be utilized to selectively interact with specific cancer cell pathways. A phase I clinical trial examining cotreatment of glioblastoma with G207 and temozolomide is currently in development.

It has been demonstrated that both radiation therapy and chemotherapy can be complementary and synergistic with oncolytic HSV-1 treatment of glioblastoma. The mechanisms are not limited to upregulation of a single pathway and, in fact, may differ depending upon the molecular profile of the particular tumor in question. Many questions remain to be answered including the possible involvement of inflammatory cytokine release and apoptosis that is associated with both radiation and chemotherapy.

However, there is sufficient justification to examine these combinations in clinical trials and to work toward incorporating oncolytic HSV-1 into the standard therapies for glioblastoma.

Adenovirus

Adenoviruses are also very suitable for gene therapy and gene delivery. They can be grown in high titer and can bear large transgenes. The *Adenoviridiae* family is composed of 47 serotypes. Ad5 is commonly associated with a self-limiting febrile respiratory illness in humans. The structure of an adenoviral particle includes an icosahedral capsid around an inner nucleoprotein core. The capsid bears hexon proteins and the vertices consist of a pentose base that joins the fiber protein [48]. The Ad5 genome consists of double-stranded DNA with short inverted terminal repeats, with gene expression occurring in early (E) and late (L) phases. All early regions, except E3, are required for adenovirus replication. Therefore, replacement of an early adenoviral gene with an analogous but cancer cell-specific gene or an element whose transcription is driven by tissue or tumor-specific promoters are strategies of targeting adenoviral replication to tumor cells. Furthermore, adenoviral infectivity is dependent upon cellular expression of the coxsackie and adenovirus receptor (CAR), which leads to the normal entry process. CAR is expressed on many cell surfaces but less commonly on cancer cells. Manipulating the pathway of adenoviral entry into cells is another strategy by which an adenovirus can be engineered to be selectively replicating within cancer cells.

Onyx-015, also known as dl1520, is one such conditionally replicating adenovirus that has been used to target glioblastoma cells [49]. Tumor selectivity is achieved by deleting the viral genomic region encoding E1B 55 kd. EIB 55 kd typically functions to prevent p53-mediated processes that lead to cell death after adenoviral infection, including induction of growth arrest via induction of genes such as cyclin-dependent kinase p21/WAF1/Cip1 or induction of apoptosis via Bax. Thus, EIB-deleted vectors are engineered to replicate in cells with mutant p53, which includes a variety of malignancies. One-third to one-half of glioblastoma patients either express mutated p53 or harbor a defect in a downstream molecule [50]. However, studies have demonstrated that the ability of Onyx-015 to replicate, to produce viral DNA, and to lyse cells does not depend entirely on p53 status [51]. Rather, tumor cells may substitute another E1B function, that is, late viral RNA export [52].

Onyx-015 was delivered to glioblastoma patients in a recent phase I clinical trial [53]. In this dose-escalation study, 4 cohorts of 6 recurrent malignant glioma patients underwent open resection of tumor. At the completion of tumor removal, the surgeon injected 100 microliters of Onyx-015 into each of 10 sites around the periphery of the resection cavity. No treatment-associated adverse effects were identified. An antitumor effect could not be detected. Median time to progression was 46 days. Onyx-015 was either ineffective or the amount was insufficient; the maximally tolerated dose was not achieved. 1×10^{10} plaques of adenovirus were injected at the highest dose level. Onyx-015 has also been employed in clinical trials for other solid tumors, including pancreatic carcinoma [54], hepatobiliary carcinomas [55], colorectal carcinoma [56], and oral carcinomas [57].

Delta-24 adenovirus (Ad5-Delta 24) is similarly constructed to take advantage of cellular defects in cancer cells. A mechanism of self-preservation employed by wild-type adenovirus is its ability to interfere with cellular Rb. Rb is a tumor suppressor protein that binds to E2F transcription factors and controls the transition from G1 to S cell cycle phases. Ad5-Delta 24 is engineered by deletion of 24 base pairs in the E1 region that are responsible for adenoviral interference with Rb, and, thus, renders normal cells resistant to infection. Cells deficient in Rb, however, including those in 80% of gliomas, are permissive, and the virus conditionally replicates and is oncolytic. In the first studies with Ad5-Delta 24, intratumoral injection into human glioma cell lines was very effective, both *in vitro* and *in vivo* in athymic mice [58].

However, some glioma cell lines were relatively resistant to infection with Ad5-Delta 24. An impediment for adenoviral treatment of cancer, including glioblastoma, is the requirement that target cells express the coxsackie-adenovirus receptor (CAR) as a prerequisite for infection. Glioma cells variably express CARs and, therefore, cell killing with Ad5-Delta 24 was inconsistent in the above-mentioned study. Ad5-Delta 24, therefore, was modified to infect cells via integrins expressed on cancer cells rather than CARs [59]. Insertion of the RGD-4C sequence into the fiber knob protein of Ad5-Delta 24 allowed the virus to enter cells expressing integrins αvβ3 and αvβ5. "Delta-24-RGD" infected a wider range of glioma cells and was also more cytopathic than Delta-24, both *in vitro* and *in vivo*.

The potential exists for developing synergistic interaction between oncolytic adenoviral infection of tumors and standard cancer therapies. Adenovirus safeguards its own replicaton within infected cells by preferentially entering them while quiescent and, then, inducing transition to S phase, at which time viral DNA synthesis occurs [60]. Delta 24 infection, likewise, causes accumulation of cells in S phase and, therefore, may render cancer cells more susceptible to chemotherapy agents that impact at that time.

Topoisomerase I inhibitors, such as irinotecan (CPT-11), prevent DNA unwinding during DNA replication and RNA transcription and lead to DNA strand breaks that trigger apoptosis [61]. Adenoviral infection increases cellular topoisomerase I levels, providing further rationale for combination therapy with agents targeting cells in S phase. Treatment with topoisomerase I inhibitors, however, can drive cells into G_2M phase, which might diminish the supposed benefits of combination therapy.

Gomez-Marzano *et al.* confirmed that combining adenoviral infection with irinotecan enhances antitumor effect in human glioblastoma cell lines [62]. In excess of 70% of glioma cells accumulated in S phase after infection with Delta 24, similar to the response to wild-type adenoviral infection. Furthermore, cells treated with the combination remained in S phase, suggesting that Delta 24 was able to override irinotecan-associated transition to G_2M. Treatment with Delta 24 followed 48 hours later with irinotecan was more effective against glioma cells both *in vitro* and *in vivo* in glioma cell lines, including in a murine intracranial tumor model. As opposed to the example of synergy between oncolytic HSV-1 G207 and temozolomide, treatment of glioblastoma cells with irinotecan had no effect on viral replication. Rather, pretreatment with Delta 24 sensitized glioblastoma cells to treatment with certain cytotoxic drugs by driving cells in to S phase and by increasing expression levels of the targeted enzyme, topoisomerase I.

In an excellent review on oncolytic adenoviral therapy for glioblastoma, Sonabend *et al.* [48] categorize engineering strategies as follows: "(1) deletion of viral genomic regions that are not needed for replication in cancer cells with specific pathway alterations; (2) facilitation of viral transduction in neoplastic cells; and (3) transcriptional targeting of viral genes or transgenes using tumor-specific promoters." Onyx O15 and Ad5-Delta 24 are examples of the first category. As described above, Ad5-Delta 24-RGD combines principles of the first and second categories, i.e., it selectively replicates in cells with defects in the Rb pathway and it efficiently infects cells bearing integrins particular to glioblastoma or other cancer cells. Another example of a surface receptor on glioblastoma is the epidermal growth factor receptor (EGFR). EGFR is highly expressed on many tumors and is commonly amplified in glioblastoma [63]. Targeting adenovirus to EGFR is also appealing because it leads to activation of PI3-K, upon which adenoviral entry into cells is dependent [64]. In a very thorough set of experiments, Miller *et al.* [65] developed a bispecific antibody conjugate (Fab-425), composed of the Fab fragment of a neutralizing, antifiber knob domain monoclonal antibody and mAb 425, which binds to human EGFR. This bispecific antibody was able to prevent adenovirus from binding to cells via the fiber knob and CAR and redirected it to EGFR, at once making attempted adenoviral infection of tumors in the brain more specific and more effective.

The above studies measured efficiency of gene transfer using EGFR targeting. Van Beussechem *et al.* [66] engineered an oncolytic adenovirus with a delta

24 mutation to produce the bispecific single-chain antibody against the adenovirus fiber knob and the EGFR. This construct replicated in CAR-deficient glioma cell lines and was more effective at killing them than the parent Delta 24 adenovirus. This was also true in primary human glioblastoma cells. In an *in vitro* model for solid tumors, oncolytic adenovirus expressing the bispecific antibody caused significant cell death in cancer cell "spheroids," demonstrating significant lateral spread. In contrast, parental Delta 24 – mutated adenovirus – infected spheroids showed patchy infection. These models have not yet been examined *in vivo*.

The use of tumor-specific promoters to drive oncolytic adenovirus replication and glioblastoma targeting has been studied in more depth than in the case of oncolytic HSV-1. The midkine promoter, for example, is highly active in glioblastoma cells, with little activity in normal brain cells. Kohno *et al.* have designed a midkine promoter-driven conditionally replicating oncolytic adenovirus that effectively killed midkine-expressing glioma cells in culture but was ineffective versus midkine-negative brain cells [67]. Oncolysis was effective in an *in vivo* model as well with this virus.

Engineered incorporation of transgenes responsive to the E2F promoter represents another method by which the Rb pathway can be exploited for adenoviral glioblastoma specificity. Rb represses E2F-responsive promoters. Therefore, in tumor cells with mutated Rb, E2F promoters are "derepressed" and, thereby, more active than they are in normal cells. An adenovirus containing transgenes driven by the E2F-1 promoter was very effective at curing established intracerebral gliomas in an *in vivo* model [68].

Survivin is an inhibitor of apoptosis protein (IAP) that has high activity in multiple cancers and is integral to cancer cell survival and malignant progression [69]. Survivin is not typically detectable in adult tissues but is overexpressed in roughly 80% of astrocytomas and in up to 90% of glioblastomas. Survivin expression in gliomas is also associated with prognosis and resistance to chemotherapy and radiation [70,71]. Van Houdt *et al.* have engineered a conditionally replicating adenovirus for which E1 gene expression is driven by the survivin promoter [72]. In this study, the authors demonstrated that the survivin promoter is active both in glioma cell lines and primary tumors, that promoter activity correlates with survivin gene expression, and that the targeted adenoviral vector could effectively kill astrocytic tumors. Furthermore, the construct was not toxic to hepatocytes, an important feature for clinical translation, as hepatotoxicity has been a side effect of adenoviral gene therapy.

Other glioma-specific transcriptional regulators have been studied, e.g., hTERT [73] and may be candidates for adenoviral targeting.

Replicating RNA viruses

Host response to the presence of intracellular double-stranded RNA includes activation of PKR, which, in turn, blocks protein synthesis and leads to apoptosis. Also, double-stranded RNA provokes an antiviral type I interferon response from infected cells, which may also lead to cell destruction. Generally speaking, malignant cells may have defective PKR expression or may be unable to mount an interferon response, thereby rendering them susceptible to RNA virus infection. For these two reasons, therefore, RNA viruses may have inherent tumor cell selectivity.

Reovirus

Reovirus is an example of an RNA virus that has demonstrated oncolytic capacity against glioblastoma. Reovirus, a double-stranded RNA virus that can be isolated from human respiratory and gastrointestinal tracts, makes use of the activated Ras-signaling pathway that is a feature of many tumor cells and, therefore, conditionally replicates in Ras-transformed malignancies. The Ras pathway is stimulated by ligation or activation of the EGF receptor. Activated Ras inhibits PKR activation, allowing the reovirus life cycle to continue unabated. Nontransformed cells are not susceptible to productive reovirus infection, and viral infection is

nonpathogenic in immune-competent animals. Wilcox *et al.* examined the efficacy of reovirus as an oncolytic agent against experimental human gliomas [74]. Viral replication led to effective cell killing in 20 of 24 human malignant glioma cell lines in culture. Resistant cell lines did not demonstrate phosphorylated mitogen-activated protein kinases (MAPK) – a downstream effector of RAS. However, although MAPK was activated in 90% of susceptible cell lines, it was not activated in two of them, suggesting an alternative mechanism. Reovirus was also effective at killing a variety of human primary glial tumors, including glioblastoma, anaplastic astrocytoma, low-grade astrocytoma, and oligodendroglioma, but was ineffective against benign meningiomas.

In SCID mice, reovirus caused regression of subcutaneous and intracranial tumors, associated with significant immune-associated toxicities. These side effects were mitigated in the less immunocompromised nude mouse model, but survival was greatly enhanced and cures were effected in 67–82% of glioma-bearing mice.

Reovirus is currently being examined in a phase I/II clinical trial for patients with recurrent malignant gliomas, as well as in other cancer types. By unpublished report of the phase I component for brain tumors, treatment was well-tolerated, and reoviral therapy is now being examined in combination with low-dose irradiation (www.oncolyticsbiotech.com/tech.html, accessed May 20, 2007).

Newcastle disease virus (NDV)

NDV is another RNA virus that is not pathogenic in humans and has oncolytic properties. NDV acts in cancer cells by causing apoptosis triggered by the intrinsic mitochondrial death pathway [75]. NDV is also attractive because infection induces danger signals, such as dsRNA, that can be associated with generation of antitumor immunity [76]. A series of small clinical trials against glioblastoma have been reported [77,78,79,80]. In all, therapy has been well-tolerated, including with intravenous administration [78]. A few durable antitumor responses have been reported. An "oncolysate" composed of irradiated NDV-infected glioblastoma cells generated an antitumor immune response when given as a subcutaneous vaccine to glioma patients. Observed immune effects included increase delayed-type hypersensitivity, increased tumor-reactive memory T cells, and $CD8^+$ T lymphocyte infiltration in "secondary" tumors [80].

Poliovirus

Poliovirus is a nonenveloped positive-strand RNA virus of the *Picornoviridae* family that is highly neuropathogenic and is tropic to motor neurons in the brain stem and spinal cord, resulting in a characteristic syndrome of flaccid paralysis (see also Chapter 1). Polio neurotropism is likely derived from two features: (1) targeting via the poliovirus receptor of the Ig superfamily, CD155, highly expressed on cells of neuronal origin [81], and (2) the cell type-specific function of the poliovirus internal ribosomal entry site (IRES) element in cells of neural origin. The polio IRES is part of the 5′ nontranslated region that governs initiation of translation in a 5′ independent manner. The consequence is cell type-specific restriction of poliovirus propagation [82]. Creation of an "intergeneric" recombinant using IRES elements from human rhinovirus type 2, known as PV1(RIPO), yields a poliovirus construct with poor growth in neural cell lines and avirulence in CD155 transgenic mice [82].

While PV1(RIPO) does not propagate in neural tissue, it does so quite efficiently both in glioma cell lines and primary glioma tissue, with a strong oncolytic cytopathic effect on these cells, which also highly express CD155 [83]. In athymic mice, intratumoral injection of glioma tumors implanted either in the flank or in the brain allowed for 80% survival at 50 days. Intramuscular injection of PV1(RIPO) did not affect growth or survival. Intravenous injection did delay symptoms and death up to 11 days, demonstrating that polio targeting to human glioma cells is promising enough to consider systemic delivery. Similarly, when flank tumors were treated with intratumoral injection of PV1(RIPO), both the

directly injected and synchronous contralateral flank tumors regressed, suggesting the possibility that the progeny from the directly infected tumor were numerous enough to track systemically to distant targets. Intracranial astrocytomas, however, were not affected by systemic delivery of poliovirus, perhaps, underscoring the need to alter the blood-brain barrier for this to be effective.

Intrathecal delivery of oncolytic poliovirus engineered by replacement with the human rhinovirus type 2 IRES was safe in rats and prolonged survival in a model of multifocal or leptomeningeal glioblastoma [84].

The use of poliovirus to treat glioblastoma is a unique approach in the field of oncolytic viruses in that tumor cell expression of a single marker molecule, CD155, is necessary and sufficient for poliovirus entry and cellular lysis. CD155 seems to be expressed universally in malignant glioma cell lines and in primary cultures, and toxicity studies with the IRES-modified poliovirus constructs demonstrate safety. Clinical trials have not yet been performed. Further issues include whether pre-existing immunity to poliovirus – common in almost all humans in the developed world – impairs the antitumor response. Preliminary studies suggest that the effects of intratumoral injection of poliovirus are not adversely affected by immunity [85], and it is not thought that vaccinated patients have neutralizing antibodies within the CNS.

Measles

Measles is a negative-strand RNA paramyxovirus that is associated with significant cytopathic effect, including cellular syncitiae formation, followed by apoptotic cell death (see also Chapter 2, Subacute sclerosing panencephalitis). The viral H protein binds to and enters cells through SLAM (signaling lymphocyte activation molecule), predominantly on lymphocytes, or CD46. CD46 is a complement regulatory protein that protects against complement-mediated lysis, is expressed on most nucleated cells, and is overexpressed on many tumors [86]. Attenuated strains of measles are used as vaccines and have well-established safety profiles, including in the setting of direct intracerebral injection [87,88].

Measles virus can target glioma cell lines, which have been demonstrated to display abundant levels of CD46. Phuong *et al.* have demonstrated that the attenuated Edmonston strain, which enters cells chiefly via CD46 rather than SLAM, replicates very well in cultured glioma cells with effective cytopathic and oncolytic effect [89]. The virus was engineered to express carcinoembryonic antigen (CEA), used as a marker of viral activity. Intravenous injection of virus effectively achieved growth reduction in subcutaneous U87 malignant glioma cells in mice, and intratumoral injection of virus in intracranially implanted U87 was also effective. In both models, CEA levels in the blood surged with treatment and went back to baseline shortly thereafter. Also, tumor volume correlated tightly with serum CEA levels.

CD46, however, while upregulated in tumors, is widely expressed on many cell types, so more precise targeting of measles virus to tumors may be safer. Engineered mutations in the measles H protein can abrogate viral ability to bind to CD46 and SLAM and, therefore, its ability to infect and kill cells. When further engineered to include genes for single-chain antibodies to EGFR and EGFRvIII, targeted measles virus was able specifically to infect and kill EGFR and EGFRvIII – expressing cell lines when delivered by intravenous injection. In a metastatic tumor model, tumor deposits at many sites, including in the brain, had evidence of oncolytic measles infection after IV administration. Recovered virus from these tumors had not converted back to CD46 tropism but remained specific to the engineered target [90].

The same group from the Mayo Clinic, upon demonstrating the ability to engineer and recover high titers of H protein-mutated virus, has demonstrated efficacy in intracranial EGFR and EGFRviii-positive tumors [91,92]. Futhermore, intracranial intratumoral injection of EGFRviii-targeted measles was as effective as unmodified virus and was associated with no neurotoxicity when examined in measles replication-permissive transgenic mice. Targeting measles virus to molecules expressed

specifically on glioblastoma effectively widens the therapeutic window.

Systemic delivery of oncolytic measles virus, however, may be compromised by widespread antiviral immunity. Iankov *et al.* [93] have devised a viral delivery system wherein infected autologous peripheral blood mononuclear cells or endothelial cells could deliver measles to tumors after intravenous or intraperitoneal injection. In an ovarian carcinoma model, the efficacy of oncolysis and tumor control was less affected by coadministration of measles-neutralizing antibodies than when virus was directly injected into the bloodstream.

Phase I trials examining oncolytic measles virus are underway for ovarian carcinoma, multiple myeloma, and glioblastoma, each at the Mayo Clinic.

Vesicular stomatitis virus

Oncolytic VSV achieves tumor replication selectivity by preferentially dividing in and propagating in cells with impaired antiviral interferon responses, i.e., in cancer cells. VSV is a negative-strand RNA virus of the *Rhabdoviridae* family that replicates in the cytoplasm and has an extremely rapid life cycle, releasing progeny virus from infected cells within 2–3 hours. Wild-type VSV is lethal to tumor cells but is also extremely toxic to normal tissues. Administration of exogenous interferon at the time of wild-type VSV infection abrogates this toxicity and prevents viral-associated death in immunocompromised mice. Stojdl *et al.* [94] selected mutant VSV strains by picking two variants that produced small plaques on interferon-responsive cells. Infection with these strains induced 20 to 50 times more interferon production than did wild-type infection. Mice were able to tolerate intravenous injection of up to eight-fold more of this attenuated virus. These strains differed chiefly in the M-protein, which, in wild-type VSV, disables the host antiviral immune response, at least partially by blocking the nuclear export of interferon-β mRNA. In this study, the authors demonstrated that attenuated VSV was effective at killing interferon nonresponsive cancer cells *in vitro* by direct inoculation into tumors in nude mice and in widely disseminated tumors when delivered intravenously in immunocompetent mice. Attenuated VSV was examined versus the NCI-60 panel of cancer cell lines and was highly oncolytic in four of five CNS lines, three of which had interferon α/β defects.

Lun *et al.* [95] used a mutant VSV, $VSV^{\Delta M51}$, that has a deletion of methionine in the 51 position within the gene for the VSV M protein and, therefore, as described above, allows the cellular interferon response to proceed in normal cells. Systemic administration of $VSV^{\Delta M51}$ was effective at killing *in vivo* U87 cells, implanted both unilaterally and bilaterally. Intravenously administered $VSV^{\Delta M51}$ infected and slowed the growth of intracranially implanted U87 glioblastoma cells and was, furthermore, able to treat multifocal disease and invasive tumor cells that had migrated beyond the main tumor masses, demonstrating unique potential for targeting these very invasive malignancies. Despite the defective M-protein, intracerebral injection of $VSV^{\Delta M51}$ was very toxic; systemic administration appeared safe and had clear effects against intracerebral tumors.

In an organotypic brain tissue-glioma coculture model, wild-type VSV was very effective at killing tumor cells but was also toxic to neurons. Pretreatment with interferon-β did not impact the tumoricidal effects but did prevent damage to normal cells. However, pretreatment with interferon-β did not completely prevent development of cytopathologic effect on neurons. Therefore, a "replication-restricted" VSV with the G-protein deleted, rVSV-ΔG, was engineered [96]. The VSV G-protein is an envelope-associated molecule that is required for viral attachment to the cell membrane and fusion of the viral envelope with the endosomal membrane and is required for infectivity. Propagation of virus, therefore, only occurs in cells that are transiently expressing the viral glycoprotein. Otherwise, viral progeny are noninfectious. RVSV-ΔG maintained efficacy against gliomas in the aforementioned three-dimensional coculture model but was completely nontoxic against normal tissues, including neurons.

Wollman *et al.* developed a "glioblastoma-adapted" oncolytic VSV by serial passage of virus on

glioblastoma cell lines and have called it "VSV-rp30a." VSV-rp30a replicates more quickly and effectively than wild-type VSV in glioblastoma cell lines. In this same study, VSV was most effective among a panel of nine potential oncolytic viruses against glioblastoma [97]. Building upon this work, Wollman next examined the relationship of interferon deficiency on the efficacy of VSV-rp30a [98]. Glioblastoma cell lines, human astrocytes, oligodendrocyte precursors, and primary explants from normal human brain were infected with VSV-rp30a with and without simultaneous administration of IFN-β or poly I:C, an activator of innate immunity and the IFN α/β pathway that operates via binding of Toll-like receptor 3. Both IFN-β and poly (I:C) protected normal brain cells from VSV-mediated death but did not impact the oncolytic effect against glioblastoma. The authors concluded that combination therapy with VSV and exogenously administered interferon or poly (I:C) represents a potentially safe and effective therapy for glioblastomas.

Conclusions

Glioblastoma is a disease in need of novel oncolytic viral approaches and may be well-suited to them. With the current standard of care, rates of survival are dismal. As neuro-oncologists develop a deeper understanding of the genetic alterations that both lead to glioblastoma and that occur in response to radiation and chemotherapy, virologists have been able to take advantage of tumor biology in order to design and to construct "smarter" vectors. We have reviewed efforts to engineer DNA and RNA viruses that can selectively infect glioma cells and maintain the ability to replicate within and destroy tumors while being safe for direct injection into the brain or for systemic delivery. A number of oncolytic viruses have been examined in clinical trials, and safety, to date, has been the rule. Nevertheless, we must continually consider ways in which these vectors can be made safer for use in humans, more selective for malignant cells, and, at the same time, more effective at killing and spreading within tumors. Ultimately, effective treatment of malignant brain tumors may require a multipronged approach; therefore, as phase I trials using oncolytic viruses are completed, it will become necessary to integrate these treatments both with established therapies, such as radiation and chemotherapy, as well as with new ones such as angiogenesis inhibitors. The inclusion of tissue-specific promoters within these constructs and the insertion of genes for proimmune or antiangiogenic molecules are other approaches that, once safety and antitumor efficacy are properly established in preclinical models, will be important to consider in combination clinical trials. Furthermore, the role of the immune system, both with regard to antiviral immunity and antitumor immunity, must be further clarified and considered in these patients, most of whom are severely immunosuppressed.

With exercise of proper caution, continued development of the field of oncolytic viruses for the treatment of glioblastoma has great promise as an avenue toward effective therapy for these deadly tumors.

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23

Viral gene therapy for central nervous system diseases

Pedro R. Lowenstein, Kurt M. Kroeger, and Maria G. Castro

Viruses as therapeutic agents: science fiction becomes reality

The idea of using genes as medicines was initially proposed in 1972 by Friedmann and Roblin before it was possible to identify specific genes within genomes, before the discovery of restriction enzymes to cut and paste DNA, and before the development of efficient gene delivery vehicles such as viral vectors [1]. The idea of using genes as medicines to treat diseases was a logical outcome of the identification of complex diseases resulting from mutations in single genes. If complex phenotypes were the result of mutations in a single gene, gene replacement into the right tissue at the right developmental stage should suffice to prevent or even reverse the disease progression. The implementation of this originally simple idea has ushered in a new and exciting era of therapeutic molecular medicine (i.e., gene therapy). Over the past 15 years, hundreds of gene therapy clinical trials have been implemented demonstrating therapeutic results in a growing number of genetic disorders, from relatively simple monogenic inborn errors of metabolism to complex diseases such as cancer.

The techniques required to implement gene transfer began to appear in the early 1980s with the development of viral vectors (i.e., disabled viruses that could function as gene delivery vehicles). Mouse leukemia retroviruses were among the first viral vector systems that were converted into effective gene transfer vectors. By replacement of viral genes with a potentially therapeutic gene the virus was rendered incapable of replication and thus, producing disease. After the development of these first efficient gene transfer tools, further vector systems were engineered from such divergent viral families as adenoviruses, adeno-associated viruses, herpesviruses, vaccinia virus, and SV40. Very quickly thereafter came laboratory proof that the introduction of foreign genes with such vectors could indeed lead to stable expression of new therapeutic functions in cells and the correction of *in vitro* defects [2]. A series of additional preclinical studies in animal models of disease supporting the notion that the introduction of normal genes could indeed reverse or prevent the development of a disease phenotype eventually led to the first human clinical studies in 1989 [3].

Concomitant with the technical development of the molecular tools and techniques necessary for gene therapy, scientists discussed the ethical boundaries of gene therapy as well as the foundations for the ethical and scientific review of clinical trials in gene therapy. The Food and Drug Administration established a Cell and Gene Therapy section, and the National Institutes of Health created the Recombinant DNA advisory committee (RAC) to review the science and ethics of clinical trials in gene therapy.

The biology of virally mediated gene transfer and therapy: turning viruses into therapeutic delivery vehicles

Viruses cause disease by infecting target host cells, transferring their own genetic material into the host cells, and hijacking the host cellular machinery

responsible for DNA replication and protein synthesis to replicate themselves. To turn viruses into therapeutic carriers, viruses were genetically engineered to contain foreign therapeutic genes. Such viral vectors will deliver foreign, therapeutic DNA sequences into affected cells within diseased tissue without themselves causing disease. Vector genomes were engineered in such a way to remove sequences encoding for pathogenic functions and generally to abolish the replication of the parent wild-type viruses. However, in some cases viral replication has been redirected to tumor cells to selectively kill them (i.e., oncolytic viruses).

Retroviral and lentiviral vectors

One of the most important properties of retroviruses and lentiviruses is their ability to integrate their genomes into the host's DNA. Integration of the retroviral/lentiviral viral genome into the DNA of target cells permits stable long-term expression of therapeutic transgenes. Integration of retroviruses is only accomplished in dividing cells as retroviruses use the breakdown of the nuclear membrane to access the host cells' chromosomes. Lentiviral-derived vectors, however, have evolved the capacity to cross the intact nuclear membrane and thus integrate into cells independently of their division status. Therefore, lentiviral vectors are able to transduce cells such as neurons and mature hepatocytes [4]. For that reason lentiviruses are now among the favorite candidates for gene therapy approaches for long-term treatment of neurological diseases. Lentiviral vectors are commonly referred to as minimal self-inactivating (SIN) vectors because the vector genome that becomes inserted into the host genome cannot be "rescued" or excised to resume viral replication. Usually the viral genome is deleted of all viral coding regions. To complement for vector production, replication enzymes (Gag and Pol) and an envelope protein (Env) are provided in *trans* from separate expression cassettes that are cotransfected together into a cell line (293T) modified to support lentiviral production. Lentiviral vectors are "pseudotyped" by using envelope glycoproteins of nonlentiviral origin that are selected to increase tissue tropism toward tissues and organs of therapeutic interest. The envelope of the vesicular stomatitis virus (VSV-G) is usually used due to its tropism across a wide variety of both species and tissues [5].

HSV-1-derived vectors

Herpes simplex virus type 1 (HSV-1) can infect and express genes in both dividing and non-dividing cells [6,7]. HSV-1 is a large neurotropic human virus containing 152 kb of linear double-stranded DNA that naturally establishes lifelong latent asymptomatic infections of the nervous system, with periodic epidermal manifestations, without the need of integrating the viral chromosome into the host genome (see also Chapters 1 and 11). However, the virus can reactivate, causing disease recurrence. The life cycle and infectious properties of HSV-1 provide an indirect approach for targeting gene transfer to cells within the central nervous system (CNS) that are therefore difficult to reach directly but whose terminal fields are accessible, such as dorsal root ganglion neurons that can be reached from their axonal terminals in the skin. Long-term transgene expression (up to 6 months) has been shown using HSV-1-derived vectors, even during latency, when the LATP2 promoter is used [8]. Alternative strategies are also available to achieve long-term expression.

Two types of vectors are derived from HSV-1: recombinant viral vectors and amplicon vectors. Recombinant viral vectors (rHSV) contain the full viral genome mutated in one or more viral genes to eliminate replication, reduce toxicity, and provide space for transgenes in the range of 30–50 kb. An alternative HSV vector system is the "HSV amplicon" that relies on introducing the gene of interest into a plasmid, which has an HSV origin of replication and packaging signal and is grown with a helper HSV virus [9]. The advantages of these vectors are that they are essentially non-toxic or antigenic, as they express no virus proteins, albeit low levels of contaminating recombinant replication-competent virus during

packaging. They also have a large transgene capacity (theoretically up to 150 kb), relatively high titers (up to 10^8 t.i / ml with the current packaging modalities), high infectivity for cells of the nervous system, and retention for several months in nondividing cells [10]. In fact, the transgene capacity of HSV amplicon vectors is so large that recently the group of R. Wade-Martins demonstrated successful gene transfer and expression of the entire 135 kb human Friedreich ataxia (FRDA) genomic locus to human patient cells with a defective FRDA genetic locus. Defects in the FRDA gene cause Friedreich's ataxia, the most common recessive ataxia for which there is currently no cure or treatment. Wade-Martins' group was able to show functional complementation and a restoration of the wild-type phenotype in patient cells transduced with the HSV amplicon vector carrying the FRDA genomic locus [11].

Adenovirus-derived vectors (Ad)

The major clinical interest in adenovirus vectors stems from their broad host range, high infectivity for most human cells, and the ability to infect and transfer genes to quiescent and dividing cells. Adenoviral vectors can be divided into three broad categories: E1 deleted (first, second, and third generation), gutless, and oncolytic adenoviral vectors.

First-generation adenovirus vectors

The most common adenoviral vectors developed for human gene therapy are derived from adenovirus serotypes 2 and 5 that were made replication-defective through deletions in the E1 region. Simultaneously, this procedure creates space for transgene insertion. A human embryonic kidney-derived 293 cell line was used to provide the E1A and E1B functions *in trans* to permit vector replication and growth [12]. One of the most important characteristics is their relative ease for scale up of titers reaching above 10^{12} IU/ml (infectious units/ml). Other attractive features include the ability to infect many different cell types, both dividing and non-dividing, and having an extremely low probability of random integration into the host chromosomes [13]. In spite of the early region deletions, first generation Ad vector genomes retain low level residual expression of viral genes. This can lead to a host adaptive immune response that leads to high titer, neutralizing anti-capsid antibodies that inhibit reinfection with the same serotype of Ad vector [14], as well as a cytotoxic T lymphocyte (CTL) response directed against various proteins expressed from the wild-type genome sequences.

Second-generation Ad vectors

To overcome the immunological insults caused by proteins expressed from the wild-type sequences within adenoviral vector genomes, a series of Ad vectors with further deletions and complementing cell lines was developed. Second-generation vector systems include E1/E4-deleted Ad vectors [15], E1/E2a or E1/E2b-deleted Ad vectors [16], 100K, protease-deleted Ad vectors [17], and targeted vectors containing capsid modifications that ablate the vector's native tropism targeting the vector to specific tissues [18,19] , containing chimeric fibers to avoid pre-existing antiadenovirus-neutralizing antibodies [20, 21,22], or most recently, vectors derived from alternative "fastidious" serotypes [23].

Helper-dependent adenoviral vectors

In spite of statistical improvements to toxicity and longevity of expression with second-generation adenoviral vectors, immune responses were not abolished, and toxicity from remaining wild-type sequences could not be eliminated. Helper-dependent adenoviral vectors (also known as high-capacity, "gutless" vectors; HC-Ad) have been developed that are devoid of all viral coding sequences, greatly expanding the capacity of foreign DNA that can be inserted [24]. HC-Ads are copropagated with an E1-deleted helper virus, which provides in *trans* all of the proteins required for the propagation of the vector.

Several systems have been developed to prevent packaging of the helper viral genomes during the HC-Ad vector rescue/amplification process in order

to minimize the helper virus contamination including the Cre/loxP-based system [25] and Flp/frt-based [26]. The HC-Ad system has been fine-tuned where 1×10^{13} viral particles can be easily produced from 3 liters of cells within 2 weeks of vector rescue with specific yields of >10,000 vp/cell and with exceedingly low helper virus contamination of 0.001–0.1% [27]. Due to the large cloning capacity of HC-Ad vectors, there is enough cloning space to contain large regulatory regions. The HC-Ad vectors have the added advantage of increased cloning capacity, reduced toxicity and immune responses, and prolonged, stable transgene expression *in vivo* [28,29].

Oncolytic vectors (adenovirus, HSV-1, and vaccinia)

One potential limitation in effective gene transfer into growing tumor tissues is that vector administration is a terminal event because most vectors have been made replication-incompetent to increase their safety (see also Chapter 22). While the inability to replicate *in vivo* improves the safety of the vector, it also limits the efficacy and distribution of the vector throughout the diseased tissues. By taking advantage of the lytic properties of adenovirus, oncolytic adenoviral vectors have been developed to circumvent this problem and specifically kill tumor cells. Oncolytic adenoviral vectors have been engineered to only replicate in p53-defective tumor cells with mixed results in human clinical trials for various cancers [30]. Vaccinia virus has also been used as a replication-competent oncolytic vector to treat prostate cancer and breast cancer in human clinical trials [31]. Moreover, the vaccine strain of the measles virus is being developed as another potential candidate gene therapy vector for targeting tumor-associated vasculature for the treatment of solid tumors [32].

Adeno-associated vectors (AAV)

Adeno-associated virus (AAV) is a small human parvovirus having a 4.7 kb linear single-stranded DNA genome. It has not been associated with any human disease and its genome is integrated in a site-specific manner, thereby allowing stable transgene expression without the risk of mutation caused by random integration, which makes this virus a good candidate for a gene therapy vector [33]. Various regions of the brain have successfully been transduced by rAAV vectors [34,35,36,37] with apparently no toxicity.

The advantages of rAAV are that (1) it is an integrating vector, with the potential of persistent transgene expression following integration, (2) it efficiently transduces a wide range of host cells, and most importantly, (3) wtAAV is nonpathogenic. Initial experiments investigating the immune response against rAAV vectors in the muscle of mice showed only mild and transient inflammation. The main drawbacks of rAAV are (1) limited cloning capacity (4.5 Kb) available for the transgene, (2) difficulty producing high titers, (3) lack of helper virus in purified stocks, and (4) the fact that total elimination of the helper virus may modify some AAV properties, such as the infection of non-dividing cells or integration into the host genome. Several gene therapy clinical trials utilizing AAV vectors have been performed, including applications for several devastating and untreatable neurodegenerative diseases, such as Canavan's and Batten's disease. These studies are relatively early, and definitive demonstration of clinical benefit has not yet been proven.

Simian virus-40 vectors (SV-40)

The properties that make SV-40 a lucrative gene therapy vector include: ability to infect both dividing and nondividing cells, ability to achieve high titer preparations, potential for integration into cellular DNA, and a viral entry pathway that bypasses the target cells' antigen-processing apparatus. Currently, SV-40 vectors are being evaluated in preclinical models for correction of inherited hepatic deficiencies, treatment of inherited and acquired diseases of the CNS, and inhibition of several viral infections [38].

Vector regulatory expression systems

Tight regulation of therapeutic gene expression is of critical importance when considering implementation of gene therapy to the human trials. One of the most effective of such regulatory systems involves the use of the antibiotic tetracycline to turn gene expression on or off reversibly and quantitatively through the expression of an inducible transcriptional activator. In the tet-off system, the expression of the target transgene is off in the presence of tetracycline, while in the tet-on system, the transgene is expressed in the presence of the drug. The latest regulatory switch composed of a tetracycline response promoter coengineered with mutant rtTA2^{S}M2 transactivator and a tTSkid repressor allows tight regulation of transgene expression in mice, rates, and nonhuman primates [39,40,41].

Applying the tet-on regulatory switch system to the HC-Ad vector provides a strong gene therapeutic tool. This vector will express high levels of the transgene while being tightly regulated to produce the therapeutic gene at specific times and therefore minimize side effects caused by overexpression of the transgene. Excellent disease candidates for which this regulatable switch system can be utilized might include neurodegenerative diseases such as Parkinson's disease (PD) or similar diseases in which therapeutic doses may require adjustment as disease progresses.

Viral vector-based treatments of neurodegenerative diseases

Alzheimer's disease

Alzheimer's disease (AD) is characterized by deposition of extracellular β-amyloid plaques, intracellular neurofibrillary tangles, synaptic loss, and neurodegeneration with symptomatic presentation including progressive cognitive decline and memory loss. No early diagnosis is currently possible and the only approved treatments delay the onset of memory loss by administration of cholinesterase inhibitors without substantially altering disease progression. Thus, rather than attempting to alter any of the genetic defects that lead to the pathological features of AD, most proposed therapeutic approaches aim to reduce amyloid load, or using gene transfer of neurotrophic factors to reduce the death of brain cells. Attempts have also been made to induce immune responses that could potentially clear amyloid deposits from the brain by immunizing against β-amyloid. Initial clinical trials of this approach have run into serious side effects, such as brain inflammation, and possibly reductions in amyloid deposits in the brain. Brain inflammation is thought to have been the result of stimulating a T cell response. As a consequence several groups are attempting to improve on the early immunization protocols to avoid the activation of T cells and brain inflammation while maintaining a strong antibody response that could help clear the brain's amyloid load.

The recent discovery of mutations in the SORL1 gene that may underlie pathology in so called "sporadic" cases of AD may offer more direct attempts to manipulate the genetic causes of nonfamilial AD, the most common type of disease [42]. The neurotrophic activity of nerve growth factor (NGF) [43] has led to the use of NGF itself to attempt to rescue degenerating basal forebrain cholinergic neurons [44]. However, injections of NGF into the ventricles of patients with AD not only did not have striking therapeutic effects but also had serious toxic effects including pain and weight loss [45]. As a consequence, an *ex vivo* gene therapy approach was developed that grafts autologous fibroblasts transduced *ex vivo* with a retroviral vector-expressing NGF into the fimbria-fornix lesioned rat brain [44].

Following appropriate safety and feasibility testing in nonhuman primates, Tuszynski and colleagues initiated a phase I trial in a small number of patients with mild AD that showed no adverse effects 22 months after transplantation of autologous fibroblasts obtained from skin biopsies of eight probable early-stage AD patients infected with an NGF-expressing retroviral vector and stereotactically

implanted into the nucleus basalis of Meynert [46]. The remarkable lack of adverse events is most likely attributed to the lack of migration of the *ex vivo* transduced fibroblasts from the transplantation site in the basal forebrain. This absence of migration restricted the secretion of NGF to the degenerating cholinergic neurons rather than diffusing throughout the brain and spinal cord [47]. Clinical follow-up has suggested an apparent slowing in the rate of cognitive decline and a significant increase in cortical glucose consumption after treatment. A phase I/II dose-escalating, randomized study of an AAV vector-encoding NGF is currently ongoing. Results from longer term follow-up studies and controlled clinical studies are expected to clarify the clinical impact of these approaches.

Other gene therapy strategies for AD include gene transfer of apoE2 [48], a lipid-binding protein involved in the deposition or clearance of Aβ in the brain, to reduce the Aβ burden and development of neuritic plaques. More recently, neprilysin, an extracellular enzyme that degrades Aβ has been proposed as an alternative approach for gene therapy, and as such, has been tested in a number of different experimental models [49]. Also, IGF-1 has been shown to be effective in experimental models of neuronal degenerations in mouse models of amyotrophic lateral sclerosis [50]. Even though questions remain on how IGF-1 may be delaying the death of affected mice in this model, the strong experimental results in a disease that is otherwise untreatable has led to an accelerated development of significant clinical trials of Huntington's disease, dominant inherited ataxias, and torsion dystonia [51]. *Ex vivo* gene therapy has also been proposed as a potential therapeutic approach to treat AD.

Parkinson's disease

The second most common neurodegenerative disorder is Parkinson's disease (PD), which occurs both in sporadic form and, far less commonly, in familial form. In patients suffering from PD, there is a progressive loss of dopaminergic neurons in the substantia nigra and other brain stem nuclei. There are about 400 000 dopaminergic neuron cells in the midbrain of humans [52]. Patients with PD suffer from various motor impairments including resting tremor, bradykinesia, and rigidity but also balance problems, autonomic nervous dysfunction, and at late stages, cognitive and psychiatric symptoms. Currently there are 11 gene loci linked to the familial form of Parkinson's disease named PARK1 through PARK11. Only six of the genes have been identified and are named as follows: α-synuclein, Parkin, UCH-LA, Pink1, DJ-1, and LRRK2 [53].

Gene therapy for PD was first developed in rat models using gene transfer of tyrosine hydroxylase [34,54]. In the past 10 years, gene therapy approaches for PD have been further developed in three main directions: (1) transduction of multiple genes essential for the synthesis of dopamine, to restore dopamine levels; (2) transduction of genes encoding growth factors, differentiation factors, transcription factors, and antiapoptotic proteins to prevent ongoing neurodegeneration of nigrostriatal dopamine neurons; and (3) improvements and further developments of vector and promoter systems to reduce toxicity, immune responses, increase longevity of expression, and regulation of transgene expression. Alternatively a series of immune-based protocols has been attempted to use antibodies to lower the brain levels of Aβ. A number of immunization protocols were attempted. While some intriguing positive results were obtained, uncontrolled inflammation forced a re-evaluation of this approach. As a result, new approaches focused on the stimulation of selective Aβ antibodies without stimulation of T cells is being attempted. Some of these immunization protocols aim to use gene therapy approaches for vaccination.

The exact mechanisms of dopaminergic neuron degeneration are not fully understood. The production and release of dopamine is dependent on the coexpression of multiple proteins, and recent gene therapy clinical trails have been implemented to deliver these proteins to improve the levels, synthesis, and regulation of dopamine production. A clinical trial for PD is ongoing using an AAV vector encoding the therapeutic gene glutamic acid

decarboxylase (GAD) to manage the tremors associated with late stage PD. This therapeutic approach aims to stimulate the motor-inhibitory γ-aminobutyric acid (GABA)-ergic pathway following gene transfer of GAD into the subthalamic nucleus [55]. An AAV vector carrying the therapeutic gene aromatic-L-amino-acid decarboxylase (AADC) is being evaluated in clinical trials combined with the administration of L-dopa, the current standard of care for the treatment of the dyskinesia associated with PD. Outstanding efficacy was observed in non-human primates when AAV-mediated gene transfer of AADC is combined with gene transfer of tyrosine hydroxylase (TH1) and GTP cyclohydrolase I (CH1) encoded on two other AAV vectors [56]. To circumvent the small cloning capacity of AAV vector systems, a lentiviral vector system is being used to encode a tricistronic expression cassette containing all three therapeutic genes and is currently under development for human clinical trials [5].

Neuroprotective gene therapy should be especially useful in early PD stages when a significant number of nigral neurons remain and could be protected from further degeneration. It has been shown that gene transfer of glial cell-derived neurotrophic factor (GDNF) [57], brain cell-derived neurotrophic factor (BDNF) [58], the differentiation faction sonic hedgehog [59], the transcription factor Gli [60], and neurturin [61] protect nigrostriatal neurons from neurotoxic insults in rat and primate models of PD. For potential clinical application, uncertain consequences of long-term growth factor expression, such as downregulation of TH [62] and questions regarding timing and regulation of therapy need to be addressed. A double-blind phase I, open-label study of and AAV vector-encoding neurturin is currently in process and recruiting patients (http://www.clinicaltrials.gov/ct/show/NCT00400634?order = 1).

Other paradigms of gene therapy for PD that are currently being tested in animal models include the transduction of dopaminergic neurons with JNK-interacting protein-1 (JIP-1); sonic hedgehog, a secreted neurodifferentiation factor [63]; apoptosis protease activating factor-1 (APAF-1) [64] dominant negative inhibitor; neuronal apoptosis inhibitor protein (NAIP) [65]; Hsp70 [66]; and Parkin [67].

Gliomas

Gliomas are the most common primary intracranial neoplasms and are divided into astrocytomas, oligodendrogliomas, oligoastrocytomas and glioblastoma [68]. Gliomas affect about 5 per 100 000 people each year with a median survival time of less than 1 year [69]. Glioblastoma multiforme grade IV is the most fatal and most common primary brain neoplasm. Molecular lesions in glioma cells include deregulation of the cell cycle, alterations of apoptosis and cell differentiation, endothelial proliferation, neovascularization, and tumor cell migration and invasion.

The most important challenge for therapy of glioblastoma is the heterogeneity of the tumor tissue with highly proliferative tumor areas alongside with parts of necrosis and nondividing tumor cells migrating into the surrounding edematous tissue. Therefore, further developments of gene therapy for glioblastoma are concentrating on the following:

1. the combination of different therapeutic genes for synergistic action;
2. a combination of viral therapy with gene and immunotherapy;
3. improved methods of vector application based on convection-enhanced delivery;
4. imaging-based control of vector application and therapy readout [70].

There have been more than 40 clinical trials initiated using gene therapy approaches to treat glioma (see also Chapter 22). Adenoviral vectors, HSV-1 vectors, retroviral vectors, and oncolytic vectors (both adenoviral and HSV-1-derived) have all been used in human clinical trials for glioma [71]. Various gene therapeutic strategies have been studied in preclinical models of glioma including prodrug-activating enzymes (e.g., thymidine kinase [HSV1-TK], cytosine deaminase from various bacterial strains, guanine phosphoribosyl transferase, cytochrome P450, deoxycytidine kinase, folylpolyglutamyl synthetase,

carboxylesterase); cell-cycle regulating proteins (e.g., p53, p16, p21, PTEN, Rb, p300); proapoptotic genes (caspases, bax, Fas ligand); factors inhibiting angiogenesis (endostatin, angiostatin, antisense VEGF, dominant negative VEGF receptors, antisense EGF, dominant negative EGF receptors, antisense basic FGF and IGF1); immunomodulation (e.g., IL-2, IL-4, IL-6, IL-12, IL-13, GM-CSF, TNF-α, interferon-γ, antisense TGF-ß, TGF-ß-soluble receptors, Flt3L), and toxic proteins (*Pseudomonas exotoxin*) [72].

Some of these systems have been evaluated in clinical gene therapy protocols investigating the safety and efficiency of

1. replicating viruses (G207 [73], 1716 [74], ONYX-015 [75]),
2. prodrug therapy (HSV-1 TK delivered with an adenoviral vector + treatment with gancyclovir or valaciclovir),
3. cell-cycle regulation (p53 gene delivered with an adenoviral vector), and
4. immunomodulation (human interferon-β or Flt3L delivered with an adenoviral vector), or (v) stem cells (neural precursors targeting infiltrating brain GBMs) [76,77].

Interestingly, some therapeutic genes have been delivered with multiple gene therapy delivery strategies. For instance, the group of S. Yla-Herttuala demonstrated greater transduction efficiency and better brain tissue penetration when delivering HSV1-TK by an adenoviral vector as compared to delivery by a retroviral vector or retrovirus producing cells to treat glioma [78]. A phase III trial of adenovirus-expressing TK for treatment of glioma is currently ongoing. It is also important to note that while clinical responses may have been lackluster in most clinical trials using gene therapy for glioma. This is similar to results obtained in other antiglioma novel therapeutic approaches. On the positive side, the gene therapy treatment procedures and therapeutic agents were generally well-tolerated with minimal adverse events [71]. Further optimizations are currently being tested in clinical trials. Data from a phase I clinical trial using convection-enhanced delivery of a protein formulation of *Pseudomonas exotoxin* (PE) was recently published with only limited enhancement in survival of patients suffering from glioma [79]. A major drawback of treatment with a protein formulation is the short half-life of the compound. This disadvantage could be circumvented by delivering a viral vector encoding the *Pseudomonas exotoxin* gene directly into the tumor mass.

Pain

Pain is a very complex symptom that can be caused by various types of pathological stimuli. Neural pain pathways have been well-mapped, with the peripheral components involving the dorsal root ganglion neurons, while more psychological aspects of pain perception involve the cerebral cortex. Theoretically, pain perception could be modified by interfering with neural pathways anywhere along the pain circuits, but most emphasis has been given to modifying the function and signaling capacity of dorsal root ganglion neurons.

Of the various types of vectors in current use, herpes simplex type 1 vectors are ideally suited for the treatment of pain at the level of dorsal root ganglion (DRG) neurons. HSV-1 establishes latency within DRG neurons, and increased understanding of the HSV-1 promoters active during latency has allowed the production of vectors that express therapeutic transgenes specifically during latency. Adenoviral vectors also have potential as gene delivery vehicles for treatment of chronic pain as they are capable of infecting both dividing and nondividing, though if administration needs to be repeated, the use of these vectors and their potential immune responses will need to be controlled [80,81,82]. There are currently proposals to initiate clinical trials in various pain syndromes utilizing HSV-1-derived vectors.

Many candidate genes are under investigation for their ability to modify pain perception. Therapeutic genes such as met-enkephalin, leu-enkephalin, GDNF [83,84,85], γ-aminobutyric acid [86], β-endorphin [87], proopiomelanocortin [88], GABA [86,89] have been used in experimental gene therapy studies for the treatment of pain. Clinical trials

for gene therapy of pain are expected to be coming forward soon.

Silencing gene expression: an approach to treat dominantly inherited diseases

The technologies developed by the gene therapy scientific community have mainly concerned the expression of genes in target tissues. However, the reduction of endogenous gene expression has been more difficult to obtain. This has left dominantly inherited diseases where the expression of a mutated protein causes the disease without being developed as targets for gene therapy. This includes diseases such as Huntington's disease, dominantly inherited ataxias, and dominantly inherited spinal cord degenerations such as the familial cases of amyotrophic lateral sclerosis.

The recent discovery of endogenous cellular mechanisms that regulate precisely and effectively the levels of endogenous mRNA in cells has opened up the possibility of using this method, generally known as siRNA, to block expression of endogenous genes [90]. These techniques have now been used by many preclinical studies as an effective way to inhibit gene expression in a therapeutic context.

Various groups have now shown that siRNAs can be expressed from various types of vectors, and thus, these have been tested in the potential treatment of brain, liver diseases, infectious diseases, cancer, and pain. The already mature nature of the gene therapy field is likely to allow the rapid translation of these findings into clinical trials. Especially, the treatment of inherited dominant diseases has been limited to palliative treatments. Successes in experimental models of Huntington's disease are thus likely to be made into treatments in shorter time frames than gene therapies for diseases that already have effective, though not curative, treatments such as Parkinson's disease.

siRNA technology has been recently implemented in the context of gene therapy to treat spinocerebellar ataxia type 1 (SCA1), a progressive neurodegenerative disorder caused by a mutation in the ataxin-1 gene. Importantly, similar kinds of mutations due to triplet repeats are also involved in the pathophysiology of Huntington's disease, as well as at least nine other ataxias. AAV vectors were engineered to express enhanced short hairpin RNAs (shRNAs) specific for mutated human ataxin-1 gene expressed in a transgenic mouse model of SCA. Animals treated with AAV vectors expressing SCA1 shRNAs had reduced ataxin-1 expression, but more importantly, improved motor performance and reduced neuropathology [91].

The future challenges of gene therapy

During the last 20 years, major strides have been made in translating the potential of gene therapy into a clinical reality. To do so, vectors have been engineered to accept large, regulated expression cassettes to target vectors to predetermined cell types. The areas that have continued to challenge the field include the many difficulties of effective and targeted delivery of genes to the CNS and the innate and adaptive immune responses to the vectors, especially the exquisite sensitivity of the immune system to detect vector-infected cells in the brain [92,93,94]. The seemingly unavoidable clashes with the immune system have now called for the use of short-term immunosuppression during the early phases of vector delivery [95] or until the vectors uncoat and deliver their genomes to the nuclei of transduced cells. Alternative ingenious ways to overcome this with novel vectors, and steps to hide or reprogram the immune system, have also made significant progress.

Even if the challenge of immune responses against viral vector capsids is eventually resolved, the potential immunogenicity of transgenes will force the development of tolerance induction in gene therapy [96]. Thus, the field of gene therapy for the treatment of neurological diseases remains extremely active, busy, challenging, and is now close to significant clinical efficacy in various areas described above. The progress will undoubtedly continue, although with likely disappointments and setbacks, but certainly with a positive trajectory.

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