Carol Shoshkes Reiss *Editor*

Neurotropic Viral Infections

Volume 1: Neurotropic RNA Viruses

Second Edition

Springer

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Preface

This is one of two books that comprise a total of 29 cutting-edge review articles written by leaders in the basic or clinical and translational fields working on viruses that infect the central nervous system (CNS). Book 1 focuses on those pathogens with an RNA genome. Book 2 includes chapters on retroviruses, DNA viruses, prions, immunity, transmission, and beneficial uses of neurotropic viruses.

In this first volume are 14 chapters on neurotropic or neuroinvasive RNA viruses that are human pathogens. Viruses capable of infecting the cells within the brain can be spread to people by many routes including ingestion (for instance, polio, chapter "Poliovirus"), the respiratory route (one example is measles, chapter "Measles Virus and Subacute Sclerosing Panencephalitis"), and insect (Japanese encephalitis virus is spread by mosquitoes, chapter "Japanese Encephalitis Virus: Molecular Biology to Pathology") or animal (bats can transmit rabies, chapter "Measles Virus and Subacute Sclerosing Panencephalitis") bites.

Some viruses cause CNS disease in a small subset of people infected, and this may be due to many factors including variants in host genes, underlying chronic health conditions, or mutations in the virus (West Nile virus is one example, chapter "Neurotropic Flaviviruses"). To become successful pathogens, many neurotropic viruses have become masters of evasion of host innate or adaptive immune responses.

Viral infections can be prevented by avoiding exposure or by some excellent vaccines. For instance, in 2015, aggressive and deliberate use of the vaccine enabled Nigeria to eradicate endemic poliovirus infections (chapter "Poliovirus"). A new equine vaccine has been developed against the *Hendra* virus, and therefore people who care for horses are protected (chapter "Henipaviruses").

This book is restricted to RNA viruses. RNA viruses range from small, extremely simple agents in the picornavirus family that have a capsid and are relatively resistant to environmental conditions (chapter "Poliovirus") to more complex viruses with cell-derived membranes around the nucleic acid that can be easily disrupted by drying or soap and water (chapters "Measles Virus and Subacute Sclerosing Panencephalitis" to "Borna Disease Virus"). All these viruses can cause acute infections; some are capable of persisting in chronic infections (for instance, bornavirus in chapter "Borna Disease Virus").

This second edition of *Neurotropic Viral Infections* builds upon the highly successful first edition published in 2008 by Cambridge University Press (ISBN-13: 978-0521869645). I would like to acknowledge the generosity of Cambridge University Press in permitting us to move *Neurotropic Viral Infections* to Springer Scientific Publishers for the second edition. Arthur Smilios convinced me to undertake this volume. When he left Springer, Rita Beck ably succeeded him with the project. The book would not have reached the final stage without the fantastic production assistance by Portia Formento Wong.

New York, NY, USA Carol Shoshkes Reiss
February 19, 2016

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Poliovirus

Vincent Racaniello

Introduction

The family *Picornaviridae* includes many human and animal pathogens, such as poliovirus, hepatitis A virus, foot-and-mouth disease virus, and rhinovirus. All picornaviruses are small, non-enveloped 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. This chapter will focus on the biology and pathogenesis of poliovirus, the best studied picornavirus that causes disease of the nervous system. There are three serotypes of poliovirus which are classified in the species *Enterovirus C* within the genus *Enterovirus*. See "Measles Virus and Subacute Sclerosing Panencephalitis" chapter for a discussion of other neurotropic picornaviruses.

Virus Structure

Poliovirus particles consist of a 30 nm protein shell surrounding the naked RNA genome. The virus particles lack a lipid envelope, and consequently their infectivity is insensitive to organic solvents. These viruses pass through the stomach to gain access to the intestine and therefore must be resistant to low pH.

The capsids of polioviruses are built with 60 copies each of four structural proteins, VP1, VP2, VP3, and VP4, arranged into an icosahedral lattice (Fig. 1) (Rueckert et al. 1969). The basic building block of the poliovirus capsid is the pro-

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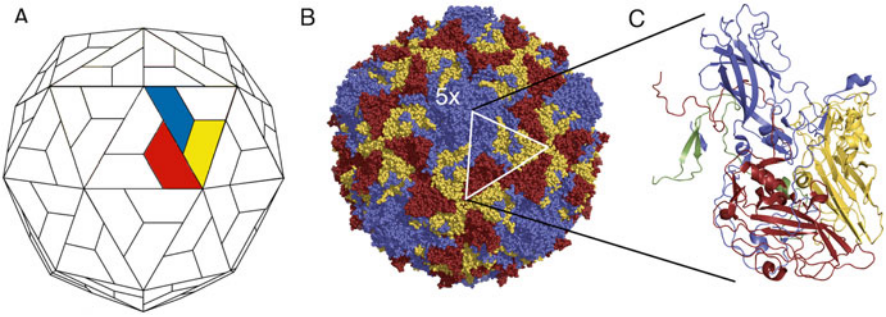

Fig. 1 Structure of poliovirus. (**a**) Schematic of the viral capsid, showing the packing arrangement of VP1 (*blue*), VP2 (*yellow*), and VP3 (*red*). VP4 is on the interior of the capsid. (**b**) Model of poliovirus type-1, Mahoney strain, based on the X-ray crystallographic structure determined at 2.9 Å (Hogle et al. 1985). At the fivefold axis (labeled) is a star-shaped mesa surrounded by the canyon, which is the receptor-binding site. (**c**) A single protomer is shown as a ribbon diagram, showing the locations of capsid proteins VP1, VP2, VP3 and VP4

tomer, 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.

Resolution of the atomic structure of poliovirus revealed that the surface of the capsid has a corrugated topography; there is a prominent star-shaped plateau (mesa) at the fivefold axis of symmetry, surrounded by a deep depression (canyon) and another protrusion at the threefold axis (Hogle et al. 1985) (Fig. 1). It was originally proposed that the canyon is the receptor-binding site, and this hypothesis has been proved for poliovirus and other picornaviruses (Belnap et al. 2000; He et al. 2000).

The Viral Genome

The genome of poliovirus, 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 genome is 7.4 kb in length and is covalently linked at the 5′ end to VPg protein (Virion Protein, genome linked) (Flanegan et al. 1977; Lee et al. 1977), which serves as a primer for viral RNA synthesis (Nomoto et al. 1977; Pettersson et al. 1978). The long (~742 nucleotide) and structured 5′-noncoding region contains 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. 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 poliovirus genome is the 3′-noncoding region (~70 nucleotides) which has been implicated in controlling viral RNA synthesis (Jacobson et al. 1993), and a 3′ stretch of poly(A) (Yogo and Wimmer 1972) that is required for viral infectivity (Spector and Baltimore 1974).

Viral Replication

Virus Entry into Cells

Poliovirus replication begins with attachment of virus particles to a cell surface receptor; for all three serotypes this molecule is CD155, a glycoprotein that is a member of the immunoglobulin superfamily of proteins (Mendelsohn et al. 1989). 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 (Koike et al. 1991a; Morrison and Racaniello 1992; Selinka et al. 1991, 1992; Aoki et al. 1994; Bernhardt et al. 1994; Morrison et al. 1994; Belnap et al. 2000; He et al. 2000; Xing et al. 2000). Alternative splicing of mRNA leads to the synthesis of two membrane-bound isoforms, CD155a and CD155d, and two isoforms that lack transmembrane domains and are secreted from the cell (Mendelsohn et al. 1989; Koike et al. 1990). The function of the secreted isoforms is unknown. The membrane-bound isoforms are adhesion molecules, participating in the formation of adherens junctions by interacting with nectin-3, an immunoglobulin-like protein related to CD155 (Mueller and Wimmer 2003). CD155 is also a recognition molecule for natural killer (NK) cells, and interacts with CD226 and CD96 on NK cells to stimulate their cytotoxic activity (Bottino et al. 2003; Fuchs et al. 2004). Cytomegalovirus evades NK cell-mediated killing because the viral UL141 protein blocks the surface expression of CD155 (Tomasec et al. 2005).

After attachment to a cellular receptor, the poliovirus capsid dissociates, releasing the RNA genome, which then enters the cytoplasm, the site of replication. Interaction of poliovirus with domain 1 of CD155 causes a conformational change in the capsid leading to release of the genome. 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 (Fricks and Hogle 1990). 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 (Bubeck et al. 2005a, b).

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 (Perez and Carrasco 1993), and arrest of the clathrin-dependent endocytic pathway using dynamin mutants that prevent clathrin-coated pit budding have no effect on poliovirus replication (DeTulleo and Kirchhausen

1998). 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 (Arita et al. 1999; Mason et al. 1993). CD155-mediated conformational changes in poliovirus are clearly important for the uncoating process.

Translation and Proteolytic Processing

After positive-strand polioviral RNA enters the cytoplasm, it is translated to provide viral proteins essential for genome replication and the production of new virus particles. The viral genome lacks a 5′-terminal cap structure, and cannot be translated by 5′-end dependent mechanisms. The 5′-untranslated region of poliovirus RNA harbors an internal ribosome entry site (IRES) that promotes internal binding of the 40S ribosomal subunit and allows 5′-end independent translation (Fig. 2). The poliovirus IRES contains extensive regions of RNA secondary structure that is crucial for ribosome binding. Translation initiation mediated by the IRES of poliovirus involves binding of the 40S ribosomal subunit to the IRES and scanning of the subunit to the AUG initiation codon. 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 poliovirus IRES requires cell proteins other than the canonical translation proteins. Such proteins have been identified by their ability to bind the IRES and restore internal initiation in reticulocyte lysates, in which

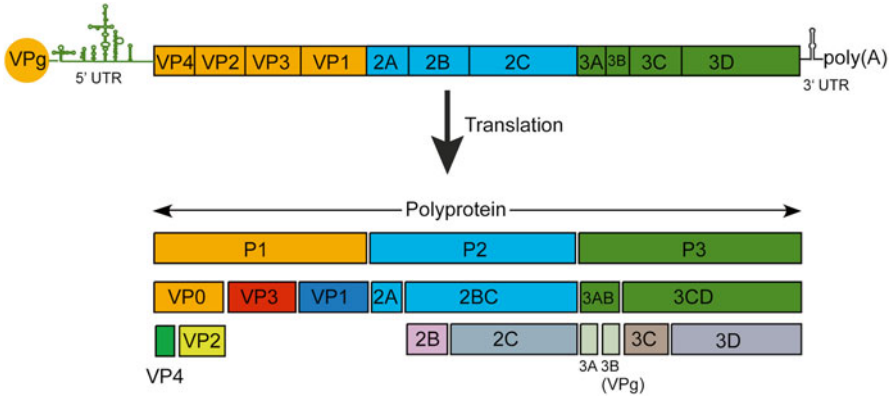

Fig. 2 Schematic of the poliovirus genome. At *top* is shown a diagram of the viral RNA with coding regions labeled. RNA structural elements include an enterovirus IRES within the 5′ untranslated region and the pseudoknot within the 3′ untranslated region. *Below* is the processing pattern of poliovirus 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

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 and stimulates its activity (Meerovitch et al. 1993; Kim and Jang 1999). Other proteins include polypyrimidine tract-binding protein, a regulator of pre-mRNA splicing (Hellen et al. 1993; Kaminski et al. 1995); unr, a RNA-binding protein with five cold-shock domains (Hunt et al. 1999); and ribosome-associated poly r(C)-binding proteins (Blyn et al. 1996, 1997; Gamarnik and Andino 1997). 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 (Jackson et al. 1995).

Poliovirus 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 poliovirus genome encodes two proteinases: $2A^{pro}$, and $3C^{pro}/3CD^{pro}$, which carry out cleavage of the polyprotein (Fig. 2).

RNA Synthesis

Poliovirus RNA synthesis is carried out by the virus-encoded RNA-dependent RNA polymerase, $3D^{pol}$, a primer- and template-dependent enzyme that specifically copies viral 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 either the 3′-poly(A), or a short RNA hairpin structure (50–100 nt), the cis-acting replication element, *cre*, located in the coding region of the poliovirus genome (Paul et al. 2000; Rieder et al. 2000; Yin et al. 2003).

The first step in genome replication is copying of 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 meditated 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 (Herold and Andino 2001). 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 the synthesis of (+) strand viral RNA. Synthesis of (+) strand viral RNA also requires uridylylated VPg (Morasco et al. 2003; Murray and Barton 2003).

Poliovirus RNA synthesis takes place on the cytoplasmic surfaces of membranous structures that are induced by viral infection (Bienz et al. 1987; Cho et al. 1994; Egger et al. 2000). Early in infection these include vesicles and tubular structures, while later in infection double-membrane vesicles predominate. 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, and involves the COPI and COPII secretory pathways, lipid kinases, and autophagy (Jackson 2014).

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 (Basavappa et al. 1994). VP0 is probably cleaved by an autocatalytic mechanism mediated by the viral RNA (Arnold et al. 1987).

The time required for a single replication cycle ranges from 5 to 10 h, depending on many variables, including the cell type, temperature, pH, host cell, and multiplicity of infection. The primary mechanism of poliovirus release from cells is by lysis, but non-lytic mechanisms have also been observed (Jackson et al. 2005; Bird and Kirkegaard 2015). Multiple viral particles appear to be packaged within phosphatidylserine lipid-enriched vesicles, and these are released without lysis from cells (Chen et al. 2015).

Pathogenesis of Poliomyelitis

General Features

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 grey and matter) was isolated in 1908 (Landsteiner and Popper 1908). At its peak in industrialized countries, poliomyelitis leads to paralysis of thousands of children each year. Research on the virus over the next 40 years leads to the development of two effective vaccines in the 1950s and 1960s. Recognition of poliomyelitis as a problem in developing countries did not take place until the 1970s. The Global Polio Eradication program began in 1988, when over 1000 children developed poliomyelitis each day. Eradication of polio now seems within grasp, although lingering pockets of disease confound this goal (Wassilak et al. 2014).

Infection with poliovirus begins when the virus is ingested and multiplies in the oropharyngeal and intestinal mucosa (Fig. 3) (Bodian and Horstmann 1965; Sabin 1956). Virus shed in the feces of infected individuals is largely responsible for transmission of infection. From the primary sites of multiplication in the

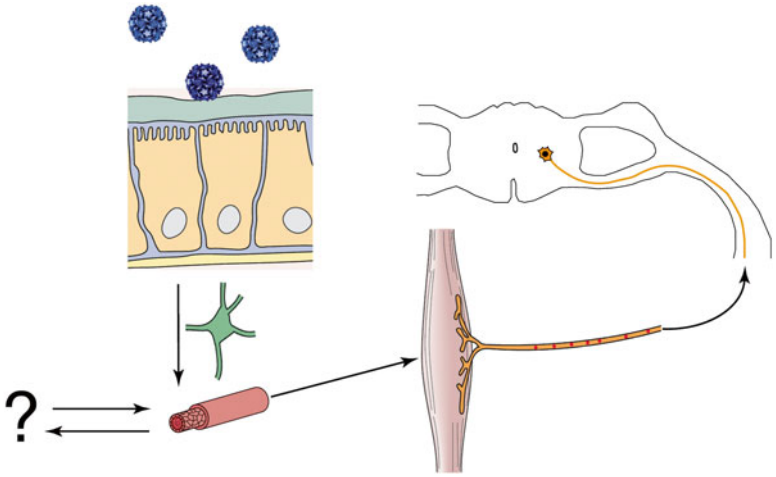

Fig. 3 Pathways of poliovirus spread in humans. Virus enters at the oropharyngeal and intestinal mucosa, replicates, and spreads to the blood through the lymph nodes, leading to viremia. Entry of virus into the central nervous system may occur either directly from the blood, or by retrograde axonal transport when virus is brought to the muscle via the blood stream. Invasion of the brain or spinal cord is preceded by viral multiplication in extraneural tissues (labeled, 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

mucosa, virus drains into cervical and mesenteric lymph nodes and then to the blood, causing a transient viremia (Bodian and Horstmann 1965). Most natural infections end at this stage with no symptoms or a minor disease consisting of nonspecific symptoms such as sore throat, fever, and malaise, followed by complete recovery. 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 (Bodian 1955; Wenner and Kamitsuka 1957; Ren and Racaniello 1992a). 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. In spinal poliomyelitis, paralysis is limited to muscles supplied by motor neurons in the spinal cord. The legs are affected more frequently than the arms. In bulbar poliomyelitis, the cranial nerve nuclei or medullary centers are affected; this form is often fatal due to respiratory or cardiac failure.

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. Transmission of poliovirus within the population, and maintenance of the virus, depends 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 (Pfeiffer and Kirkegaard 2006). When a mixture of 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. 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, e.g., they produce infectious virus after transfection with viral RNA (Holland et al. 1959a, b). The synthesis of CD155 in mouse L cells or in transgenic mice confers susceptibility to infection (Mendelsohn et al. 1989; Ren et al. 1990; Koike et al. 1991b). Orthologs of the *CD155* gene are present in the genomes of a number of mammals, including those that are not susceptible to poliovirus infection (Ida-Hosonuma et al. 2003). 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 a serial passage of viruses in non-primate hosts (Armstrong 1939; Li and Schaeffer 1953). Other poliovirus strains are naturally virulent in mice (Moss and Racaniello 1991). When mice are inoculated intracerebrally with P2/Lansing, they develop a disease with clinical, histopathological, and age-dependent features that resembles human poliomyelitis (Jubelt et al. 1980a, b). The murine cell receptor that allows entry of these strains into 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 (Murray et al. 1988). This six amino acid sequence is part of capsid protein VP1, on the surface of the virion at the fivefold axis of symmetry (Lentz et al. 1997), near the binding site for CD155 (Belnap et al. 2000; He et al. 2000; Xing et al. 2000). 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 (Bodian and Horstmann 1965). Poliovirus has been isolated from human tonsillopharyngeal tissue, the wall of the ileum, and mesenteric lymph nodes (Sabin and Ward 1941). However, removal of tonsils or adenoids does not reduce the level of poliovirus multiplication in the throats of humans (Sabin 1956). Consequently, it is not known if poliovirus replicates in lymphoid tissues or 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 (Ren and Racaniello 1992a). 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 (Iwasaki et al. 2002). 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 have been interpreted to 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 (Iwasaki et al. 2002).

CD155 transgenic mice are not susceptible to oral infection with poliovirus (Ren et al. 1990; Koike et al. 1991b). CD155 protein is present at very low levels in the intestinal epithelium of these mice, and absent in the Peyer's patches (Zhang and Racaniello 1997; Iwasaki et al. 2002). 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 (Zhang and Racaniello 1997). However, disruption of the type-I interferon gene in CD155 transgenic mice leads oral susceptibility to infection (Ohka et al. 2007). After oral infection, virus replication takes place in many tissues, including esophagus, nasopharynx-associated lymphoid tissue, small intestine, and spinal cord.

The susceptibility to oral poliovirus infection of CD155 transgenic IFNAR$^{-/-}$ mice has lead to an examination of the role of the intestinal microbiota in replication (Robinson et al. 2014; Kuss et al. 2011). Treatment of mice with antibiotics to reduce the intestinal microbiota leads to reduced susceptibility to poliovirus infection by the oral route, and lower viral replication in the intestine. Viral infectivity is enhanced by incubation with gut bacteria or surface polysaccharides such as lipopolysaccharide and peptidoglycan. These results demonstrate that poliovirus has evolved to benefit from the gut microbiota which enhances viral replication and transmission.

Although poliovirus is believed to be transmitted by fecal–oral contamination, in countries with high standards of hygiene, virus may be transmitted by the respiratory route. The source of virus for this mode of transmission is the tonsils and pharynx.

Replication at these sites usually occurs after virus replication in the intestine and spread 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.

Spread in the Host

After replication at primary sites in the mucosal epithelium, poliovirus drains into deep cervical and mesenteric lymph nodes and then to the blood, causing a transient viremia (Bodian and Horstmann 1965). Viral replication in extraneural tissues is thought to maintain viremia beyond the primary stage, and is required for viral invasion of the central nervous system (Bodian and Horstmann 1965). However, the sites at which viral replication occurs in humans is not known. In experimentally infected chimpanzees, high concentrations of virus are detected in brown fat (Nathanson and Bodian 1961), and in lymph nodes, axillary fat, adrenal glands, and muscle of monkeys (Wenner and Kamitsuka 1957). There is also evidence that virus may replicate in cells of the reticuloendothelial system and in the vascular endothelium of monkeys (Blinzinger et al. 1969; Kanamitsu et al. 1967). Poliovirus replication has been observed in skeletal muscle, brown adipose tissues, and nasal mucosa (Ren and Racaniello 1992a).

There is evidence that poliovirus may enter the central nervous system in two ways: from the blood, or by entering a peripheral nerve and being carried to the central nervous system by axonal transport (Fig. 3). 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 (Bodian and Horstmann 1965). The results of experiments in CD155 transgenic mice have provided 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 would be expected based on the vascular volume of the organ (Yang et al. 1997). Furthermore, the distribution of poliovirus in the brain of transgenic and non-transgenic 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 is unknown.

Poliovirus infections in humans and monkeys have provided 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 (Hurst 1936). 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 (Nathanson and Bodian 1961). Children who received incompletely inactivated poliovaccine in 1954 (the Cutter incident) developed a high frequency of initial paralysis in the inoculated limb

(Nathanson and Langmuir 1963). 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 limb that is inoculated; poliovirus is first detected in the lower spinal cord, and sciatic nerve transection blocks infection of the spinal cord (Ren and Racaniello 1992b; Ohka et al. 1998). The rate of poliovirus transport along the sciatic nerve was determined to be >12 cm per day, independent of virus replication (Ohka et al. 1998). 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." For example, in Oman, intramuscular injections have been linked to cases of vaccine-associated poliomyelitis (Sutter et al. 1992). Provocation poliomyelitis has been reproduced in CD155 transgenic mice (Gromeier and Wimmer 1998). In mice, skeletal muscle injury stimulates retrograde axonal transport of poliovirus to the spinal cord (Kuss et al. 2008).

The observation that the cytoplasmic domain of CD155 interacts with Tctex-1, the light chain of the retrograde motor complex dynein (Mueller et al. 2002; Ohka et al. 2004) suggests a hypothesis for the mechanism of axonal transport of poliovirus (Fig. 4). At the interface of muscle and motor neuron, 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

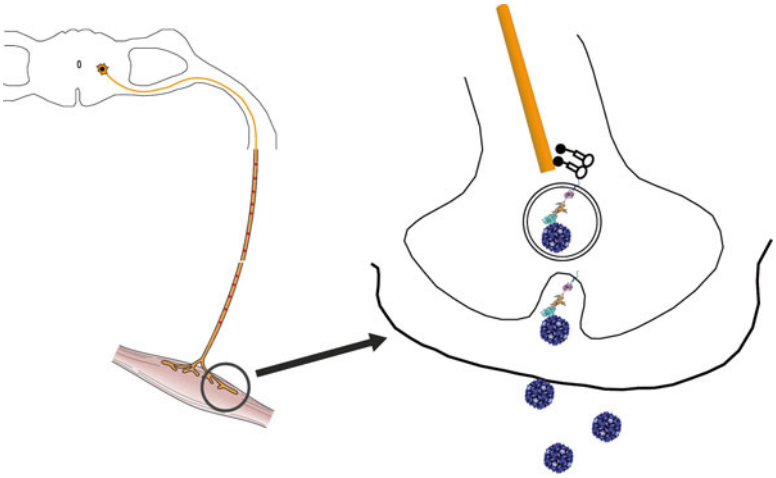

Fig. 4 Hypothetical mechanism of poliovirus axonal transport. Virus particles are transported to the muscle via the blood. At the neuromuscular junction, virus binds to its receptor, CD155, at the presynaptic membrane and is taken into the cell by endocytosis. The cytoplasmic domain of CD155 interacts 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

released into the cytoplasm and virus replication begins. In support of this hypothesis, CD155 has been detected at the neuromuscular junction of human muscle (Leon-Monzon et al. 1995), and it has been shown that poliovirus-containing vesicles are brought to the spinal cord by axonal transport dependent upon Tctex-1 (Ohka et al. 2004). 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 (Fricks and Hogle 1990). 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 (DeTulleo and Kirchhausen 1998).

Tropism

Poliovirus replicates only in specific cells and tissues in primates, even though the virus reaches many organs during the viremic phase (Bodian 1955; Sabin 1956). 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 (Holland 1961). The 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 (Mendelsohn et al. 1989; Freistadt et al. 1990; Koike et al. 1990). CD155 RNA and protein expression are also observed in many tissues of CD155 transgenic mice, including those where poliovirus does not replicate (Ren and Racaniello 1992a; Koike et al. 1994). 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 (Ohka and Nomoto 2001; Gromeier et al. 1996; Yanagiya et al. 2003; Borman et al. 1997). 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 (Kauder and Racaniello 2004). 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) (Garcia-Sastre et al. 1998; Ryman et al. 2000). 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 (Ida-Hosonuma et al. 2005). 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 sites of poliovirus replication. 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 play a role in determining whether or not the virus invades the central nervous system. As discussed earlier, poliovirus replication at the entry portal leads to viremia, which allows virus to reach an unidentified extraneural site. Replication at this site appears to be required for virus entry into the central nervous system. In 99 % of infections, the IFNα/β response may limit 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 non-neural sites followed by invasion into the central nervous system.

Histopathology of Poliomyelitis

In experimentally infected primates, lesions in the central nervous system consist of neuronal changes and inflammation. Viral replication leads to destruction of neurons and the inflammatory process follows as a secondary response (Bodian and Horstmann 1965). There is little evidence for viral replication in other cell types in the central nervous system. The relative contributions of polovirus-induced cell lysis and immunopathology to neuronal damage have not been determined. It is widely assumed that because poliovirus leads to lysis of most cells in culture, then it must also have the same effect on neurons in vivo. However, it would be informative to determine the outcome of infection of CD155 transgenic mice lacking specific components of the immune system, such as B-cells and/or T-cells.

Two factors determine the characteristic pattern of poliomyelitis lesions: the susceptibility of nervous centers to infection, and restricted movement of the virus along nerve fiber pathways (Bodian and Horstmann 1965). The motor neurons in the anterior horns of the cervical and lumbar regions are most sensitive to infection, followed by neurons in motor nuclei of cranial nerves in the brainstem. Lesions in the spinal cord are largely restricted to the anterior horns, but may also be observed in the intermediate, intermediolateral, and posterior gray areas (Bodian and Horstmann 1965; Bodian 1959) and may extend to the sensory spinal ganglia. Lesions in the brain are mainly in the brain stem, from the spinal cord to

the anterior hypothalamus, while those in the forebrain are generally mild and limited to the motor cortex, the thalamus, and the globus pallidus. More severe lesions may be found in the cerebellar vermis and the deep cerebellar nuclei (Bodian and Horstmann 1965).

In CD155 transgenic mice, the sites of poliovirus replication in the central nervous system are parallel to those observed in primates (Koike et al. 1991b; Ren et al. 1990; Ren and Racaniello 1992a). Motor neurons of the ventral horns of the cervical and lumbar regions are most sensitive to infection. A difference is that poliovirus replicates in the hippocampus of CD155 transgenic mice and causes damage to that area. Viral replication has been observed in neurons in the ventral and dorsal horns of the spinal cord of CD155 transgenic mice (Ren and Racaniello 1992a). However, neuronal degradation and inflammation is localized to the ventral horns. Histopathology is observed in the posterior horn (analogous to the dorsal horn of mice) in the spinal cord of humans and monkeys, but less frequently than in the anterior horn (Bodian 1959).

Post-poliomyelitis Syndrome

Survivors of poliomyelitis may develop muscle weakness, atrophy, and fatigue many years after poliovirus infection. These late consequences of infection, also observed in non-paralytic infections, were collectively called post-polio syndrome (PPS) and accepted in the 1980s as a new medical condition (Halstead 2011). Post-polio syndrome may develop in 20–75 % of infected patients 15 to over 60 years after the original infection, even in non-paralytic cases (Nee et al. 1995). With an estimated 15–20 million polio survivors throughout the world, the disease is the most common to affect anterior horn neurons. No specific and effective therapy for the syndrome is available.

The cause of post-polio syndrome is unknown. It has been suggested that a small number of PPS patients harbor fragments of the poliovirus genome, but infectious virus has not been recovered and the sequences of these genomes are not known (Baj et al. 2015). Another hypothesis for the etiology of PPS is that the loss of neurons during previous viral infections renders the patients susceptible to muscle weakness as age-related loss of neurons takes place. Alternatively, the large motor neuron units that form after recovery from poliomyelitis might degenerate, consistent with disintegration of these units in patients (Baj et al. 2015). The lack of an animal model of PPS has hindered an understanding of the mechanism of this syndrome.

Vaccines

Two highly effective vaccines to prevent poliomyelitis were developed during the 1950s. Inactivated poliovaccine (IPV), developed by Jonas Salk, consists of wild-type poliovirus strains that are treated with formalin to destroy infectivity without

altering the antigenic properties of the capsid. This vaccine was licensed in 1955 in the US and reduced the number of cases of paralytic disease from about 20,000 per year to ~2000. Despite this success, it was debated whether the use of a non-replicating vaccine such as IPV could eradicate the disease. Consequently, the infectious, attenuated oral poliovaccine, OPV, developed by Albert Sabin was licensed in the 1960s (Sabin et al. 1954). 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. Therefore, these vaccine strains were selected by the World Health Organization for the campaign to eradicate global poliomyelitis by 2000 (Organization WH 1993). When the eradication initiative began, wild-type poliovirus was endemic in over 125 countries, and over 350,000 cases of polio were reported annually. In mid 2015, polio was endemic in only three countries: Nigeria, Pakistan, and Afghanistan, and no cases of polio have been reported in Nigeria so far in 2015. 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 (Kawamura et al. 1989; Evans et al. 1985; Ren et al. 1991). 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 (Evans et al. 1985; Westrop et al. 1989; La Monica et al. 1987). This mutation has been shown to cause a translation defect in vitro and in cultured cells of neuronal origin (Gutierrez-Escolano et al. 1997; Svitkin et al. 1990; Haller et al. 1996). 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 (Ohka and Nomoto 2001; Gutierrez-Escolano et al. 1997; La Monica and Racaniello 1989). 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 non-neuronal cells and tissues (Kauder and Racaniello 2004) 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 (Kauder and Racaniello 2004). 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 (Nkowane et al. 1987). Vaccine-associated poliomyelitis occurs

due to reversion of the mutations in the viral genome that confers 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 (Evans et al. 1985). Because the Sabin strains undergo reversion in the gastrointestinal tract of nearly all recipients (Martinez et al. 2004), 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. Determination of patient genome sequences should be done to determine whether mutations in innate immune genes are associated with vaccine-associated poliomyelitis.

Circulating vaccine-derived polioviruses (cVDPV) pose a challenge to the eradication effort because they are capable of spreading from person to person and causing poliomyelitis. The first outbreak caused by cVDPV was in Hispaniola in 2001, where 22 cases of poliomyelitis were identified (Kew et al. 2002). 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 Phillipines (2001), Madagascar (2002 abd 2005), China (2004), and Indonesia (2005) (Kew et al. 2004; Katz 2006). It has also been reported that all cases of poliomyelitis occurring in Egypt between 1988 and 1993 were caused by vaccine-derived strains (Yang et al. 2003). These outbreaks call into question the WHO plan to stop immunization after polio eradication is achieved. In the post-immunization world, cVDPV strains will continue to circulate, posing a threat to the increasingly non-immune population.

One reason why polio was believed to be an eradicable disease was the belief that there are no chronic human carriers or non-human reservoirs of the virus. This notion was dispelled by the discovery of prolonged excretion of VDPV strains from individuals with defects in humoral immunity (Abo et al. 1979). 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 (Minor 2001). 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.

Since 1988, when the World Health Organization announced the polio eradication plan, it had relied exclusively on the use of OPV. The polio eradication and endgame strategic plan announced in 2014 includes at least one dose of IPV (Organization 2014). The rationale for including a dose of IPV was to avoid outbreaks of vaccine-derived type-2 poliovirus. This serotype had been eradicated in 1999 and had consequently been removed from OPV. However, IPV, which is injected intramuscularly and induces highly protective humoral immunity, is less effective in producing intestinal immunity than OPV. This property was underscored by the finding that wild poliovirus circulated in Israel during 2013, a country which

had high coverage with IPV. Furthermore, in countries that use only IPV, over 90 % of immunized children shed poliovirus after oral challenge. This shortcoming of IPV is problematic, in view of the recommendation of the World Health Organization to gradually shift from OPV to IPV. Even if the shift to IPV occurs after eradication of wild-type polioviruses, vaccine-derived polioviruses will continue to circulate because they cannot be eradicated by IPV. These concerns are mitigated by new results from a study in India which indicate that IPV can boost intestinal immunity in individuals who have already received OPV. This study shows that a dose of IPV is more effective than OPV at boosting intestinal immunity in children who have previously been immunized with OPV. Both IPV and OPV should be used together in the polio eradication program.

As of this writing, all cases of polio caused by wild-type virus are serotype-1; type-2 has been declared eradicated and type-3 will soon be as well.

Antiviral Drugs

Antiviral compounds have not played a role in control of poliovirus infections; none have been licensed for use in humans. However, one class of antiviral drugs has been useful in elucidating mechanisms of virus entry into cells. These are the WIN compounds, originally produced by Sterling-Winthrop (Smith et al. 1986), and similar molecules produced by Schering-Plough (Kenilworth, NJ) and Janssen Pharmaceuticals (Titusville, NJ) (Andries et al. 1990; Cox et al. 1996). The compounds bind tightly in a hydrophobic tunnel that is located within the core of VP1, just beneath the canyon floor of many picornaviruses. In poliovirus type-1 and -3, the pocket appears to contain sphingosine (Filman et al. 1989). 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 CD155 does not lead to the production of A particles (Fox et al. 1986; Zeichhardt et al. 1987). WIN compounds block poliovirus infectivity by preventing CD155-mediated conformational alterations needed for uncoating. Poliovirus mutants have been isolated that are not infectious unless WIN compounds are present (Mosser and Rueckert 1993). Such WIN-dependent mutants spontaneously convert into 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 CD155 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 (Pevear et al. 2005). One problem that has prevented licensing of these compounds is the problem of resistance: 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, the approach that has been successful in controlling infections with human immunodeficiency virus (Chen et al. 2007). 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 National Research Council of the US recommended that anti-poliovirus drugs should be developed in the event of a post-eradication outbreak. Assuming multiple drugs could be administered to overcome the problem of resistance, the use of 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 (Crowder and Kirkegaard 2005; Tanner et al. 2014). If antiviral drugs could be identified that produce dominant negative proteins, then the replication of drug-resistant genomes should 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 % 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.

The polio eradication effort has lead to a renewed effort to discover anti-poliovirus compounds that could be useful for limiting outbreaks in a post-vaccine era. Examples include the capsid inhibitor pocapavir (McKinlay et al. 2014), and itraconazole, an anti-fungal agent that blocks poliovirus replication (Strating et al. 2015).

Perspectives

If the eradication of poliomyelitis 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 that are infected by poliovirus in the alimentary tract, and precisely how the gut microbiota assists in poliovirus replication. 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, or the immune response, or both?

Poliovirus has been studied for over 100 years, first as the etiologic agent of a significant human disease, then as a model for RNA virus infections of the central nervous system. Once research on poliovirus ceases, attention will likely 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 for unraveling many of these fundamental problems. Which picornavirus will emerge to replace poliovirus as a model system?

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Measles Virus and Subacute Sclerosing Panencephalitis

Lauren A. O'Donnell and James F. Bale Jr.

Introduction

The measles–mumps–rubella (MMR) vaccine has nearly eliminated subacute sclerosing panencephalitis (SSPE), a rare complication of measles, and other measles-virus-related neurological disorders in populations with compulsory vaccination programs (http://www.cdc.gov/measles/about/history.html). Nonetheless, the neurological complications of measles, including SSPE, remain threats to unimmunized persons, especially children who live in measles-endemic regions and acquire measles at young ages. This chapter summarizes current information regarding the epidemiology, virology, clinical manifestations, diagnosis, and management of measles and its neurological complications, focusing on SSPE.

Epidemiology

Measles

The paramyxoviruses, a family of animal and human viruses, can be associated with serious and life-threatening disorders of the central nervous system (CNS). While measles virus is a human pathogen, virologists speculate that measles descended from rinderpest virus, a paramyxovirus that infects cattle and other cloven-hoofed

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ruminants, or from a more ancestral virus shared with rinderpest (Baron and Barrett 1995; Furese et al. 2010). Thus, measles may have emerged in settings where livestock and humans lived in close proximity (Furese et al. 2010). Recently emergent paramyxoviruses, such as Nipah and Hendra viruses, appear to use fruit bats as reservoirs (Chua et al. 2002; Halpin et al. 2000) and may occasionally crossover into domestic animals and perhaps directly into humans (Epstein et al. 2006; Hsu et al. 2004).

Estimates of the divergence of measles virus from rinderpest virus suggest that measles emerged between the eleventh and twelfth centuries, corresponding to the period of time when human populations were sufficient to maintain the virus (Furese et al. 2010; Black 1966). Measles virus adapted quickly to replication in human cells, circulated efficiently between people, and demonstrated significant genetic stability (Schrag et al. 1999). While circulating strains are relatively stable, the World Health Organization continues to monitor the origin of measles outbreaks and track elimination of measles virus in different geographical regions (Rota et al. 2011). Measles virus, a highly contagious pathogen, requires substantial herd immunity to prevent spread within communities (Moss and Griffin 2006). Humans currently serve as the only reservoir of measles virus. Persons with measles, most contagious during the catarrhal or secretory phase of infection, transmit measles virus via infected respiratory droplets when coughing and sneezing. Fomites can also participate in the transmission of the measles virus; the measles virus can survive as long as 2 h on inanimate surfaces (http://www.cdc.gov/measles/about/transmission.html).

Prior to the development and licensure of the measles vaccine and implementation of immunization programs, measles occurred worldwide, often in major outbreaks in the winter and spring every 2–4 years (Johnson 1998; American Academy of Pediatrics 2012). While potentially associated with serious illnesses and occasional deaths in populations in which the measles virus circulated historically (http://www.cdc.gov/measles/about/history.html), the measles virus decimated certain native populations, including the Native Americans, Canadian First Nations, and Pacific Islanders, when explorers, traders, or missionaries introduced the measles virus into populations which had never encountered the virus (http://www.deephawaii.com/hawaiianhistory.htm; Shulman et al. 2009). 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 other Europeans, a phenomenon attributable to the introduction of the measles virus and other human pathogens (http://www.deephawaii.com/hawaiianhistory.htm; Shulman et al. 2009). Measles often had tragic consequences. In 1824, the Hawaiian king and queen (King Kamehameha II and Queen Kamamalu) contracted measles during a diplomatic visit to England and died from its complications (Shulman et al. 2009). Prior to 1978, when the US Centers for Disease Control and Prevention (CDC) established measles elimination as a national goal (http://www.cdc.gov/measles/about/history.html), three to four million people had measles annually in the US. Measles accounted for approximately 50,000 hospitalizations and 500 deaths annually in the US, primarily among young children (http://www.cdc.gov/measles/about/history.html).

Measles virus vaccine, an attenuated, live-virus vaccine developed in the 1960s, dramatically reduced the numbers of measles cases in the US and other resource-rich regions with compulsory immunization programs. By the mid-1980s,

the US CDC was receiving reports of <4000 cases annually. Periodically since, however, the United States has experienced measles outbreaks, indicating that the virus continues to circulate. During 1989–1991 a major US outbreak occurred, causing nearly 60,000 cases and approximately 125 measles-related deaths largely among unvaccinated Hispanic and African-Americans in Milwaukee, Dallas, Houston, Los Angeles, and New York City (Centers for Disease Control and Prevention 1991). In the winter of 2014–2015 another widespread outbreak began following exposure to a single measles-virus-infected person at a US theme park (Zipprich et al. 2015). Such resurgences emphasize the importance of adhering to vaccination recommendations, which include a second measles vaccination be given in late childhood or early adolescence. Because of this strategy and effective measles vaccination campaigns, the US has achieved a >99 % reduction in the incidence of measles.

By contrast, measles remains a major source of morbidity and mortality among children and adolescents in many of the world's regions, especially in Asia and sub-Saharan Africa (http://www.who.int/mediacentre/factsheets/fs286/en/), as well as in certain resource-rich nations, such as the United Kingdom, that have witnessed declines in vaccination coverage (Simone et al. 2014). In 2013, the World Health Organization estimated that 145,000 persons worldwide died from complications of measles, with the majority of deaths among children less than 5 years of age (http://www.who.int/mediacentre/factsheets/fs286/en/). Although the number of measles-related deaths remains substantial, the current number represents a >90 % reduction in deaths since 1980 when measles vaccination became a worldwide priority (http://www.who.int/mediacentre/factsheets/fs286/en/). More than 80 % of the world's children currently receive at least one dose of measles vaccine by 1 year of age.

Subacute Sclerosing Panencephalitis

Prior to 1980, subacute sclerosing panencephalitis (SSPE) affected approximately three of every ten million persons under the age of 20 years in the US, corresponding to approximately ten cases of SSPE per 100,000 cases of measles (Johnson 1998; Modlin et al. 1979). With the introduction of compulsory measles vaccination, however, SSPE has nearly disappeared in the US and other resource-rich nations (Modlin et al. 1979; Dyken et al. 1989; Abe et al. 2012; Schönberger et al. 2013). The CDC currently receives few reports of SSPE in the US annually, and the incidence of SSPE in Japan was only 0.03 cases/million during 2001 to 2005 (Abe et al. 2012). From 2003 through 2009 only 31 cases of SSPE were treated in Germany, and of these, fewer than one-half of the children acquired measles virus in Germany (Schönberger et al. 2013). These authors estimated that the risk of developing SSPE in Germany was 1:1700 to 1:3300 when measles virus infection occurred prior to 5 years of age. By contrast, SSPE persists in areas with low rates of measles vaccination. Papua, New Guinea, which has a measles vaccine coverage of <70 %, has 50–100 cases of SSPE/million inhabitants (Manning et al. 2011).

SSPE typically affects children between the ages of 5 and 15 years, with a median age of onset between 7 and 9 years in most studies (Sonia et al. 2009), but cases with onset in adulthood have been reported (Singer et al. 1977). In nearly all reported case series, boys outnumber girls by a ratio of approximately 2.5:1 or greater (Modlin et al. 1979; Sonia et al. 2009). A history of measles is almost always elicited, and approximately 50 % of patients with SSPE have had measles before the age of 2 years (Schönberger et al. 2013; Jabbour et al. 1972). The interval between measles virus infection and the onset of SSPE averages 6–10 years (Johnson 1998; Dyken et al. 1989; Sonia et al. 2009; Jabbour et al. 1972; Zilber et al. 1983; Anlar et al. 2001a), although the interval can exceed 20 years in adults with SSPE.

The role of wild measles virus infection in the pathogenesis of SSPE is undisputed, but some debate persists regarding the role of measles vaccine, an attenuated, live virus, and SSPE. Occasional reports, including some from the US, suggest that vaccination was the only known exposure to the measles virus in cases of SSPE (Dyken et al. 1989; Zilber et al. 1983). The risk of SSPE after wild measles virus infection is approximately 1 per million, whereas the risk of SSPE after vaccination is *at least* an order of magnitude less (Zilber et al. 1983; Gascon 1999). The virtual disappearance of SSPE from the US and other regions with vaccination programs argues against a substantial role for the measles vaccine in the etiology of SSPE (Gascon 1999; http://www.who.int/vaccine_safety/committee/topics/measles_sspe/Jan_2006/en/).

Virology

A viral origin was long suspected for SSPE based on the identification of inclusion bodies in the nuclei and cytoplasm of cells in brain tissues from patients with the disorder. Measles became the primary suspect for SSPE when electron micrographs of brain biopsies revealed inclusions that were similar to measles virus grown in cell culture (Bouteille et al. 1965). A subsequent series of studies in the 1960s demonstrated the presence of immunoreactive staining for measles antigens in brain tissue and high titers of anti-measles antibodies in the CSF and serum (Connolly et al. 1967; Legg 1967). However, measles virus was not propagated from SSPE tissues until 1969, when measles was recovered through co-culture with permissive tissue culture cells (Chen et al. 1969; Payne et al. 1969). In the years since the connection between measles and SSPE was established, considerable effort has been expended studying the pathogenesis of measles virus infection of the CNS.

Like other member of the paramyxovirus family, measles virus is an enveloped, negative sense, single-stranded RNA virus. The viral genome encodes for eight proteins (Fig. 1), including replication factors [RNA dependent RNA polymerase (L) and phosphoprotein (P)], structural proteins [nucleoprotein (N), matrix (M), hemagglutinin (H), and fusion (F)], and two accessory proteins that are important for immune evasion and virulence (C and V) (Poole et al. 2002; Rodriguez et al. 2003). The viral ribonucleoprotein complex (RNP) is contained within the viral particle, and includes the ~16 kb RNA genome associated with N, L, and P. The M protein

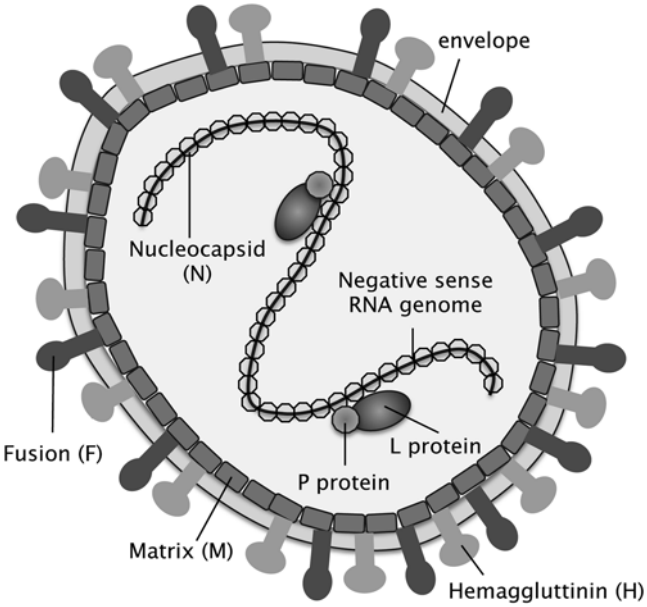

Fig. 1 Structure of the measles virus particle. Measles virus is an enveloped virus that expresses fusion (F) and hemagglutinin (H) proteins on its surface. Matrix (M) proteins line the interior of the viral membrane and provide a structural link between the envelope proteins and the ribonucleoprotein (RNP) complex. The RNP is comprised of the single-stranded, negative sense RNA genome, which is encapsulated in nucleocapsid (N) proteins. The Large protein (L), which serves as the RNA polymerase, and the phosphoprotein (P) are attached to the genome. In viral isolates from the brains of SSPE patients, mutations are often found in M, H, and F proteins

acts a bridge between the RNP and the envelope proteins (Manie et al. 2000; Vincent et al. 1999), and is required to transport H and F to the plasma membrane before budding (Naim et al. 2000). The viral envelope includes spikes that are comprised of multimers of the H and F proteins. To initiate infection, the measles virion attaches to the host cell via the H protein, triggering a conformational change in H that reveals the stalk domain of the F protein (Jardetsky and Lamb 2014). The F protein then mediates fusion of the viral and host cell membranes and facilitates the release of the RNP into the host cell cytoplasm.

The measles virus H protein binds to the host cell via one of three known measles virus receptors: CD46, CD150/signaling lymphocyte activation molecule (SLAM), and nectin 4 (Delpeut et al. 2012). Laboratory-adapted and vaccine strains of measles virus interact with CD46, a complement receptor that protects cells from complement-mediated attack (Dorig et al. 1993; Naniche et al. 1993; Russell 2004). CD46 is expressed widely on nucleated cells, and the nearly ubiquitous expression contrasts with the more limited tropism of measles virus in vivo. Later, researchers discovered that wild-type measles strains can utilize CD150/SLAM as a cell entry receptor (Tatsuo and Yanagi 2000). CD150/SLAM is expressed on various immune cells, including activated dendritic cells, macrophages, and B and T cells, which

mirrors the ability of measles virus to target immune cells during an infection. The most recently discovered measles virus entry receptor is Nectin-4 (Noyce et al. 2011; Mühlebach et al. 2011), which also serves as a receptor for canine distemper virus (Noyce et al. 2013). Nectin-4, an adherens junction protein, is expressed by epithelial cells, including cells in the respiratory epithelium. Wild-type measles virus strains utilize Nectin-4 as an epithelial entry receptor, helping to explain why measles virus is ultimately shed from the respiratory epithelium during infection (Delpeut et al. 2012).

Measles virus first infects alveolar macrophages and dendritic cells (DCs) in the respiratory tract (Noyce et al. 2013; De Swart et al. 2007; Ferreira et al. 2010; Lemon et al. 2011). The infected macrophages and DCs migrate to lymph nodes and other secondary lymphoid organs, where the virus spreads to activated-T- and B-cells (Ludlow et al. 2015). The amplification of the virus in lymphoid tissues facilitates hematogenous dissemination of measles virus to systemic organs. Ultimately, infected lymphocytes migrate to the lung, passing the virus to the basolateral side of the respiratory epithelium and leading to the shedding of virus from the respiratory tract (Delpeut et al. 2012). Once the infection is resolved, lifelong immunity against the virus is established.

In rare instances, measles virus causes serious CNS complications, including SSPE. The mechanism and timing of viral entry into the CNS is, however, unknown. Some investigators propose that measles enters the brain via a "Trojan Horse" method through infiltration of infected leukocytes or through cerebral endothelial cells via an unknown receptor (Buchanan and Bonthius 2012). Regardless of the route of entry, measles virus replicates and spreads in neural cells, particularly in neurons and oligodendrocytes. Astrocytes and infiltrating lymphocytes can also be infected (Allen et al. 1996; Kirk et al. 1991). CD150 expression has not been detected on neural cells, and staining of SSPE tissue only shows CD150 expression on subsets of infiltrating leukocytes (McQuaid and Cosby 2002). The viral life cycle also differs in infected neurons, with little release of extracellular virus and no evidence of viral budding from the neuronal membrane.

Brain isolates of measles virus show extensive mutations, particularly in the M, H, and F proteins (Ayata et al. 1989; Baczko et al. 1984, 1986, 1988; Cattaneo et al. 1986, 1988a, b, 1989; Schmid et al. 1992; Wong et al. 1989; Yoshikawa et al. 1990). Mutations in the M protein are clonally expanded throughout the brain, suggesting that measles enters the CNS early in the course of infection (Baczko et al. 1993). The overall effect of these mutations is to interfere with assembly of new viral particles and budding, leading to defective viruses that spread by the RNP instead of complete viral particles. The RNP may spread through the brain via transynaptic spread between neurons. Evidence for transynaptic spread of measles virus has been shown in cultured hippocampal neurons in vitro (Lawrence et al. 2000; Ehrengruber et al. 2002) (Fig. 2), in mouse models of measles infection (Duprex et al. 2000) and in SSPE brains (Sawaishi et al. 1999). The measles RNP is transported in a retrograde manner along the axon of the infected neuron. At the synapse, it is hypothesized that the RNP is transported to the adjacent neuron via interactions between the viral F protein and neurokinin-1, a neuronal receptor located at the synaptic cleft

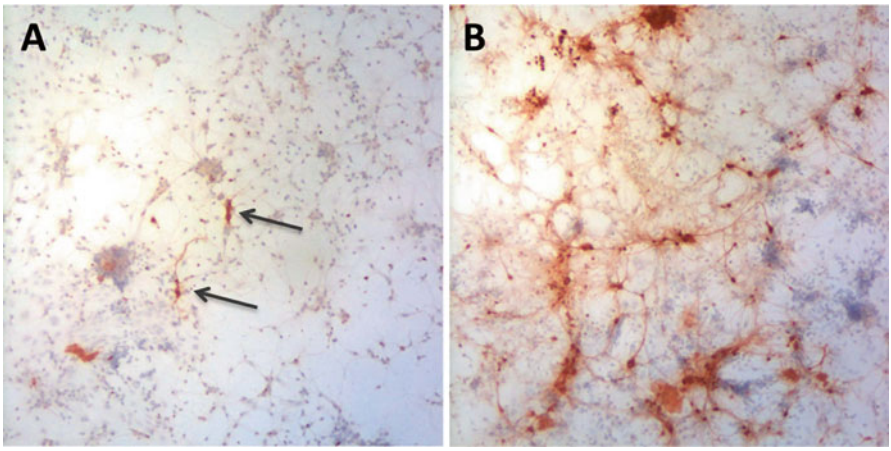

Fig. 2 Measles virus spreads between neurons via transynaptic spread. Murine hippocampal neurons expressing the human isoform of CD46 were infected with measles virus (brown stain) for 24 h (**a**) or 72 h (**b**). *Arrows* indicate measles-infected cells at 24 h post-infection, showing single neurons that are expressing viral proteins. By 72 h, measles has spread through groups of interconnected neurons. Studies have demonstrated that measles spread between neurons occurs by crossing at the synapse

(Makhortova et al. 2007). Since the RNP is the minimal infective unit for measles virus (Rosenblatt et al. 1979), the spread of the RNP would be sufficient to cause infection in neighboring neurons.

While measles virus clearly causes SSPE, measles does not always induce SSPE when it gains access to the brain. Autopsy studies on individuals without SSPE have shown that ~20 % had detectable measles RNA present in the brain (Katayama et al. 1995, 1998). Though further studies would be required to confirm these findings, these suggest that measles may enter the brain more frequently than would be assumed, based on the number of SSPE cases, and that neuroinvasion by measles virus may not lead to neurological disease in every case.

Although immune responses are active during SSPE, host immunity ultimately fails to control measles within the CNS. Anti-measles antibodies are found in the CSF and the serum of SSPE patients, and measles-specific plasma cells can be detected within the brain (Burgoon et al. 2005). Substantial lymphocyte infiltration is also observed, with CD4+ helper T-cells in perivascular regions and CD8+ cytotoxic T-cells in the brain parenchyma (Anlar et al. 2001b). Despite the robust infiltration of immune cells into the CNS, deficits in cell-mediated immunity have been observed in SSPE patients. CD8+ T-cells derived from SSPE patients show reduced cytotoxic T-lymphocyte (CTL) responses to measles-infected target cells, but not to target cells infected with other viruses (Dhib-Jalbut et al. 1989). Peripheral blood mononuclear cells from SSPE patients also show limited production of interferon-gamma (IFNγ), an important anti-viral cytokine, in response to measles virus (Hara et al. 2000). SSPE patients could be stratified based upon relative levels of IFNγ production, with low IFNγ-producers experiencing more rapid disease

progression and loss of receptive function. These findings, along with reports of lower reactivity to measles and decreased responses to mitogens (Aysun et al. 1984; Derakhshan et al. 1981), suggest that SSPE patients experience deficits in CTL or cytokine responses to the measles virus.

Numerous uncertainties regarding the neuropathogenesis of SSPE persist. The replication status of the virus between acute measles virus infection and the development of SSPE years later is unknown. Measles may be slowly but persistently replicating during that period, or the virus may undergo a replicative burst years after the initial infection that then leads to SSPE. Some investigators have postulated that unidentified cofactors facilitate the appearance of SSPE (Halsey et al. 1980). The effects of measles virus on neuronal function and mechanisms of neuronal loss during SSPE also remain undefined. Such questions are challenging to answer in human studies, as SSPE samples are rare and typically available only from late stages of the disease. Animal models of measles CNS infections have provided insights into interactions between measles-infected neurons and the host immune response (Oldstone 2009). Rodent-adapted virus strains and transgenic mice expressing human measles virus receptors provide models for addressing viral persistence in the brain (Carsillo et al. 2004; Schubert et al. 2006), age-dependent neuropathogenesis (Schubert et al. 2006; Lawrence et al. 1999), and immune factors related to viral control in the CNS (Patterson et al. 2002; Tishon et al. 2006; Kim et al. 2013). The animal models should provide additional insights into the neuropathogenesis of measles virus-induced CNS disease, including SSPE.

Clinical Manifestations

After an incubation period of approximately 10 days, persons with measles experience fever, cough, congestion and conjunctivitis (American Academy of Pediatrics 2012; Carole 1987). Within 3–5 days of the onset of these symptoms, Koplik spots, a grayish-white, sand-like enanthem, appear on the buccal mucosa adjacent to the lower molars. Over the next 48 h, the measles exanthem, consisting of erythematous, maculopapular lesions a few millimeters in diameter, appears on the face, neck, and upper trunk and then spreads diffusely over the entire body. The exanthem, commonly accompanied by fever to 40 ° C, can be petechial or purpuric, occasionally resembling the rash associated with meningococcemia or Rocky Mountain spotted fever (Carole 1987). The rash lasts 5–7 days, and gradually assumes a brownish coloration before fading entirely. The severity of measles does not predict, however, the likelihood of SSPE.

Severe cases of measles can be associated with pneumonia, mastoiditis, encephalitis, or acute disseminated encephalomyelitis. Acute CNS disease complicates approximately 1 per 1000 cases of acute measles (Johnson 1998; Buchanan and Bonthius 2012). Encephalitis, reflecting direct viral invasion of the CNS, and encephalomyelitis, an immune-mediated post-infectious process, produce headache, irritability, seizures, somnolence, or coma (Johnson 1998; Buchanan and Bonthius 2012); some affected children have choreoathetosis, ataxia, Guillain-

Barré-like paralysis or bladder/bowel dysfunction. Although most patients with measles encephalitis or encephalomyelitis recover spontaneously, some deteriorate and die because of intractable seizures or cerebral edema.

Patients with congenital or acquired disorders of cell-mediated immunity can have a progressive, often fatal measles-virus-induced neurological disorder after wild-type virus infection or immunization known as measles inclusion body encephalitis or subacute measles encephalitis/encephalopathy (Johnson 1998; Murphy and Yunis 1979; Mustafa et al. 1993; Freeman et al. 2004). The temporal profile of this disorder, onset 1–6 months after exposure to the measles virus, distinguishes this disorder from both acute encephalitis and encephalomyelitis, which usually begin within 3 weeks of measles, and SSPE, which usually begins years after infection with the measles virus (Johnson 1998). Inclusion body encephalitis/subacute measles encephalopathy produces incoordination, cognitive dysfunction or seizures, and progresses to debility and coma in typical cases. Death usually ensues, although some patients can improve with anti-viral therapy (Mustafa et al. 1993).

SSPE begins insidiously with behavioral or intellectual deterioration (Johnson 1998; Murphy and Yunis 1979), and early on, these features, which include irritability, emotional lability or attention-deficient hyperactivity disorder-like symptoms, can be confused with a neurobehavioral or psychiatric disorder (Garg 2008); SSPE can also begin with visual symptoms, particularly in the cases with onset in adulthood (Garg 2008; Singer et al. 1977; Haddad et al. 1977; La Piana et al. 1974). Myoclonus ensues, although this can be subtle during the early stages and may consist only of eye blinking or head nodding (Gascon 1999). Myoclonus, often provoked by excitement or sensory stimuli, typically intensifies thereafter, involving the extremities, head, or trunk. Generalized tonic-clonic or absence seizures can occur at this time, as well.

As SSPE progresses, myoclonus worsens, affecting gait or other motor activities, and the patient's speech, coordination, and intellectual abilities deteriorate. The patient's myoclonus can assume a periodic pattern (Gascon 1999). Choreoathetosis, bradykinesia or rigidity may appear at this time. Eventually, patients with SSPE become completely debilitated with autonomic instability, bulbar dysfunction, paralysis and profound dementia. Approximately one-half of the patients have visual signs or symptoms, consisting of chorioretinitis, optic atrophy, nystagmus, cortical visual impairment, or visual field defects (Garg 2008). Occasional patients have atypical SSPE and deteriorate acutely with seizures, focal deficits, and increased ICP (Silva et al. 1981).

The generally characteristic and relatively predictable course of SSPE can allow patients to be assigned to reasonably well-defined clinical stages (Johnson 1998; Gascon 1999; [99]). According to one staging schema (Table 1), Stage 1 denotes the early neurobehavioral features of the disorder; Stages II and III correspond to phases of neurological deterioration; and Stage IV denotes the preterminal vegetative/debilitated state (Gascon 1999). Clinical categorization can facilitate therapeutic trials, and subcategories can be assigned to identify specific clinical manifestations. However, the clinical course of SSPE can also be highly variable, making precise predictions of progression and analysis of therapeutic trials very difficult (Prashanth et al. 2006).

Table 1 Clinical Staging of subacute sclerosing pancephalitis

Stage	Clinical features
IA	Changes in behavior, personality, and cognition
IB	Myoclonic spasms
IIA	Mental deterioration; generalized myoclonus; independent ambulation
IIB	Apraxia, agnosia, independent ambulation still possible
IIIA	Seizures, prominent myoclonus, unable to ambulate independently
IIIB	No spontaneous speech, blind, bedridden
IV	Neurovegetative state

Diagnosis

Measles virus infection can be confirmed by (1) detecting measles virus-specific immunoglobulin (Ig) M or rising titers of measles virus-specific IgG in serum, (2) isolating measles virus from urine, blood, saliva or nasopharyngeal secretions (American Academy of Pediatrics 2012), or (3) detecting measles virus RNA in body fluids or brain tissue using reverse transcription (RT) polymerase chain reaction (PCR). Measles-virus-specific IgM can persist in serum for up to 1 month after acute infection; molecular studies can differentiate vaccine and wild measles strains (American Academy of Pediatrics 2012).

The cerebrospinal fluid (CSF) in children with measles virus encephalitis or encephalomyelitis usually has a lymphocytic pleocytosis, mildly elevated protein content, and normal glucose content. The electroencephalogram (EEG) may show diffuse slowing or epileptiform discharges in either condition. Magnetic resonance imaging (MRI), the most sensitive means to detect measles-virus-related brain lesions, can be normal in measles virus encephalitis or show white matter lesions compatible with acute disseminated encephalomyelitis in children with measles virus encephalomyelitis (Buchanan and Bonthius 2012). In measles inclusion body encephalitis, a rare disorder of immunocompromised hosts (Murphy and Yunis 1979; Mustafa et al. 1993; Freeman et al. 2004), the CSF is usually normal, whereas EEGs may show diffuse slowing or epileptiform features. MRI can be normal early in the course of the condition and later show cerebral cortical atrophy or edema of deep nuclear structures.

SSPE, although a rare disorder, should be considered in children with progressive encephalitis and a history suggesting measles at a young age, in persons who spent their childhood in measles-endemic regions, or when the cause of progressive encephalitis has not been identified (Honarmand et al. 2004). Routine laboratory studies in patients with SSPE are typically unrevealing, and the cerebrospinal fluid (CSF) usually contains no leukocytes and has normal protein and glucose content. The diagnosis of SSPE is established by detecting high titers of measles-virus-specific IgG in serum and CSF. The CSF immunoglobulin levels are typically elevated, reflecting active synthesis of measles-virus-specific immunoglobulin, and

oligoclonal IgG bands can be detected (Gascon 1999). Measles virus RNA can be detected in the CSF or brain tissues of some SSPE patients by using RT-PCR (Nakayama et al. 1995).

The electroencephalogram (EEG), often the initial clue to the diagnosis of SSPE, shows bilaterally synchronous high amplitude slow or spike-wave bursts that may correspond to clinical myoclonus. As SSPE progresses, the background activity of the EEG becomes diffusely suppressed, frontally predominant epileptiform features are identified, and a burst-suppression pattern eventually appears (Demir et al. 2013). Neuroimaging studies show non-specific abnormalities or diffuse atrophy, although signal abnormalities can be detected on T2-weighted magnetic resonance imaging (MRI) symmetrically in subcortical white matter or cortex (Fig. 3) (Krawiecki et al. 1984; Lum et al. 1986; Anlar et al. 1996). Diffuse atrophy can be identified by computed tomography or MRI during the later stages of SSPE. Magnetic resonance spectroscopy may show reductions in the *N*-acetylaspartate peak and elevations of myoinositol, choline, and lactate (Fig. 4) (Kato et al. 2002). An international consortium established specific criteria to standardize the diagnosis of SSPE (Gascon 1999), taking into account the clinical, virologic, and neuroimaging features of the disorder (Table 2).

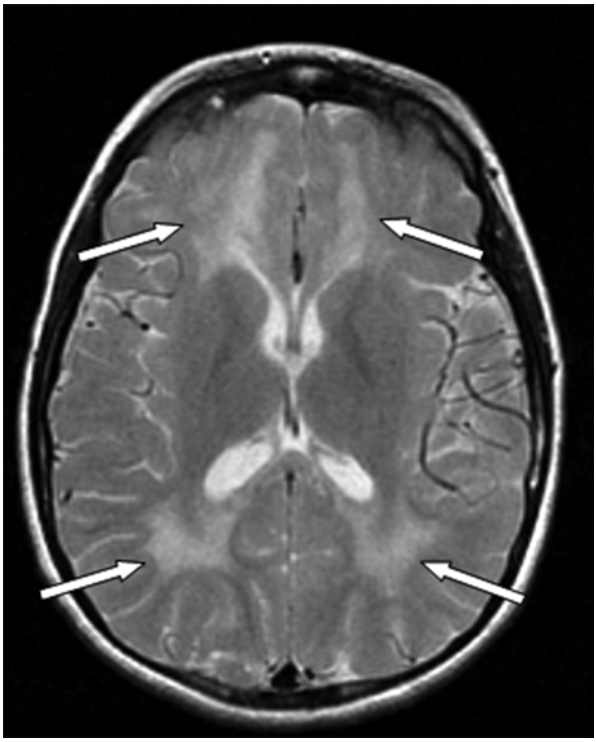

Fig. 3 Axial, T2-weighted magnetic resonance image in a teenager with SSPE shows symmetrical signal abnormalities in both frontal and occipital white matter (*arrows*)

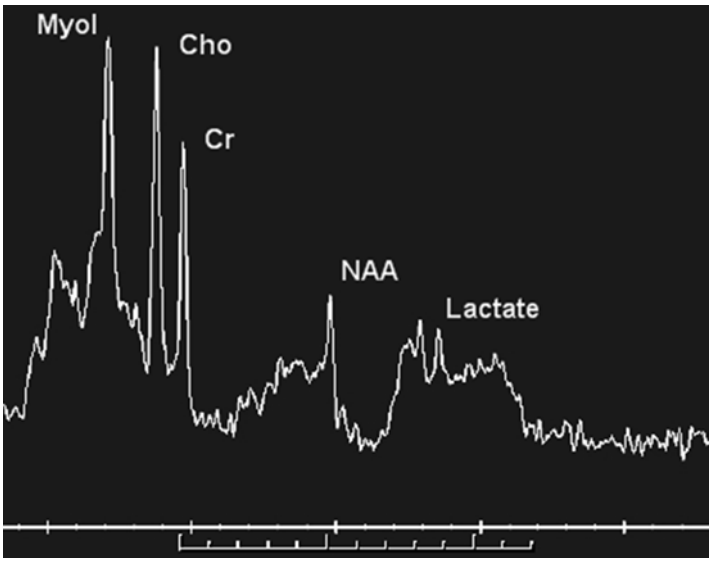

Fig. 4 Magnetic resonance spectroscopy, obtained within a boxel corresponding to the occipital white matter lesions, shows elevation of the myoinositol (MyoI) peak, reduction of the *N*-acetylaspartate (NAA) peak, and the presence of a lactate dublet (lactate). Together, these changes reflect neuronal and myelin degeneration

Table 2 Diagnostic criteria for SSPE

1. Typical or atypical clinical history
a. Typical clinical course
i. Acute progressive
ii. Subacute progressive
iii. Subacute remitting and relapsing
iv. Chronic progressive
v. Chronic remitting and relapsing
b. Atypical clinical course
i. Early seizures
ii. Unusual age
2. Increased measles-virus-specific antibody titers in CSF
3. Increased serum and CSF measles-virus-specific γ-globulin (increased IgG synthesis rate)
4. Typical EEG showing periodic slow wave complexes in stage II
5. Brain biopsy or post-mortem showing typical pathology or detection of measles virus
6. Molecular diagnosis techniques showing mutations of wild-type measles virus

Criteria 1 and 2 are necessary and specific for SSPE (Gascon 1999). Criteria 3–5 assist in identifying atypical cases

Treatment and Prognosis

SSPE remains a fatal neurodegenerative disorder with variability in the clinical course and no proven cure. The majority (60–80 %) of patients with SSPE have relentless neurological deterioration that leads to death within 2 years (Johnson 1998; Gascon 1999). Approximately 10 % have a rapid, fulminant course with death within a few months, and another 10 % have a chronic or relapsing course with exacerbations or remissions that can persist for several years (Johnson 1998; Gascon 1999; Kato et al. 2002). The variability in the clinical course of SSPE complicates the prediction of progression (Prashanth et al. 2006; Malik et al. 2010) and the evaluation of therapeutic interventions (Prashanth et al. 2006; Risk and Haddad 1979; Malik et al. 2010).

During the past 40 years, several therapeutic regimens, employing anti-viral or immunomodulating agents individually or in combination, have been used to treat patients with SSPE (Garg 2008). Beginning with Huttenlocher and Mattson's observations in the 1970s (Huttenlocher and Mattson 1979), several studies suggest that patients with SSPE may benefit from therapy with Isoprinosine®, a synthetic purine composed of ββ-inosine and the *p*-acetamidobenzoic acid salt of *N*,*N*-dimethylamino-2-propanol (Newport Pharmaceuticals, LTD. Dublin, Ireland) (Ginsberg and Glasky 1977). In the initial open-label trial in 15 patients, Huttenlocher and Mattson observed that one-third experienced neurological improvement or stabilization that persisted for 2 years. Subsequent studies by Huttenlocher and colleagues (Jones et al. 1982), involving nearly 100 patients from the United States and Canada, and Fukuyama and colleagues (Fukuyama et al. 1987), involving 89 patients in Japan, reached similar conclusions. However, both studies had methodological limitations, including a retrospective or nonrandomized design and the utilization of historical controls. Subsequent studies or case series describe the use of Isoprinosine® alone or in combination with β or α interferon and anti-viral agents, such as ribavirin or lamivudine (Anlar et al. 1998; Solomon et al. 2002; Gascon et al. 1994). Stabilization or improvement has been reported in some instances.

In 2003 Gascon and colleagues conducted the most detailed and well-designed therapeutic trial to date (Gascon and the International Consortium on Subacute Sclerosing Panencephalitis 2003). In this multicenter trial, 122 patients with SSPE were randomized to either oral Isoprinosine® 100 mg/kg/day (maximum 3 g daily) alone or in combination with intraventricular interferon-α2b, 100,000 U/m^2/day initially, escalating to 1,000,000 U/m^2 twice weekly. A placebo control group was not included, however, since the investigators deemed this unethical, given the results of previous studies (Fukuyama et al. 1987; Anlar et al. 1998). Neurological status was rated by blinded observers using a neurological disability index. Of the 122 potential subjects, 67 had evaluable data. Overall, 35 % of the subjects in each group improved or stabilized during therapy, a rate substantially greater than historical remission rates of 5–10 %. Several escape regimens, including interferon, corticosteroids intravenous immunoglobulin and amantadine, were used in non-responders, but none substantially improved the outcome of SSPE (Gascon and the International Consortium on Subacute Sclerosing Panencephalitis 2003).

Based on the currently available data, patients with proven or highly suspected SSPE require supportive care and can be treated with Isoprinosine® 100 mg/kg/day (maximum 3 g/daily) in three equally divided doses orally. Potential adverse effects include hyperuricemia and nephrolithiasis (Gascon and the International Consortium on Subacute Sclerosing Panencephalitis 2003). Although many clinicians may include therapy with interferons, Gascon and colleagues observed no added benefit from intraventricular interferon-α2b (Gascon and the International Consortium on Subacute Sclerosing Panencephalitis 2003). Patients treated with intraventricular interferons can experience serious side effects (Sato et al. 2009). More effective therapies for SSPE, using novel immunomodulating agents, anti-apoptotic compounds, or interfering molecules, are clearly needed (Tatli et al. 2012).

Additional Resources

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

Centers for Disease Control and Prevention
http://www.cdc.gov/measles/about/complications.html

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Henipaviruses

Christopher C. Broder and Kum Thong Wong

Introduction

The genus *Henipavirus* in the family *Paramyxoviridae* is presently represented by three known virus isolate species Hendra virus (HeV), Nipah virus (NiV) and CedPV (CedPV) and are enveloped, single-stranded negative-sense RNA viruses (Wang et al. 2013b; Marsh et al. 2012). HeV and NiV are bat-borne disease-causing zoonoses while CedPV also resides in the same bat species as does HeV in nature. Studies have shown that CedPV is not pathogenic in animals susceptible to HeV and NiV disease, nor is it known to be zoonotic. To date, bats appear to be predominant natural reservoir hosts for henipaviruses (Clayton et al. 2013) and recently, by nucleic acid based detection surveys, there has been a significant species expansion of the *Henipavirus* ranks including at least two full genome sequences, and also a report of one henipavirus from a rodent, but to date HeV, NiV, and CedPV are the only virus isolates that have been reported (Wu et al. 2014; Drexler et al. 2012).

Central pathological features of both HeV and NiV infection in humans and several susceptible animal species is a severe systemic and often fatal neurologic and/or respiratory disease (Abdullah and Tan 2014; Wong and Ong 2011; Playford et al. 2010). Of additional concern in people, both viruses, but particularly NiV, can also manifest as relapsing encephalitis following recovery from an acute infection resulting from a recrudescence of virus replication in the central nervous system

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(CNS) (Wong and Tan 2012; Wong et al. 2009). Spillovers of HeV have continued to occur in Australia since its identification, as does NiV in Bangladesh and India, since its recognition in Malaysia, which continue to make these henipaviruses an important transboundary biological threat (Broder et al. 2013). NiV in particular possesses several features that highlight a pandemic potential, such as its ability to infect humans directly from natural reservoirs or indirectly from other susceptible animals along with a capacity of limited human-to-human transmission (Luby 2013). Several henipavirus animal challenge models have been developed which has aided in understanding how HeV and NiV invade the central nervous system (Geisbert et al. 2012; de Wit et al. 2014), and successful active and passive immunization strategies against henipaviruses have been reported which target the viral envelope glycoproteins (Middleton et al. 2014; Broder 2012; Broder et al. 2012).

Emergence of Henipaviruses

A new paramyxovirus was isolated and identified in 1994 in an outbreak of fatal cases of respiratory disease in horses and humans in the Brisbane suburb of Hendra, Australia, and was shown to be distantly related to measles virus and other morbilliviruses (Murray et al. 1995a). Thirteen horses and their trainer succumbed to the infection by this previously unknown virus, along with the non-fatal infection of seven other horses and a stable hand. In an unrelated and only retrospectively identified spillover of this same virus near Mackay in central Queensland, ~1000 km north of Brisbane, a farmer experienced a brief aseptic meningitic illness after caring for and assisting at the necropsies of two horses that were only later shown to have died from this virus infection (Hooper et al. 1996; Rogers et al. 1996). Thirteen months later this individual suffered severe fatal encephalitis resulting from that initial virus infection characterized by uncontrolled focal and generalized epileptic activity (O'Sullivan et al. 1997). The virus was provisionally termed equine morbillivirus but was later re-named HeV where the initial recognized outbreak had occurred. To date, HeV has since reemerged in Eastern Australia on 55 occasions with more than 97 horse deaths, 2 HeV antibody positive euthanized dogs, and 4 of 7 human case fatalities (Broder et al. 2013; Anonymous 2012, 2013a, b, 2014a, b). Although HeV infection was detected in two dogs in recent years, the extent of HeV transmission from bats to dogs in Australia is unknown, and all recognized HeV spillovers and all cases of confirmed human infections, the horse has served as an intermediate host between the virus-shedding bat reservoir and humans. The epidemiological features and potential mechanisms at play of HeV emergence and continued spillovers have been examined (Plowright et al. 2011) and reviewed elsewhere (Field et al. 2007, 2012).

NiV emerged just a few years later following the initial recognition of HeV. A large outbreak of encephalitis among pig farmers in Peninsular Malaysia began in 1998 and continued into the next year (Chua et al. 1999). This outbreak was initially attributed to Japanese encephalitis virus because it occurred among people in close

contact with pigs. However, several features distinguished this outbreak from Japanese encephalitis such as patients were primarily adults not children, along with household clustering of cases being noted, and many of those afflicted had previously been vaccinated against Japanese encephalitis (Chua et al. 1999). A syncytia-forming virus in Vero E6 cell culture was obtained from the cerebrospinal fluid (CSF) of two patients which cross-reacted with antibodies against HeV and several patients had IgM antibodies in their CSF that were reactive against HeV (Chua et al. 1999). Later molecular genetic studies confirmed the close relationship of this new paramyxovirus, termed NiV, to HeV (Chua et al. 2000a). There were at least 265 cases of human infection with 105 fatalities in Malaysia along with an additional 11 cases and 1 fatality among abattoir workers in Singapore (Chua et al. 2000a; Paton et al. 1999). The chronology of the events and the epidemiological features of this outbreak, including potential causes and the factors that exacerbated this outbreak, as well as the pathological observations made in both animals and humans have been critically reviewed and recently examined elsewhere (Wong and Tan 2012; Wong and Ong 2011; Chua 2003; Pulliam et al. 2012). NiV has not reappeared in Malaysia, however nearly annual outbreaks of NiV infection have now been recognized since 2001, occurring primarily in Bangladesh but also India. The most recent cases of human infections occurred in early 2015 with two fatalities (Anonymous 2015). The spillovers of NiV in Bangladesh and India have had lower numbers of human infections; however the fatality rates have been notably higher from 75 to 100 %. In addition, direct transmission of NiV from bats to humans from the consumption of contaminated date palm sap along with significant human-to-human transmission has now been documented (Rahman et al. 2012; Homaira et al. 2010a, b; Luby et al. 2009b). The epidemiological details of the spillovers of both HeV and NiV into people since their emergence and recognition have recently been reviewed and summarized in detail (Luby and Gurley 2012; Luby and Broder 2014). There have been ~613 human cases of NiV infection with 315 fatalities (reviewed in Luby et al. 2009b; Broder 2012; Anonymous 2014c, 2015). Both HeV and NiV are highly pathogenic in a number of mammalian species and possess several characteristics that distinguish them from all other known paramyxoviruses and are classified as Biosafety Level-4 (BSL-4) agents.

Finally, although not associated with a zoonotic event, the third recognized henipavirus species as a virus isolate was recently identified (Marsh et al. 2012). Urine sample collecting for PCR and virus isolation experiments were being carried out as part of field studies on HeV genetic diversity and infection dynamics in flying-fox populations in Queensland, Australia. From these studies a syncytia-inducing virus was identified in *Pteropus* bat kidney cell culture isolated from samples collected in September 2009 from a flying-fox colony in Cedar Grove, South East Queensland (Marsh et al. 2012). Molecular analysis indicated that this virus was a new paramyxovirus most closely related to HeV and NiV and the virus was named CedPV after the location of the bat colony sampled. Animal challenge studies with CedPV in guinea pigs and ferrets which are susceptible to infection and disease with HeV and NiV, revealed that while CedPV replication occurred and induced neutralizing antibodies, no clinical disease was apparent (Marsh et al. 2012).

Reservoir Discovery and Diversity

Soon after the discovery and isolation of HeV, a state-wide serologic survey of 2411 horses reported no evidence of infection and only horses involved in the initial Brisbane outbreak were positive (Ward et al. 1996). This was followed by a large serological survey conducted across eastern Queensland, Australia in an effort to identify the potential natural host(s) of the virus, and 5264 sera samples across 46 species, mostly wildlife, were screened and no evidence of HeV neutralizing antibody was found (Young et al. 1996). However, the additional screening of potential animal reservoirs that overlapped the two initial but distant HeV spillover events led to the testing of the four fruit bat species (flying foxes) native to mainland Australia, and here serological evidence was found in all four species of *Pteropus* fruit bats (Young et al. 1996). HeV was later isolated from the gray-headed flying fox (*Pteropus poliocephalus*) and the black flying fox (*P. alecto*) (Halpin et al. 2000).

Following the first appearance of NiV in Peninsular Malaysia, a serological surveillance study on samples from 324 bats across 14 species revealed the presence of NiV neutralizing antibodies in Island flying-foxes (*P. hypomelanus*) and Malayan flying foxes (*P. vampyrus*) (Yob et al. 2001). A follow-up study focusing on virus isolation by collecting pooled urine samples from Island flying foxes, as well as partially eaten fruit, reported the isolation of NiV (Chua et al. 2002). NiV has since been isolated from the urine of *P. lylei* in Cambodia (Reynes et al. 2005). Serological assays as a means of detection of the presence of NiV and/or HeV in nature, from wildlife, domestic animals and human populations, is more readily achievable as compared to either virus isolation or nucleic acid detection (McNabb et al. 2014). A number of serological surveys have been carried out over the past several years to screen for the presence of henipaviruses in bats, domestic livestock and people. The preponderance of data indicates that the *Pteropus* bat species appear to be the major natural reservoir hosts for henipaviruses (Sendow et al. 2013; Yadav et al. 2012; Wacharapluesadee et al. 2010; Epstein et al. 2008; Iehle et al. 2007) and all bat isolates of HeV, NiV and also CedPV have been derived from *Pteropus* bats (Halpin et al. 2000; Chua et al. 2002; Reynes et al. 2005; Rahman et al. 2010; Marsh et al. 2012) (see also Chap. 26). Further, as natural hosts, a lack of any observed overt disease in wild bats is also in agreement with a lack of elicited clinical signs in experimentally infected pteropid bats (Middleton et al. 2007; Williamson et al. 1998, 2000; Halpin et al. 2011). *Pteropus* bat species are distributed as far west as Madagascar, through the Indian subcontinent to Southeastern Asia and Australia, and eastwards through Oceania (Clayton et al. 2013; Breed et al. 2013; Field et al. 2001).

However, there is evidence of henipaviruses in wide variety of other bat species in both Megachiroptera and Microchiroptera suborders (Hayman et al. 2008; Peel et al. 2012, 2013; Hasebe et al. 2012; Wacharapluesadee et al. 2005; Li et al. 2008; Drexler et al. 2009, 2012). Most recently, a novel henipa-like virus, Mojiang paramyxovirus (MojV), was identified in rats (*Rattus flavipectus*) in China by nucleic acid analysis, with a genome length of 18,404 nt; however no virus isolate was obtained (Wu et al. 2014). Also, serological and/or nucleic acid evidence of henipa-

viruses in domestic livestock and in human populations have been reported providing evidence of sporadic henipavirus spillover events and also suggesting the existence of less pathogenic-related henipavirus. These findings included henipavirus presence in domestic pigs in Ghana, West Africa; cattle, goats, and pigs in Bangladesh; horse and humans in the Philippines, and human populations in Cameroon, Africa (Ching et al. 2015; Pernet et al. 2014; Chowdhury et al. 2014; Hayman et al. 2011). Only the incident in the Philippines was associated with a disease outbreak with evidence of horse-to-human and human-to-human transmission with NiV as the likely cause (Ching et al. 2015).

Genomic sequence analysis revealed that HeV isolates obtained from horses and a fatal human case in 1994 were essentially identical and both were highly similar to genomic sequences later obtained from *P. poliocephalus* and *P. alecto* 2 years after the initial outbreak (Halpin et al. 2000; Murray et al. 1995b). Also, sequence analysis of five HeV isolates obtained from horses in Australia; Murwillumbah, in New South Wales (2006), and Peachester (2007), Clifton Beach (2007), Redlands (2008), and Proserpine (2008) all in Queensland, revealed identical genome lengths of 18,234 nt and sequence variation across the full genomes was <1 % (Marsh et al. 2010). Similarly, in the initial Malaysian outbreak of NiV, both pig and human isolates were genetically similar to those obtained some years later from Island flying-foxes (*P. hypomelanus*) (AbuBakar et al. 2004; Chan et al. 2001; Chua et al. 2002; Harcourt et al. 2000). However, a greater diversity among NiV isolates is seen when comparisons are made between the Malaysian isolates to the more recent NiV isolates from other areas of Southeast Asia.

The first NiV isolate from outside of Malaysia came from Bangladesh (Harcourt et al. 2005). Characterization of the genome of NiV-Bangladesh revealed a length of 18,252 nt, 6 nt longer than the prototype NiV-Malaysian isolate, with a genome homology between them of 91.8 % (Harcourt et al. 2005). Also, in that study, four NiV-Bangladesh isolates were examined showing a 99.1 % nt homology with inter-strain nucleotide heterogeneity suggesting multiple spillovers of NiV-Bangladesh into people from varying bat sources. A third lineage of NiV was isolated from Lyle's flying fox (*P. lylei*) in Cambodia and nucleocapsid (N) gene sequence analysis revealed this isolate to be more closely related to NiV-Malaysia than to NiV-Bangladesh (Reynes et al. 2005; Wacharapluesadee et al. 2010) whereas an analysis of nucleic acid sequences of NiV derived from human sources from an outbreak in Siliguri, India in 2001 revealed an isolate similar to NiV-Bangladesh (Chadha et al. 2006) and a full NiV genome amplified from patient lung tissue from an outbreak in 2007 in West Bengal, India showed 99.2 % nt with the NiV-Bangladesh isolate from 2004 (Arankalle et al. 2011). More recently, partial genome sequence analysis of NiV derived from an Indian flying fox (*P. giganteus*) obtained from Myanaguri, West Bengal, India, revealed an N gene with 100.0 % homology with NiV sequences from those prior outbreaks in India and with NiV-Bangladesh sequences, and a 96.0 % identity with NiV isolates from Cambodia and Malaysia (Yadav et al. 2012). In addition to the demonstration of at least three distinct virus isolate lineages of NiV; Malaysia, Bangladesh and Cambodia (Wang et al. 2013b), other nucleic acid based studies have significantly expanded the genus Henipavirus (Drexler et al. 2012).

Nineteen newly identified virus species classified into the genus Henipavirus have been identified, along with one full genome sequence, 18,530 nt, (GH-M74a) from a bat spleen (*Eidolon helvum*) from Ghana confirmed classification in the genus Henipavirus (Drexler et al. 2012).

CedPV is the third recognized species of henipavirus as a virus isolate (Marsh et al. 2012). CedPV was isolated from pooled urine samples from a colony of predominantly *P. alecto* also with some *P. poliocephalus*. The CedPV genome is 18,162 nt and its organization was shown to be similar to that of HeV and NiV. Also, some antigenic cross-reactivity of the CedPV N protein was noted with that of NiV and HeV; and CedPV was shown to utilized ephrin-B2 as entry receptor (discussed in the next section).

Henipavirus Biology

Virion, Genome Organization, and Proteins

Henipavirus particles are enveloped and pleomorphic, with a size ranging from 40 to 1900 nm and can vary from spherical to filamentous forms when imaged by electron microscopy (Hyatt et al. 2001; Goldsmith et al. 2003; Murray et al. 1995b). The viral envelope carries surface projections composed of the viral transmembrane-anchored fusion (F) and attachment (G) glycoproteins (Fig. 1). Henipavirus genomes are unsegmented, single-stranded, negative-sense RNA (Wang et al. 2013b). At the time of their discovery, the genomes of NiV and HeV were the largest amongst all members of the *Paramyxoviridae* family, a factor considered in their classification into their own genus, *Henipavirus* (Wang et al. 2000). This increase in genome length is primarily attributable to additional nucleotides in 3′ untranslated regions of each transcription unit except the large/polymerase (L) gene (Wang et al. 2000, 2001; Harcourt et al. 2000). As with all characterized members of the subfamily *Paramyxovirinae*, the HeV, NiV and CedPV genomes and are divisible by six, conforming to the "rule of six" which relates to the way each N protein molecule interacts with every six nucleotides (Lamb and Parks 2013; Wang et al. 2013b). The RNA genome in association with the N protein is also referred to as the ribonucleoprotein core that has a characteristic herringbone appearance by electron microscopy (Wang et al. 2013b) and is contained within a lipid bilayer (envelope) that is derived from the infected host cell during virus assembly and budding (Fig. 1).

The relative gene order is conserved as compared to other paramyxoviruses, with the N gene being first, followed by the P (phosphoprotein), M (matrix), F, G and L genes in a 3′ to 5′ order (Fig. 1). Gene transcription occurs in a gradient manner because of a failure of the RNA polymerase to reinitiate transcription at downstream genes and those genes located towards the 3′ end are transcribed more abundantly than genes towards the 5′ (Lamb and Parks 2013). The N, P, and L proteins form a complex that is responsible for replication of viral RNA; polymerase activity resides within the L protein (Lamb and Parks 2013). In addition to the full-length unedited P gene product, the

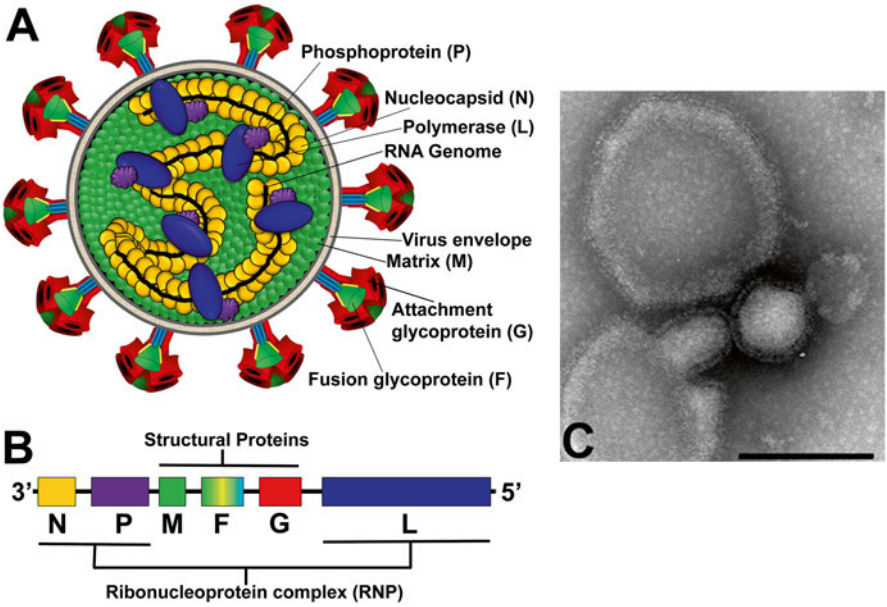

Fig. 1 Structural and genomic organization of henipaviruses. (**a**) Structural organization of the pleomorphic henipavirus virion. The virus particle is formed by the structural elements (M, F, G) and the non-structural elements of the ribonucleoprotein complex (RNP) composed of viral genome, N, P, and L. (**b**) Diagram of the henipavirus negative-sense RNA genome. The genetic features are shown, proportionally, including 3′- and 5′-untranslated regions, intragenic regions, and the ORFs encoding the nucleocapsid, N; phosphoprotein, P; matrix, M; fusion glycoprotein, F; attachment glycoprotein, G; and RNA-dependent RNA polymerase, L proteins. (**c**) Negatively stained HeV virions, bar, 200 nm. Image courtesy of the AAHL Biosecurity Microscopy Facility, Australian Animal Health Laboratory (AAHL) Livestock Industries CSIRO, Australia

Henipavirus P gene (the largest among the paramyxoviruses) also encodes the V and W proteins which are produced through a transcriptional editing mechanism involving addition of nontemplated G nucleotides, as well as the C protein, which is encoded by an alternative start site within the P gene (Lamb and Parks 2013).

Products of the P gene can antagonize both double-stranded (ds) RNA signaling and interferon (IFN) signaling (reviewed in Shaw 2009; Basler 2012). The V protein functions in anti-IFN induction or dsRNA signaling, similar to that of other paramyxoviruses, by targeting the helicase encoded by the melanoma differentiation-associated gene 5 (MDA5). Whereas the NiV W protein could also inhibit dsRNA signaling but does so by nuclear translocation, targeting interferon regulatory factor 3 (IRF-3) and effectively blocking both dsRNA signaling via MDA5 and through the cell surface expressed toll-like receptor 3 (TLR-3) signaling pathway. Henipaviruses also target the paracrine signal transduction pathway that is initiated by the binding of type I IFN to the two cell surface interferon alpha and beta receptors, IFNAR1 and IFNAR2 which assemble into a functional receptor complex leading to the activation of signal transducers and activators of transcription

(STAT) factors where they later direct the expression of genes possessing an interferon stimulated response element (ISRE) within the nuclease (reviewed in de Weerd et al. 2007). The henipavirus V, W and P proteins block the type I IFN signaling pathway with the NiV V and P proteins forming high-molecular weight complexes in the cytoplasm with STAT1, and the NiV W protein targeting STAT1 within the nuclease (reviewed in detail (Shaw 2009; Basler 2012)). In contrast, major difference between NiV and HeV with CedPV was noted in that the P gene lacks both RNA editing and also the coding capacity for the V protein which may be a factor that limited its observed in vitro pathogenesis (Marsh et al. 2012). The diverse ways that NiV and HeV can antagonize the host interferon responses are believed to be important factors that influence their pathogenic potential.

The henipavirus M protein, which underlies the viral membrane (Fig. 1), plays a key role in organization of viral proteins during the process of virion assembly and budding from the host cell, and the NiV M protein possesses the ability to bud from expressing cells independent of any other viral proteins forming virus-like particles (Ciancanelli and Basler 2006; Patch et al. 2007). Sequence motifs with the M protein have been identified that may act as trafficking signals to facilitate the budding process (Patch et al. 2008; Ciancanelli and Basler 2006; Harrison et al. 2010). Finally, the G and F envelope glycoproteins are located on the surface of the virion, appearing as spikes projecting from the envelope membrane of the viral particle (Fig. 1) and are essential for the binding and entry steps of the virus into permissive host cells (reviewed in Bossart et al. 2013; Steffen et al. 2012). The henipavirus G glycoprotein is a homo-tetramer and responsible for attachment of the virion to entry receptors on the host cell and the F glycoprotein is a homotrimer responsible for facilitating the fusion of the viral membrane with that of the host cell (reviewed in Steffen et al. 2012). Additional details of the henipavirus envelope glycoproteins will be discussed below with regard to cellular tropism and as the targets of antiviral strategies.

Host Range, Cellular Tropism, and Virus Entry

The exceptionally broad species tropism of henipaviruses, as represented by NiV and HeV, distinguishes them from all other known paramyxoviruses (Wang et al. 2013b). In addition to their principle natural hosts, pteropid bats, NiV is known to have naturally infected pigs, horses, cats, dogs and humans, and experimental infections with disease in guinea pigs, cats, hamsters, ferrets, squirrel monkeys and African green monkeys have been demonstrated. In addition, NiV can also productively infect chicken embryos with severe pathology (Tanimura et al. 2006). HeV in nature appears less transmissible and naturally acquired infections have been observed only in bats, horses, dogs and humans; however, experimentally, HeV can infect and cause disease in guinea pigs, cats, hamsters, ferrets, mice and African green monkeys (reviewed in Geisbert et al. 2012) Taken together, henipavirus infections seven orders (six mammalian and one avian).

The henipavirus membrane anchored envelope glycoproteins (G and F) are the mediators of virus attachment and host cell infection and a major determinant of cellular tropism. The G glycoprotein is the henipavirus attachment glycoprotein and has neither hemagglutinating nor neuraminidase activities; activities associated with many other paramyxovirus attachment glycoproteins known as hemagglutinin–neuraminidase (HN) or the hemagglutinin (H) protein (Wang et al. 2013b; Lamb and Parks 2013). The NiV and HeV G glycoprotein engage host cell membrane proteins as entry receptors and bind to ephrin-B2 and ephrin-B3 (Negrete et al. 2005, 2006; Bonaparte et al. 2005; Bishop et al. 2007). The ephrin-B2 and -B3 molecules are members of a large family of cell surface expressed glycoprotein ligands that bind to Eph receptors, the largest subgroup of receptor tyrosine kinases (Drescher 2002; Poliakov et al. 2004). The Eph receptors and their ephrin ligands comprise an important group of bidirectional signaling molecules in a variety of cell–cell interactions including those of vascular endothelial cells and are modulators of cell remodeling events within the nervous, skeletal and vascular systems (Pasquale 2010; Lackmann and Boyd 2008). Ephrin-B2 expression is prominent in arteries, arterioles and capillaries in multiple organs and tissues (Gale et al. 2001) while ephrin-B3 is found predominantly in the nervous system and the vasculature (reviewed in Poliakov et al. 2004; Pasquale 2008). The ephrin-B2 and -B3 molecules are highly sequence conserved across susceptible hosts including human, horse, pig, cat, dog, mouse and bat with amino acid identities of 95–96 % for ephrin-B2 and 95–98 % for ephrin-B3 (Bossart et al. 2008). The identification of ephrin-B2 as a major receptor for NiV and HeV has aided in the understanding and clarification of both their broad species and tissue tropisms, as well as the resultant pathogenic processes that are seen in humans and animal hosts (reviewed in Hooper et al. 2001; Wong and Ong 2011).

Similar to most paramyxoviruses, the henipaviruses have two membrane-anchored glycoproteins that are required for virus entry. The henipavirus attachment glycoprotein (G) is a type II membrane protein with the amino (N)-terminus oriented towards the cytoplasm and the carboxy (C)-terminus extracellular (Bossart et al. 2013). The G glycoprotein is comprised of a stem (or stalk) and a globular head domain which binds ephrin receptors. The native conformation of G is a tetramer, which is comprised of a dimer of dimers (Bossart et al. 2005). The crystal structures of both NiV and HeV G globular head domains have been determined both alone and in complex with the ephrin-B2 and -B3 receptors, revealing the exact G-receptor interactions and identical receptor binding sites; with four binding pockets in G for the residues in the ephrin-B2 and -B3 G-H loop that are highly conserved (Bowden et al. 2008a, b, 2010; Xu et al. 2008, 2012). The second protein is the fusion (F) glycoprotein that facilitates the fusion of the viral and host cell membranes. F is a type I membrane glycoprotein with an extracellular N-terminus and is a class I viral fusion protein sharing several conserved features with other viral fusion glycoproteins (Bossart et al. 2013). F is initially expressed as a precursor (F_0) which forms an oligomeric trimer that is cleaved into two disulfide bond-linked subunits (F_1 and F_2) by the endosomal protease cathepsin L (Pager and Dutch 2005). Unique to the henipaviruses, the processing of F_0 into its biologically active form is

a multi-step process requiring recycling of F_0 from the cell surface into an endosomal compartment, mediated by an endocytosis motif present in the cytoplasmic tail of F (Meulendyke et al. 2005; Vogt et al. 2005). After cleavage, the homotrimer of disulfide bond-linked F_1 and F_2 subunits is trafficked back to the cell surface. The F glycoprotein contains two α-helical heptad repeat domains that are involved in the formation of a trimer-of-hairpins structure which facilitates membrane merger and peptides corresponding to either heptad repeat domains can inhibit the fusion activity of F when present during the fusion process (reviewed in Bossart et al. 2013).

The henipavirus G and F glycoproteins work cooperatively to mediate membrane fusion and particle entry into the host cell. Following virus attachment to a receptor-bearing host cell, the fusion-promoting activity of the G glycoprotein is initiated by engaging ephrin receptors and the G glycoprotein then facilitates the triggering of conformational changes in F, transitioning F conformation from a pre-fusion to post-fusion form driving the membrane fusion process between the virion and plasma membranes, resulting in delivery of the viral nucleocapsid into the cytoplasm (reviewed in Aguilar and Iorio 2012; Lee and Ataman 2011). In a related process, virus-infected cells expressing attachment and fusion glycoproteins on their surface can fuse with receptor-bearing cells leading to the formation of multinucleated giant cells (syncytia)—a hallmark of many paramyxovirus infections including the henipaviruses (Wang et al. 2013b).

Clinical Manifestations

Hendra Virus

The incubation period of human NiV and HeV infections ranges from a few days to about 3 weeks (Goh et al. 2000; Mahalingam et al. 2012). To date, there have been only seven known cases of human HeV infection, so much less is known about its clinical manifestations compared to NiV infection. Following an influenza-like illness (fever, myalgia, headaches, lethargy, vertigo, cough, pharyngitis, and cervical lympadenopathy), the majority developed severe disease and died; only two patients survived (Mahalingam et al. 2012; Selvey et al. 1995; Playford et al. 2010). Thus the mortality was about 60 %. Three patients had an acute encephalitic syndrome characterized by drowsiness, confusion, ataxia, ptosis, dysarthria and seizures and died soon after. One patient had an acute pulmonary syndrome described as a pneumonitis with chest radiograph findings of diffuse alveolar shadowing (Selvey et al. 1995). Although clinical acute encephalitis was never suspected, apart from pulmonary pathology, this patient's brain at autopsy also showed features of acute encephalitis (Wong et al. 2009). Interestingly, abnormal chest radiographs were also described in two other clinical encephalitis cases. In one patient following relatively mild aseptic meningitis associated with headache, drowsiness, vomiting and neck stiffness, clinical features of probable meningoencephalitis, he presented 13 months later with full blown fatal encephalitis (O'Sullivan et al. 1997). In retrospect, this

was the first case of relapsing henipavirus encephalitis. The brain magnetic resonance (MR) scans available in three acute encephalitis patients showed multifocal hyperintensive lesions in the cerebrum and brainstem, and leptomeningeal enhancement. In the case of relapsing encephalitis, extensive, predominantly cortical hyperintense lesions were observed (Mahalingam et al. 2012).

Nipah Virus

Based on a large cohort of 94 patients with NiV infection from a single institution, the main features of acute infection was fever, headache, dizziness, and vomiting (Goh et al. 2000). A majority of patients had reduced consciousness levels and signs of brainstem dysfunction. Other distinctive clinical signs included segmental myoclonus, areflexia, hypotonia, hypertension, and tachycardia. The cerebrospinal fluid obtained from lumbar puncture showed elevated leukocyte counts and protein levels. Electroencephalogram abnormalities consisting of diffuse slow waves (continuous or intermittent) with or without focal sharp waves were observed, and in general correlated with disease severity. Brain MR scans (Sarji et al. 2000) of acute NiV infection were characterized by disseminated, multiple hyperintense lesions mainly in subcortical and deep white matter of the cerebrum with no associated edema or mass effect or correlation with severity of neurological signs. Chest radiographs were reported to be abnormal in some patients (Goh et al. 2000; Paton et al. 1999). The risk factors for severe disease and poor prognosis included abnormal doll's eye reflex, tachycardia, and the presence of virus in the cerebrospinal fluid (Chua et al. 2000b), and diabetes mellitus (Chong et al. 2001b).

A small number, probably <10 %, of patients with acute NiV infection developed a late-onset encephalitis (in symptomatic patients with no previous encephalitis or patients with asymptomatic seroconversion) or a relapsing encephalitis (in patients with previous encephalitis) a few weeks later. Although potentially fatal, the mortality at about 18 % is considerably lower that acute encephalitis (Tan et al. 2002). The clinical features of late-onset encephalitis and relapsing encephalitis are similar to acute encephalitis. However, some features like fever, coma, brainstem signs, segmental myoclonus and meningism were less commonly observed, while seizures and focal cortical signs were more frequent. Cerebrospinal fluid pleocytosis was common but no virus could be isolated. The brain MR scans showed confluent geographical abnormalities, especially in the cortical gray matter that is strikingly different from acute NiV encephalitis (Sarji et al. 2000). Although most NiV-infected human patients presented with acute encephalitis, some 25 % of patients also presented with respiratory signs, some cases also presented as a non-encephalitic or asymptomatic infection with seroconversion (Chua 2003).

NiV infection could also take a chronic and quiescent course with neurological disease occurring later (>10 weeks) following a non-encephalitic or asymptomatic infection. A recrudescence of neurological disease, also termed relapsing encephalitis, was also observed in some patients who had previously recovered from an acute encephalitic infection. Here, there is a recrudescence of virus replication in

the CNS. Most reported cases of relapsed encephalitis presented from a few months to approximately 2 years following the initial acute infection, however two cases of relapsed encephalitis were observed in 2003 4 years later (Wong et al. 2001; Chong and Tan 2003; Tan and Wong 2003) and the longest reported case of NiV encephalitic recrudescence is 11 years (Abdullah et al. 2012). This recrudescence of henipavirus encephalitis was first noted in the second fatal human case of HeV infection which presented with similar findings (O'Sullivan et al. 1997; Wong et al. 2009). Interestingly, evidence of recrudescence of NiV infection in pteropus bats has also been reported (Sohayati et al. 2011) as well as HeV infection modeling in flying-fox populations (Wang et al. 2013a). There is no evidence of HeV shedding in people who have recovered from infection (Taylor et al. 2012).

Persistent neurological deficits have been observed in >15 % of NiV infection survivors (Bellini et al. 2005). In addition, recent studies have also assessed the long-term neurologic and functional outcomes of >20 individuals surviving symptomatic NiV infection in Bangladesh (Sejvar et al. 2007). In Bangladesh, the outcomes among 22 of 45 serologically confirmed cases of NiV infection revealed neurological sequelae in survivors, and patients who initially had encephalitis could continue to exhibit neurological dysfunction for several years (Sejvar et al. 2007). Both persistent and delayed-onset neurological sequelae were noted, including a higher proportion of persistent behavioral disturbances including violent outbursts and increased irritability among pediatric patients (Sejvar et al. 2007). Viral persistence and/or recrudescence within the CNS are suspected to be at play in these individuals. The mechanisms that allow NiV and HeV to escape immunological clearance for such an extended period and later result in disease are unknown, and this characteristic of NiV and HeV has important implications for therapeutics development.

Pathology

Human Pathology

HeV spillovers in Australia have occurred annually since 2006 and to date there have been seven human cases of which four have been fatal (Playford et al. 2010). All human cases of HeV infection was the result of exposure and transmission of the virus from infected horses to humans. The first human case presented as an acute severe respiratory disease but no clinical evidence of acute encephalitis. At autopsy, the lungs showed macroscopic evidence of congestion, hemorrhage and edema (Selvey et al. 1995) associated with focal necrotizing alveolitis and evidence of syncytia and multinucleated giant cell formation, and viral inclusions. Focal vasculitis was also noted in some pulmonary vessels. Viral antigens were localized by immunostaining to alveolar type II pneumocytes, intra-alveolar macrophages and blood vessels (Wong et al. 2009). Although clinical encephalitis was apparently

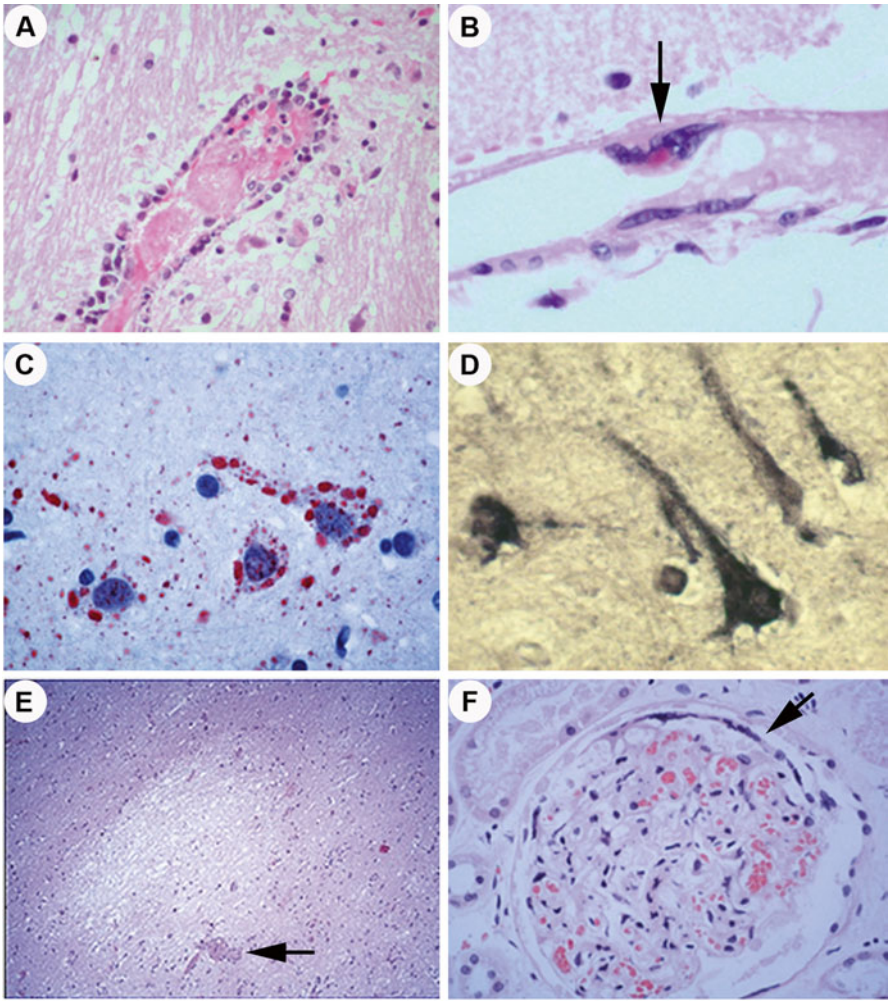

Fig. 2 Pathology of human henipavirus infection. (**a**) Vasculopathy in NiV encephalitis showing vasculitis, thrombosis and endothelial multinucleated syncytia with viral inclusion (**b**, *arrow*). (**c**) Numerous NiV inclusions/antigens within neurons, and particularly around necrotic plaques (**e**) Necrotic plaques may also have evidence of adjacent vascular thrombo-occlusion (**e**, *arrow*). (**d**) HeV RNA can be demonstrated in neurons. In the kidney infected by NiV, glomerular capillary thrombosis and multinucleated syncytia at the periphery of the glomerulus can be detected (**f**, *arrow*). Panels (**a**, **b**, **d**, **f**) from Wong and Ong (2011), panels (**c**, **e**) from Wong et al. (2002)

absent, the brain pathology clearly showed acute encephalitis characterized by mild meningitis, parenchymal and perivascular inflammation. More importantly, there was evidence of neuronal viral inclusions, vasculitis and necrotic/vacuolar plaques. Viral antigens/RNA were demonstrated in blood vessels, neurons (Fig. 2d), and ependyma. Mild inflammation could also be found in the lymph node and kidney where viral antigens were detected in glomeruli and renal tubules.

A second fatality occurred in an individual who first experienced an aseptic meningitic illness associated with drowsiness caused by HeV infection acquired after assisting at the necropsies of two horses that were only later shown to have died from HeV infection. Approximately 13 months later this individual suffered a recurrence of severe encephalitis characterized by uncontrolled focal and generalized epileptic activity. Inflammatory lesions were only found in the CNS, not in other organs obtained at (Wong et al. 2009). Extensive lesions were found mainly in the meninges and cerebral cortex, but focal lesions were also found in the cerebellum, pons and spinal cord. There was intense infiltration of the parenchyma and perivascular areas by macrophages, lymphocytes, and plasma cells together with severe neuronal loss, reactive glial, and vascular proliferation. Although viral inclusions were not prominent, viral antigens/RNA were detected in neurons, glial, and/or inflammatory cells. Interestingly, there was no evidence of vasculitis or endothelial syncytia in the CNS, as well as absence of these and other features of inflammation in all the non-CNS organs examined.

In the first NiV outbreak in Malaysia and Singapore, autopsies were conducted on >30 individuals which has afforded a better understanding of the pathology of NiV in comparison to that of HeV infection. These autopsies were mostly in individuals, including pig farm workers and farmers, who in one way or another had contact with sick pigs. The macroscopic features were generally non-specific. Perhaps the most distinctive microscopic feature is the disseminated vasculitis found in most organs examined, particularly in the CNS and lungs. The fully developed, typical vasculitic lesion comprised focal segmental inflammation of the vascular wall, endothelial ulceration and thrombosis (Fig. 2a) (Wong et al. 2002). The rare endothelial multinucleated syncytia may occasionally be found in early vasculitis (Fig. 2b). Viral antigens and nucleocapsids can be demonstrated in blood vessels. Extravascular necrotic lesions and inflammation in many organs can also be seen. In the CNS parenchyma, distinct necrotic plaques (Fig. 2e) arising from vasculitis-induced vascular obstruction, ischemia and infarction and/or neuronal infection were commonly found. Neurons in or around necrotic plaques and other inflamed neuronal areas often showed the widespread presence of viral antigens (Fig. 2c). Glial cells were much more rarely involved. Viral inclusions in neurons in the CNS and other cells in non-CNS tissues were also observed. Apart from vasculitis, inflammation, necrosis, and the rare multinucleated giant cells or syncytia involving extravascular tissue in the lung, spleen, lymph node, and kidney (Fig. 2f), were reported (Wong et al. 2002; Hooper et al. 2001; Wong 2010). The combination of disseminated, vasculitis-induced thrombosis, vascular occlusion, and microinfarction, together with direct infection of parenchymal cells suggest a unique dual pathogenetic mechanism for tissue injury in acute NiV infection. This appears to hold true for acute HeV infection as well. Certainly in the CNS, extensive virus-associated vasculopathy, with or without neuroglial infection, as a significant cause of tissue injury is probably unique.

The pathological features in the few autopsy cases of NiV relapsing or late-onset encephalitis and the single case of HeV relapsing encephalitis were similar and confined mainly to the CNS (Wong and Tan 2012; Tan et al. 2002). There was

extensive and severe meningoencephalitis with parenchymal and perivascular inflammation, severe neuronal loss and reactive gliosis. Viral inclusions, antigens/RNA could be detected but vasculitis were absent (Wong 2010). Indeed, vasculitis or other vasculopathies which were readily found in the acute infection, were absent in the CNS and extra-CNS organs.

Animal Pathology

In addition to HeV and NiV infection of bats (Middleton and Weingartl 2012), detailed reviews of the disease manifestations observed in natural and experimental infections of animals with HeV and NiV have recently been reported (Dhondt and Horvat 2013; Geisbert et al. 2012; Wong and Ong 2011). As mentioned previously, natural HeV infections have almost exclusively been observed in horses, and only recently have two dogs been reported HeV antibody positive. Whereas in addition to pigs, naturally acquired NiV infection was noted in dogs, cats and horses in the initial Malaysian outbreak (Hooper et al. 2001). Serological studies of natural NiV infection revealed that dogs in areas associated with farms in the Malaysian outbreak were susceptible to infection (Field et al. 2001). However, diseased dogs were not prevalent with only two animals examined (one dead and one sick) (Hooper et al. 2001; Wong and Ong 2011). In Bangladesh, a few cases of human NiV infection were associated with sick animal contact including cows (Hsu et al. 2004), pigs, and goats (Luby et al. 2009a), and recently serological evidence of henipavirus infection in cattle, goats and pigs in Bangladesh has been reported (Chowdhury et al. 2014).

Animal Disease Models

The development of animal models of henipavirus infection and pathogenesis has been critical for understanding henipavirus pathogenesis and also needed for the evaluation of potential vaccines and therapeutics. Several well-established animal models of HeV and NiV infection and pathogenesis have been developed and include the guinea pig (Williamson et al. 2000; 2001 #3773; Middleton et al. 2007), hamster (Guillaume et al. 2009; Wong et al. 2003), cat (Mungall et al. 2006; Middleton et al. 2002; Williamson et al. 1998), pig (Li et al. 2010; Weingartl et al. 2005; Middleton et al. 2002), ferret (Pallister et al. 2011; Bossart et al. 2009), African green monkey (AGM) (Rockx et al. 2010; Geisbert et al. 2010), squirrel monkey (Marianneau et al. 2010) and horse (Marsh et al. 2011). Among these models, the pathogenic processes of henipavirus infection in the hamster, ferret and AGM best represent the pathogenesis observed in humans; whereas the most appropriate models for livestock are the pig and horse.

The Syrian Golden Hamster

The Syrian golden hamster and NiV challenge was the first successful small animal model of henipavirus infection and pathogenesis (Wong et al. 2003). NiV infection in the hamster produced severe lesions in the brain, with animals succumbing to infection 5–9 days after intraperitoneal infection, 24 h following the development of tremors and limb paralysis. Hamsters inoculated intranasally survived ~5 days longer post-challenge, displaying progressive neurological signs and breathing difficulties. Vascular pathology was widespread, involving the brain and lung, with endothelial cell infection. The vascular and parenchyma lesions were consistent with CNS-mediated clinical signs. Another study showed that higher doses of NiV resulted in an acute respiratory distress syndrome (ARDS) while lower doses would yield the development of neurological signs and more widespread infection throughout the endothelium (Rockx et al. 2011). HeV infection of hamsters also produces both respiratory and brain pathology, with endothelial infection and vasculitis, and direct parenchymal cell infection in the CNS (Guillaume et al. 2009). Similar to NiV infection in hamsters, higher doses of HeV resulted in ARDS and lower doses produced a more neuropathogenic syndrome (Rockx et al. 2011).

The Ferret

NiV infection of ferrets produces both a severe respiratory and neurological disease along with systemic vasculitis following oral-nasal challenge by 6–10 days post-infection (Bossart et al. 2009; Pallister et al. 2009). Clinical signs in infected ferrets included severe depression, serous nasal discharge, cough and shortness of breath, and tremor and hind limb paresis. Pathological findings included vascular fibrinoid necrosis in multiple organs, necrotizing alveolitis, and syncytia of endothelium and alveolar epithelium. Severe focal necrotizing alveolitis vasculitis and focal necrosis in a wide range of tissues was observed along with significant levels of viral antigen in blood vessel walls. NiV antigen was present within the brain along with infected neurons, and virus isolation from the brain and other organs was reported. HeV challenged ferrets, also by the oral-nasal route, rapidly progressed with severe disease 6–9 days following infection with essentially identical findings as seen in NiV-challenged ferrets (Pallister et al. 2011). The henipavirus disease processes in the ferret accurately reflects those reported in NiV-infected humans and the ferret model has been used in the evaluation of vaccines and therapeutics against henipavirus infections.

Nonhuman Primates

The first successful nonhuman primate models for both NiV and HeV infection were developed using the African green monkey (AGM) (Geisbert et al. 2010; Rockx et al. 2010). Both NiV and HeV will produce a uniformly lethal disease

process following low dose virus challenge by intratracheal inoculation within 7–10 days post-infection. HeV and NiV spread rapidly to numerous organ systems within the first 3–4 days following challenge. Monkeys begin to develop a progressive and severe respiratory disease ~7 days post-infection (Geisbert et al. 2010; Rockx et al. 2010). The lungs become enlarged and with high levels of virus replication, congestion, hemorrhage, and polymerized fibrin. Widespread vasculitis with endothelial and smooth muscle cell syncytia with viral antigen, along with viral genome was detected in most organs and tissues along with associated pathology. Monkeys infected with either NiV or HeV also exhibit neurological disease signs with the presence of meningeal hemorrhaging and edema, and vascular and parenchymal lesions in the brain including infection of neurons with in the brainstem particularly involved (Fig. 3) (Geisbert et al. 2010; Rockx et al. 2010).

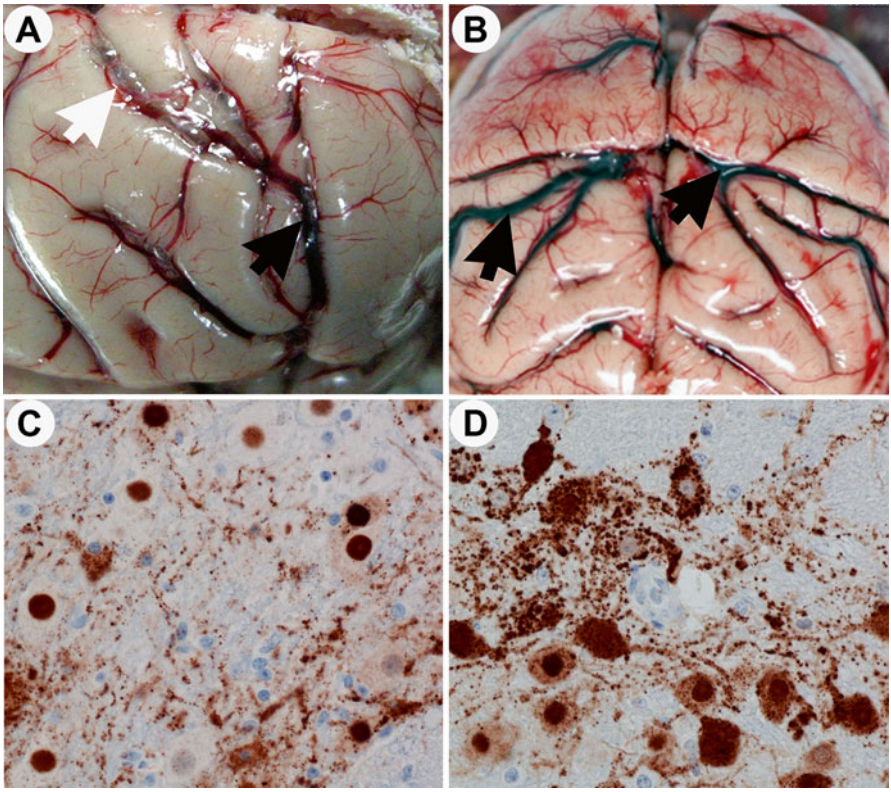

Fig. 3 Nipah virus and Hendra virus infection and pathogenesis in the nonhuman primate brain. End stage of lethal NiV and HeV infection in African green monkeys. (**a**) Brain, NiV, congestion of the brain (*black arrow*); fluid (*white arrow*) suggests mild to moderate meningeal edema; (**b**) brain, HeV, congestion of the brain (*black arrows*); (**c**) immunohistochemistry staining of NiV antigen in the brain stem; (**d**) immunohistochemistry staining of HeV antigen in the brain stem. (**c**, **d**) Strong cytoplasmic and nuclear staining of viral antigen in neurons. Panels (**a**) from Geisbert et al. (2010) and panel (**b**) from Rockx et al. (2010)

The squirrel monkey was also found to be susceptible to experimental NiV infection via intravenous and intranasal routes demonstrating findings similar to AGM and human infection (Marianneau et al. 2010). Vasculopathy and parenchymal cell infection were found in the CNS, lungs and other organs.

The Pig

NiV infection of pigs revealed the respiratory system as a major site of virus replication and pathology, with viral antigen and syncytia formation present in the respiratory epithelium (tracheal, bronchial, bronchiolar, and alveolar) and small blood and lymphatic vessels (Middleton et al. 2002; Hooper et al. 2001; Wong and Ong 2011). Virus was also observed in the kidneys and in endothelial and smooth muscle cells of small blood vessels (Middleton et al. 2002). CNS involvement was less common, with meningitis or meningoencephalitis observed as opposed to encephalitis (Middleton et al. 2002). NiV infection of piglets generally resulted in a mild clinical disease with fever and respiratory signs and virus replication noted in the respiratory system, lymphoid tissues and the CNS (Weingartl et al. 2005). Recoverable virus was recorded in the respiratory, lymphatic and nervous systems, and virus shedding in nasal, pharyngeal, and ocular fluids was reported. HeV infection of pigs also presents as a primarily respiratory disease in both Landrace piglets and older Gottingen minipigs, with possible CNS involvement observed in minipigs, and similar patterns of virus shedding (Li et al. 2010). Overall, HeV appeared to cause a more severe respiratory syndrome in pigs in comparison to NiV. Although HeV and NiV disease in pigs is often less severe in comparison to other animal models, the virus does replicate and disseminate to a variety of organs along with significant levels of virus shedding.

The Horse

Natural HeV infection in horses is often associated with severe disease and experimental infections are essentially uniformly fatal (Marsh et al. 2011). Naturally infected horses appear to have an incubation period of ~8–11 days and animals initially present as anorexic and depressed with general uneasiness and ataxia, with the development of fever and sweating. Respiration becomes rapid, shallow and labored with pulmonary edema and congestion, along with nasal discharge 1–3 days following the onset of clinical signs. In severe cases the airways of horses are often filled with a blood-tinged frothy exudate. There was hemorrhage, thrombosis of capillaries, necrosis, and syncytial cells in the endothelium of pulmonary vessels noted. Viral antigen was also observed within endothelial cells across a wide variety of organs, with recoverable virus from a number of internal organs as well as from saliva and urine. Neurologic clinical signs can also present (Rogers et al. 1996). However, in experimentally infected horses, only meningitis (with vasculitis) was noted in all animals (Marsh et al. 2011) and viral antigen was detected in the

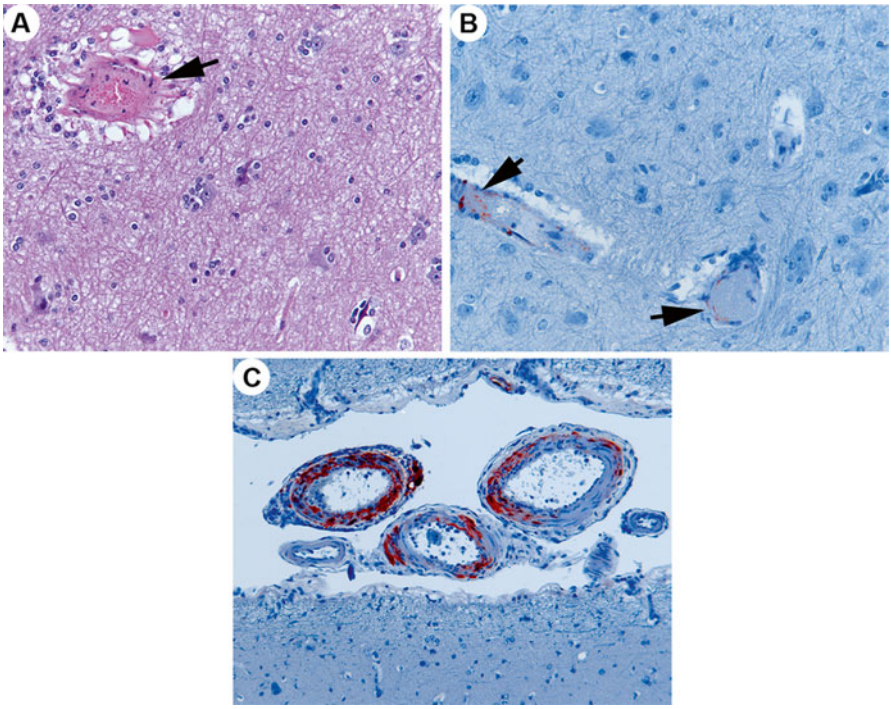

Fig. 4 Hendra virus pathology in the horse. (**a**, *arrow*) Vasculitis of blood vessels in the brain parenchyma of a HeV-infected horse. HeV antigen detected by IHC with anti-N protein polyclonal antibody within cerebral blood vessels of brain parenchyma (**b**, *arrows*) and meningeal blood vessels (**c**). Panel (**a**) from Marsh et al. (2011). Panels (**b**, **c**) courtesy of Deborah Middleton, AAHL Biosecurity Microscopy Facility, Australian Animal Health Laboratory (AAHL) Livestock Industries CSIRO, Australia

meninges of each case. One horse in this study also presented with vasculitis of blood vessels in the brain parenchyma, and HeV antigen was also identified within the cerebral blood vessels of this animal (Fig. 4) (Deborah Middleton, personal communication). Also, an experimental control horse in Middleton et al. (2014) also had vasculitis with HeV antigen in blood vessels within the brain. However, to date HeV antigen has not been reported to be present in the neurons of infected horses, but this may be a sampling artefact and/or an observation exacerbated by the fact that the horses are being euthanized and the HeV infection is not reaching its full pathogenic expression under experimental conditions. However, the meningitis and inflammation of cerebral blood vessels in the experimentally infected horses may be sufficient explanation for the clinical signs of neurological disease in naturally acquired cases of HeV infection (Deborah Middleton, personal communication). Experimental infection of horses with NiV has not been performed but the brain and spinal cord of one naturally infected horse was examined and immunohistochemical staining of viral antigen observed revealing non-suppurative meningitis (Hooper et al. 2001).

CNS Invasion

An array of viruses across many families are known to exhibit neurotropism and there are two central routes of CNS invasion; hematogenous spread or via infection of nerve cells (Swanson and McGavern 2015; Koyuncu et al. 2013). Many viruses that cause viremia following the establishment of an initial infection have an opportunity to breach the blood–brain-barrier (BBB); a highly selectively permeable barrier that separates the CNS from the peripheral blood circulation (Ransohoff et al. 2003). Once in the blood, a number of viruses including some herpesviruses, paramyxoviruses, retroviruses, picornaviruses, filoviruses, and flaviviruses can directly infect vascular endothelial cells (Koyuncu et al. 2013) which could allow passage of virus into the CNS and/or promote inflammation and breakdown of the BBB which may also facilitate virus access to the CNS (Obermeier et al. 2013). Alternatively, some viruses can infect myeloid and lymphoid cells and these infected cells can naturally traverse the BBB delivering virus into the CNS by the "Trojan horse" mechanism (McGavern and Kang 2011). A number of neurovirulent paramyxoviruses, particularly the morbilliviruses like measles virus and canine distemper virus, but also mumps virus and Newcastle disease virus, can productively infect lymphocytes (Joseph et al. 1975; Krakowka et al. 1975; Fleischer and Kreth 1982; Hao and Lam 1987) (see also Chap. 2). These infected lymphocytes serve as a cell-associated viremia which can then lead to the delivery of virus into the CNS by transmigration through BBB (Lossinsky and Shivers 2004).

CNS invasion by NiV and HeV is a key feature of their pathogenic features in humans and as discussed earlier several animal models have also demonstrated NiV and HeV CNS disease. The widespread and disseminated endothelial infection and vasculitis in henipavirus encephalitis strongly suggest that BBB disruption is an important, if not the most important route, for viral entry into the CNS. Plaque-like, groups of infected neurons were frequently observed near to infected/vasculitic vessels suggesting centrifugal viral spread from focal BBB damage.

However, although NiV was shown not to infect human lymphocytes and only low levels of monocyte infection have been reported, human lymphocytes could bind NiV and facilitate its transfer and infection to other susceptible cells (Mathieu et al. 2011). The trafficking of such cell-associated infectious NiV within a host disseminates the virus and also could potentially deliver NiV into CNS by leukocyte transmigration. In pigs, however, NiV infection of CD6+ CD8+ T lymphocyte has been observed, along with monocytes and NK cells (Stachowiak and Weingartl 2012). CD6 is a costimulatory molecule involved in lymphocyte activation and differentiation (Gimferrer et al. 2004) which engages activated leukocyte cell adhesion molecule (ALCAM/CD166) which is known to promote leukocyte migration across the BBB (Cayrol et al. 2008). In this instance, it was suggested that NiV-infected CD6+ T cells would elaborate a strong interaction ALCAM expressed on microvascular endothelial cells which could determine the observed tropism of NiV for small blood vessels and also facilitate CNS invasion by leukocyte migration. Similar studies have not been reported with HeV.

Alternatively, some neurotropic viruses can invade the CNS via infection of peripheral nerves (Swanson and McGavern 2015). For example, some neurotropic viruses begin the infection process in one cell type or tissue such as the oropharyngeal and intestinal mucosa in case of poliovirus (see also Chap. 1) or in myocytes at the bite site in the case of rabies virus (see also Chap. 4) and both later use peripheral motor neurons and retrograde transport to infect the CNS (Koyuncu et al. 2013). In the case of some herpesviruses, initial infection of sensory neurons is followed by retrograde transport and establishment of latency in the peripheral nervous system, and fortunately anterograde transport of herpesviruses to the CNS is rare (Koyuncu et al. 2013) (see also Chap. 18). Olfactory receptor neurons provide a unique opportunity for neurotropic pathogens to invade the CNS because of the direct exposure of dendrites to the environment within the olfactory epithelium, and a few members of several virus families, including flaviviruses, togaviruses, and bunyaviruses are known to invade the CNS via an initial infection of olfactory receptor neurons within the olfactory epithelium and once infected virus can gain access to the CNS by transported anterograde transport (Mori et al. 2005; Koyuncu et al. 2013).

Certain paramyxoviruses have also been shown capable of neuroinvasion via anterograde transport following infection of olfactory neurons (Rudd et al. 2006; Ramirez-Herrera et al. 1997). NiV infection in pigs is often asymptomatic as discussed above. When disease was noted in naturally infected pigs, neurological disease manifested as trembling, twitches, muscle spasms, and uncoordinated gait (Mohd Nor et al. 2000). Experimental NiV infection challenge of Landrace female piglets by the ocular and oronasal routes revealed that virus replication occurs in the oropharnyx and then spreads sequentially to the upper respiratory tract and submandibular lymph nodes, followed by replication in the lower respiratory tract, and additional lymphoid tissues, and NiV was detected in the nervous system of both sick and apparently healthy animals; including cranial nerves, trigeminal ganglion, brain, and cerebrospinal fluid. NiV invaded the CNS via cranial nerves, most importantly via the olfactory nerve, as early as 3 dpi, as well as by crossing the BBB (Weingartl et al. 2005). One report of HeV infection of Landrace and Gottingen minipig breeds by oronasal or nasal inoculations produced clinical signs that were primarily respiratory with suggestive neurological involvement seen only in the Gottingen minipig.

An aged mouse model of intranasal challenge with HeV revealed that animals could consistently develop encephalitic disease, and an anterograde route of neuroinvasion of the CNS via olfactory nerves was proposed (Dups et al. 2012), however in a follow-up study using the same model with NiV-Bangladesh and NiV-Malaysia, animals did not exhibit CNS disease (Dups et al. 2014). As was discussed earlier, in the hamster model for both NiV and HeV challenge, lower doses of virus allowed for a more neuropathogenic disease state. In an elegant spatial-temporal model of NiV infection in the hamster by intranasal inoculation (10^5 $TCID_{50}$), individual NiV-infected neurons were observed extending from the olfactory bulb by 4 dpi, demonstrating direct evidence for virus transport in the CNS via olfactory neurons (Munster et al. 2012) (Fig. 5). At 6 dpi, meningoencephalitis was observed, characterized by multifocal men-

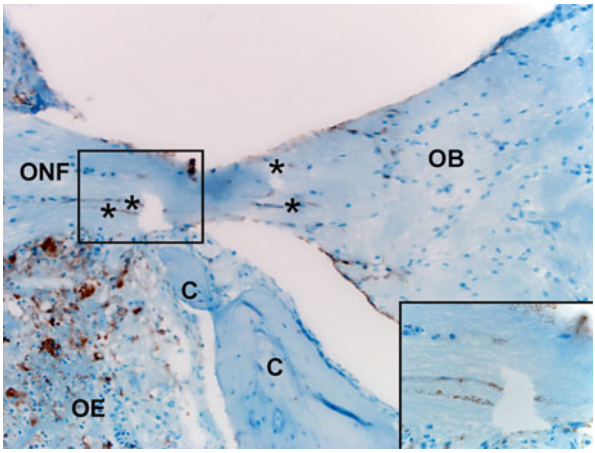

Fig. 5 Entry of Nipah virus into the CNS. A hamster model of NiV infection by intranasal inoculation revealed individual NiV-infected neurons extending from the olfactory bulb at 4 dpi. Viral antigen was detected by monoclonal antibody staining (*red–brown*) against nucleoprotein. *Asterisks* indicate positive neurons within the olfactory nerve fiber (ONF), crossing from the olfactory epithelium (OE) to the olfactory bulb (OB) through the cribriform plate (C). The inset shows a higher magnification of the *boxed area* with antigen-positive neurons. Figure 5 reproduced from Munster et al. (2012), "Rapid NiV entry into the central nervous system of hamsters via the olfactory route," licensed under a Creative Commons Attribution 3.0 Unported License. http://www.nature.com/srep/2012/121015/srep00736/full/srep00736.html

ingeal and perivascular lymphocytic infiltration, and in the olfactory bulb neurons and axons of the olfactory nerve layer, glomerular layer and external plexiform layer of the olfactory bulb were positive by NiV antigen staining. NiV dissemination from the olfactory bulb to the olfactory tubercle region was noted by 6 dpi. From olfactory tubercle region, which is highly innervated to other brain regions including the hypothalamus, thalamus, amygdala, hippocampus and brain stem, spread of NiV within the CNS is readily possible. Similarly, in oronasal challenge models of both NiV and HeV in the ferret (Pallister et al. 2011; Bossart et al. 2009), henipavirus genome and viral antigen were consistently detected in the olfactory lobe of brains along with many animals demonstrating neurological disease such as tremors and hind limb weakness or paralysis. Finally, in the AGM nonhuman model of NiV and HeV infection described earlier, consistent neurological disease was observed even though an intratracheal route of challenge is performed, with those animals surviving longer, or those challenged with lower doses of virus, showing more severe neurological disease with signs such as tremors, paralysis and convulsions (Rockx et al. 2010; Geisbert et al. 2010) (Geisbert and Broder Unpublished). However, in human NiV autopsy studies, involvement of the olfactory bulb has not been demonstrated so far (Wong et al. 2002).

Therapeutics and Vaccines

Antivirals

Presently, there are no approved therapeutics for treating HeV or NiV infection in people, but there have been a few approaches tested in animal models (reviewed in Broder 2012). Ribavirin is often a first line treatment course for suspected viral infections of unknown etiology, having antiviral activity against many RNA and some DNA viruses (Sidwell et al. 1972) and is an accepted treatment against several viruses including respiratory syncytial virus and arenaviral hemorrhagic fevers (reviewed in Snell 2001). During the initial NiV outbreak in Malaysia, some patients were treated with ribavirin and there was some evidence that this therapy may have been clinically beneficial (Chong et al. 2001a; Snell 2004). Of the recorded human HeV cases, three individuals were treated with ribavirin, and of these, two succumbed to disease and one survived (Playford et al. 2010). Chloroquine, an antimalarial drug, was shown to block the critical proteolytic processing needed for the maturation and function of the HeV F glycoprotein discussed earlier (Pager et al. 2004) and could block infection in cell culture (Porotto et al. 2009). However, chloroquine and ribavirin treatment of a HeV-infected individual had no clinical benefit (reviewed in Broder et al. 2013). Animal studies have also revealed no therapeutic benefit of either chloroquine or ribavirin. Two studies in hamsters and one study in monkeys showed that ribavirin treatment only delayed death after virus infection (Freiberg et al. 2010; Georges-Courbot et al. 2006; Rockx et al. 2010), with HeV challenge monkeys treated with ribavirin having marked increases of neurological symptoms. Chloroquine treatment was also unable to prevent NiV disease in ferrets (Pallister et al. 2009). Also, various forms of poly(I:C) are strong inducers of IFN-α and -β production, have been explored as antiviral therapies for over 40 years. Poly$IC_{12}U$ is very specific in triggering the Toll-like receptor (TLR)3 pathway (reviewed in Nicodemus and Berek 2010). Poly$IC_{12}U$ was shown capable of blocking NiV replication, and continuous administration of poly$IC_{12}U$ for 10 days beginning at the time of challenge was shown to prevent lethal NiV disease in five of six hamsters (Georges-Courbot et al. 2006), suggesting that use of TLR3 agonists such as Poly$IC_{12}U$, perhaps in combination with other antiviral strategies, should be explored. But for HeV and NiV, the development of new therapeutics and vaccines has primarily focused on targeting the attachment and infection stages mediated by the viral F and G glycoproteins.

Peptide Fusion Inhibitors

As discussed earlier, peptides, typically 30–40 residues in length that are homologous to either of the heptad repeat domains of several paramyxovirus F glycoproteins, including the henipaviruses, can potently inhibit membrane fusion by blocking the formation of

the trimer-of-hairpins structure (reviewed in Bossart et al. 2013). The first henipavirus-specific peptide fusion inhibitor was a 36 amino acid heptad repeat-2 sequence (NiV-FC2) (Bossart et al. 2001) analogous to the approved HIV-1 specific therapeutic peptide enfuvirtide (Fuzeon™). Other studies showed that a heptad repeat-2 peptide from human parainfluenza virus type-3 (hPIV3) F blocked HeV mediated fusion (Porotto et al. 2006) and a sequence-optimized and cholesterol-tagged hPIV3-based heptad repeat-2 peptide appeared effective in the NiV hamster (Porotto et al. 2010). This cholesterol-tagged antiviral peptide could also penetrate the CNS and exhibit some effective therapeutic activity against NiV. Additional in vivo efficacy testing of peptide fusion inhibitors as henipavirus therapeutics merits further investigation.

Antiviral Antibodies

Almost without exception all virus-neutralizing antibodies to enveloped viruses are directed against the viral envelope glycoproteins on the surface of the virion particle. Initial passive immunization studies were conducted in the hamster NiV-challenge model and showed that antibody immunotherapy against henipavirus infection by targeting the viral envelope glycoproteins was possible. Protective passive immunotherapy using either NiV G and F-specific polyclonal antiserums, or mouse monoclonal antibodies (mAbs) specific for the henipavirus G or F glycoproteins has been shown (Guillaume et al. 2004, 2006, 2009). These studies demonstrated a major role of viral glycoprotein specific antibody in protection from henipavirus-mediated disease (reviewed in Broder et al. 2012). Using recombinant antibody technology, henipavirus-neutralizing human mAbs reactive to the G glycoprotein were previously isolated (Zhu et al. 2006). One mAb, m102, possessed strong cross-reactive neutralizing activity against HeV and NiV and was affinity maturated (m102.4) and converted to an IgG1 format and produced in a CHO-K1 cell line (Zhu et al. 2008). The m102.4 mAb epitope maps to the receptor binding site of G and engages G in a similar fashion as the ephrin receptors (Xu et al. 2013). The m102.4 mAb can neutralize NiV-Malaysia, HeV-1994, HeV-Redlands and NiV-Bangladesh isolates (Bossart et al. 2009). In a post-exposure NiV-challenge experiment in the ferret model, a single dose of mAb m102.4 administered by intravenous infusion 10 h after lethal challenge could prevent lethal infection (Bossart et al. 2009). The therapeutic efficacy of mAb m102.4 has also been examined in monkeys against both NiV and HeV challenge with a study design reflecting a potential real life scenario that would require a post-exposure treatment (Bossart et al. 2011; Geisbert et al. 2014). In one study, animals were challenged intratracheally with HeV and later infused twice with m102.4 (~15 mg/kg) beginning at 10, 24, or 72 h post-infection followed by a second infusion ~48 h later. All subjects became infected following challenge, and all animals that received m102.4 survived whereas all control subjects succumbed to severe systemic disease by day 8. Animals in a 72 h treatment group did exhibit neurological signs but all recovered by day 16, but there was no evidence of HeV-specific pathology in any of the m102.4-treated

animals, and no infectious HeV could be recovered from any tissues from any m102.4-treated subjects. A follow-up study evaluated the efficacy of m102.4 against NiV disease in the AGM model at several time points after virus exposure by intratracheal challenge, including at the onset of clinical illness (Geisbert et al. 2014). Here, subjects were infused twice with m102.4 (15 mg/kg) beginning at either 1, 3, or 5 days after virus challenge and again 2 days later. All subjects became infected after challenge and all subjects that received m102.4 therapy survived infection, whereas the untreated control subjects succumbed to disease between days 8 and 10 after infection. Animals in the day 5 treatment group exhibited clinical signs of disease, but all recovered by day 16. Together, these studies revealed that mAb m102.4 could prevent widespread henipavirus dissemination in challenged subjects, and were the first successful post-exposure in vivo therapies against HeV and NiV in nonhuman primates.

Active Immunization Strategies

A variety of active immunization strategies for henipavirus have been examined using recombinant virus platforms, protein subunit, virus-like particles and DNA vaccines. Several of these strategies have only been examined in terms of their ability to generate a henipavirus-specific neutralizing response (Kong et al. 2012; Kurup et al. 2015; Wang et al. 2006; Walpita et al. 2011), whereas other studies examined immune response and efficacy in animal challenge models. The first report used the hamster model and the attenuated vaccinia virus strain NYVAC, using recombinant viruses encoding either the NiV F or G, both individually and in combination to immunize animals, and the study revealed that complete protection from NiV-mediated disease was achievable and that an immune response to the viral envelope glycoproteins can be important in protection (Guillaume et al. 2004). Another poxvirus-based vaccine was examined as a potential livestock vaccine using recombinant canarypox virus in pigs (Weingartl et al. 2006). Here, the NiV F and G glycoprotein genes were used to generate recombinant canarypox viruses (ALVAC) vaccine vectors and used to immunize pigs. ALVAC vectors expressing F and G were tested alone and in combination, and piglets were challenged intranasally with NiV. Here, protection from NiV-mediated disease was seen in all vaccinated pigs by either ALVAC vector alone or in combination and that vaccinated animals shed only low levels of nucleic acid detectable virus with no isolatable virus (Weingartl et al. 2006).

More recently, several viral vector-based henipavirus vaccines have also been examined in animal challenge studies; these have included immunizations using the vesicular stomatitis virus based platform (VSV) expressing either the NiV G or F glycoprotein in the hamster model (DeBuysscher et al. 2014; Lo et al. 2014) and also VSV-based vaccines using NiV F or G in the ferret model (Mire et al. 2013). All these studies demonstrated that a single dose of vaccine could induced strong neutralizing antibody responses and could afford protection from NiV challenge,

highlighting their potential usefulness as either a livestock vaccine or one suitable in an emergency use or outbreak scenario. Vaccination and challenge experiments have also been examined using an adeno-associated virus platform with NiV G showing protection against challenge in the hamster model and low level cross-protection (three of six animals) against a HeV challenge (Ploquin et al. 2013), and also a recombinant measles virus vector with NiV G which showed two of two AGMs were protected from NiV challenge (Yoneda et al. 2013).

A protein subunit vaccine strategy for henipaviruses has been extensively examined because of the inherent safety of such an approach. Soluble, secreted, oligomeric forms of the G glycoprotein (sG) from both NiV and HeV were developed (Bossart et al. 2005). The HeV-sG glycoprotein is a secreted version of the molecule with a genetically deleted transmembrane and cytoplasmic tail that is produced in mammalian cell culture systems and is properly N-linked glycosylated (Colgrave et al. 2011). HeV-sG retains many native characteristics including oligomerization and ability to bind ephrin receptors (Bonaparte et al. 2005), and it elicits potent cross-reactive neutralizing (HeV and NiV) antibody responses in a variety of animals including mice, rabbits, cats, ferrets, monkeys and horses. Studies using the HeV-sG subunit immunogen in the cat model demonstrated that it could elicit a completely protective immune response against a lethal subcutaneous NiV challenge (Mungall et al. 2006) showing that a single vaccine (HeV-sG) could be effective against both HeV and NiV. Further studies in the cat model demonstrated that pre-challenge virus-neutralizing antibody titers as low as 1:32 were completely protective from a high-dose oronasal challenge of NiV (50,000$TCID_{50}$) (McEachern et al. 2008). HeV-sG immunization studies in the ferret model using either 100, 20 or 4 μg doses of HeV-sG formulated in CpG and Allhydrogel™ could all afford complete protection from a 5000 $TCID_{50}$ dose of HeV (100 times the minimal lethal dose) with no disease or evidence of virus or viral genome in any tissues or body fluids in the 100 and 20 μg vaccine groups; and only a low level of viral genome detected in the nasal washes from one of four animals in the 4 μg vaccine group. No infectious virus could be recovered from any vaccinated ferrets. The HeV-sG subunit vaccine has also been evaluated in nonhuman primates (AGMs). In one study, doses of 10, 50, or 100 μg of HeV-sG were mixed with Allhydrogel™ and CpG and vaccine was given to three subjects in each dosing group twice, 3 weeks apart, and subjects were challenged by intratracheal administration with a tenfold lethal dose of NiV (1×10^5 $TCID_{50}$) 21 days later. Complete protection was observed in all vaccinated subjects. Some subjects had pre-challenge NiV neutralizing titers as low as 1:28. No evidence of clinical disease, virus replication, or pathology was observed. A second study examined HeV-sG vaccination and protection from HeV challenge in AGMs, and also evaluated the HeV-sG subunit (100 μg doses) in Allhydrogel™ and CpG as well as formulated with only Allhydrogel™ (Mire et al. 2014). Subjects were vaccinated twice, 3 weeks apart, and were challenged intratracheally with a tenfold lethal dose of HeV ($\sim 5 \times 10^5$ plaque-forming units) 21 days after the boost vaccination. None of the eight vaccinated animals showed any evidence of clinical illness, virus replication, or pathology. The study also clearly demonstrated that HeV-sG-Allhydrogel™ alone is capable of providing complete protection from a HeV challenge providing crucial data for supporting preclinical development as a henipavirus vaccine for use in people.

The simplicity and inherent safety of the HeV-sG subunit vaccine approach together with the numerous successful vaccination and challenge studies that have been carried out in multiple animal models, the HeV-sG subunit vaccine was chosen for the development of an equine vaccine to prevent infection in horses and also reduce the risk of HeV transmission to people. HeV-sG was licensed by Zoetis, Inc. (formerly Pfizer Animal Health) and developed as an equine vaccine for use in Australia. Horse HeV-sG vaccination and HeV challenge studies were conducted in Australia the BSL-4 facilities of the Australian Animal Health Laboratories (AAHL) in Geelong, Australia (Middleton et al. 2014). Here, HeV-sG was formulated in a proprietary adjuvant (Zoetis, Inc.) and in two initial efficacy studies in horses, either a 50 or 100 μg dose of the same sourced HeV-sG which was used in all the animal challenge studies described earlier. Two additional studies used 100 μg HeV-sG produced from clarified CHO cell culture supernatant (Zoetis, Inc.) that was then gamma irradiated. Immunizations were two 1-mL doses administered intramuscularly 3 weeks apart. Horses in the efficacy studies were exposed oronasally to 2×10^6 $TCID_{50}$ of HeV. Seven horses were challenged 28 days, and three horses were challenged 194 days, after the second vaccination. All vaccinated horses remained clinically healthy after challenge showing protection with HeV neutralizing titers as low as 1:16 or 1:32 pre-challenge. At study completion, there was no gross or histologic evidence of HeV infection in vaccinated horses; all tissues examined were negative for viral antigen by immunohistochemistry; and viral genome was not recovered from any tissue, including nasal turbinates, pharynx, and guttural pouch. In nine of ten vaccinated horses, viral RNA was not detected in daily nasal, oral, or rectal swab specimens or from blood, urine, or feces samples collected before euthanasia, and no recoverable virus was present. Only in one of three horses challenged at 6 months after vaccination, low viral gene copy numbers were detected in nasal swab samples collected on post-challenge days 2, 4 and 7, a finding consistent with self-limiting local replication, but no recoverable virus was present (Middleton et al. 2014). The horse vaccine against HeV (Equivac® HeV) is the first commercially deployed vaccine developed against a BSL-4 agent and is the only licensed treatment for henipavirus infection. To date, more than 430,000 doses of Equivac® HeV vaccine have been administered to horses (Zoetis, Inc.).

Summary and Future Directions

HeV and NiV are the first and only examples of zoonotic paramyxoviruses that can infect and cause lethal disease across a broad range of mammalian species including humans and there are currently no approved treatment modalities for people. Because of the potential environmental accessibility of HeV and NiV and their highly pathogenic characteristics, the development of effective countermeasures against these biothreats has been a major research focus over the past decade. Much of this research has focused on the virus binding and entry processes, including the processing, maturation and function of the envelope

glycoproteins and the attachment to host cellular receptors and the membrane fusion process. These efforts have led to the development and testing of potential vaccine candidates and antiviral therapeutics. In 2010, the m102.4 mAb producing cell line was provided to the Queensland Government, Queensland Health, Australia to produce the m102.4 mAb for emergency use on a compassionate basis in future cases of high-risk human HeV exposure. Queensland Health Authorities have completed in May, 2016, the first phase 1 clinical safety trial of m102.4 in human subjects (Queensland 2013). To date, 11 individuals exposed to either HeV in Australia (10 people) or NiV in the United States (1 person) have been given high-dose m102.4 therapy under emergency use protocols, and all have remained well with no associated adverse events. In addition, the vaccine against HeV (Equivac® HeV) is vaccine for horses that is also expected to provide a substantial health benefit to humans, and has fit well within the spirit of a "One Health" approach for the human and animal interface and also in respect to environmental health. Studies on NiV and HeV have also provided important model systems to examine how pathogenic viruses interact with their natural reservoir hosts and also with animals susceptible to disease, providing insight into the dynamics of virus infection and maintenance in an animal reservoir; model systems to develop a variety of intervention strategies; details on how neurotropic viruses gain access to CNS and cause disease; and will serve as tools to examine and evaluate potential therapies for virus-mediated CNS disease.

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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 encephalomyelitis, 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 originated 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 behavioural changes such as the 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 including the digestive, pulmonary and urinary systems. Once the virus has entered 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 post-exposure treatment with injection of killed rabies vaccine altogether with rabies-specific immunoglobulins. Post-exposure treatment of rabies requires public information, access to medical facilities and availability of efficient post-exposure 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.

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Human Rabies

Rabies virus causes fatal encephalomyelitis in most mammals (Warrell and Warrell 2004). Humans are infected mostly after a bite or scratches by rabid animals. Aerosol contamination is exceptional (Davis et al. 2007; Johnson et al. 2006). Incubation period is broad, ranging from a few week to 1 year or more (Smith et al. 1991; Jackson 2003). This variation could be related to the site of the inoculum or the viral load associated with the trauma.

Clinical presentations of rabies in humans can be categorized as furious (75 % of cases) and paralytic rabies (25 % of cases) (Hemachudha et al. 2003). Local prodromal signs such as aching pain near the bite site, paresthesia of the infected limb associated (or not), 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 of circulatory insufficiency, cardiac arrest and respiratory failure (Hemachudha et al. 2002; Rupprecht et al. 2002). In paralytic rabies, only one or two classical signs of furious rabies develop. Weakness of all limbs and respiratory muscles and absence of deep tendon reflexes are the initial manifestations of paralytic rabies.

Paralytic rabies can be confused with Guillain-Barre syndrome and related treatable autoimmune diseases of peripheral nerves. Misdiagnosis of rabies has led to human-to-human transmission through corneal, liver and kidney transplants. Analysis of regional distribution of rabies virus antigen (mainly in spinal cord, brainstem, thalamus and basal ganglia) revealed similar infection patterns in the two clinical forms of rabies (Hemachudha et al. 2006a). However the differential symptoms of paralytic and furious rabies suggest that dysfunction of anterior horn motor neurons and peripheral nerves occurs in the case of paralytic form, whereas cerebellum and limbic functions are altered in furious rabies. Indeed, clinical, electrophysiological studies and post-mortem histological 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 oedema) of cellular bodies in the anterior horn of spinal cord occurs in furious rabies (Mitrabhakdi et al. 2005). 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 (Hemachudha et al. 2006a; Mitrabhakdi et al. 2005). Origin of neuropain at the bite sites could be related to this dorsal root ganglionopathy. Clinical features of dog and bat acquired rabies in humans seems to be distinct (Udow et al. 2013). Once rabies virus has infected the brain stem, it gains access to the salivary glands likely by travelling along the cranial nerves (VII facial and IX glossopharyngeal) which innervates the salivary glands.

After its establishment in the central nervous system, the rabies virus reaches peripheral organs by a centrifugal spread. Rabies virus can be detected in nerves

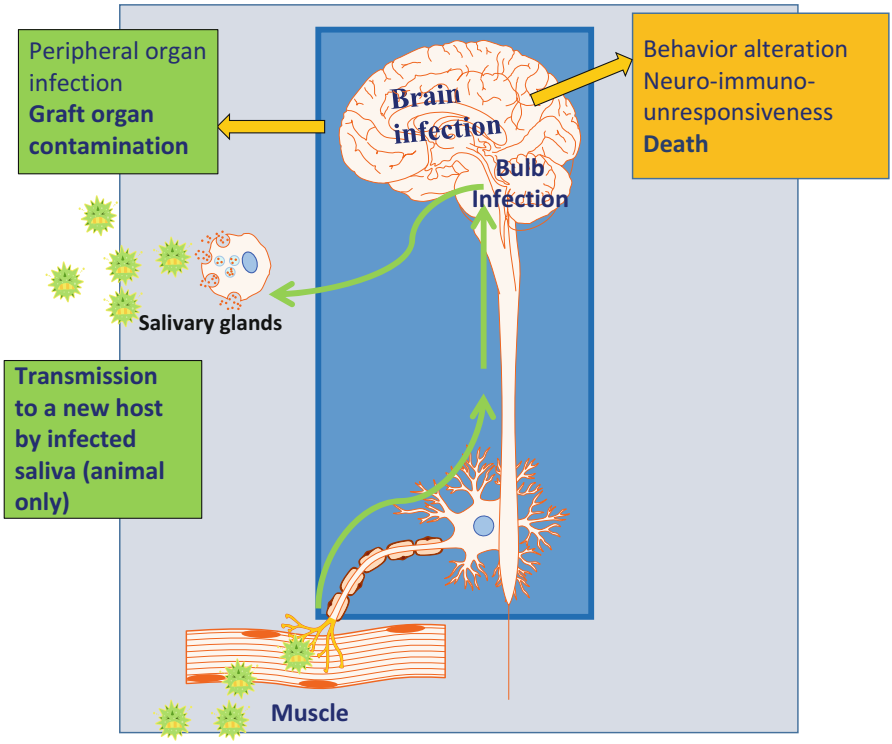

Fig. 1 Lafon rabies

plexus in multiple organs, heart, in several cell types of the gastrointestinal system, in adrenal medulla, or hair follicles of the skin (Jackson et al. 1999; Jogai et al. 2002). In contrast to transmission by organ transplantation, human-to-human transmission by bites has never been reported. Figure 1 shows the cycle of rabies virus infection.

Rabies has the highest case fatality rate of any infectious diseases known to man and is considered to be virtually 100 % once symptoms have developed. A few exception of recovery cases, one spectacular after intensive treatment have been obtained (Willoughby et al. 2005). Survivors have all in common to have developed neutralizing antibodies both in serum and cerebro-spinal fluid (Jackson 2014). Nevertheless, it is still unclear which therapeutic gestures were critical for rabies recovery (Hemachudha et al. 2006b; Jackson et al. 2007; Weli et al. 2006).

Zoonotic Rabies

Vast majority of animals infected by rabies are dogs. Canine rabies causes annually over 3.7 million disability adjusted life years (DALYS) and 8.6 billion USD of economics losses (Hampson et al. 2015). Dogs transmit classical rabies and develop

furious rabies. Less often, paralytic rabies is also observed in dogs as in humans. Behaviour modifications such as furious state (in dogs, cats, bears) or loss of natural shyness in wildlife animals (foxes) are common feature of rabies.

Bats are important reservoirs for several zoonotic viruses including rabies viruses (Calisher et al. 2006). Most members of the Rhabdovirus family have been found in bats. This is the case of classical rabies which variant of rabies viruses are harboured by vampires (Warner et al. 1999) or insectivorous bats (Silver Hair bats and Eastern pipistrelle) (Dietzschold et al. 2000) in the Americas. This is also the cases of various rabies related viruses such as Lagos Bat and Duvenhage, Shimoni viruses in Africa (Swanepoel et al. 1993; King et al. 1990; Shope 1982; Kuzmin et al. 2010), or Australian Bat (Warrilow 2005) and European bat lyssaviruses (Johnson et al. 2006; Muller et al. 2004, 2007; Marston et al. 2007; Fooks et al. 2003a, b, 2006; Brookes et al. 2005; Nathwani et al. 2003; Lumio et al. 1986) as well as Aravan, Khujand, Irkut, West Caucasian (Kuzmin et al. 2006) in Europe and Asia. Transmission of rabies virus bat variants to humans are commonly reported (Jackson 2006; Dixon 2007; Messenger et al. 2002; Warrell 1995) 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 (Johnson et al. 2006; Constantine et al. 1968, 1972). Spill-over of bat rabies can occur by cross-species transmission involving infection of terrestrial non-flying animals (skunks, foxes, marten) (Muller et al. 2004; Leslie et al. 2006; Daoust et al. 1996). It has been proposed that all rabies variants that infect terrestrial mammals originated from cross-species transmission of bat-associated variants (Badrane et al. 2001).

Rabies is usually fatal in bats as for other mammals, however healthy bat rabies carriers have been described (Aguilar-Setien et al. 2005; Echevarria et al. 2001; Jackson et al. 2008). Rabies virus mortality has been estimated to be much lower in vampire bat colonies (only 10 %) than in other mammals (Blackwood et al. 2013). This could be related to the acquisition of immunoprotection due to long-term repeated infections of bats in the colonies (Turmelle et al. 2010). Alternatively, neurotropism of bat rabies variants is possibly not as strict as for rabies variants transmitted by dogs since rabies bat variants replicate more efficiently in dermal cells and at lowest temperatures than virus variants originated from dogs (Morimoto et al. 1996).

Rabies Virus Strains for Experimental Research

While lagomorphs (rabbit) and rodents (mice and rats) are not natural hosts for rabies virus, rabies virus has been adapted to these animals in the laboratory by serial intracranial injections of brain tissue.

Most of analysis of pathogenicity and immunological reactions have been examined in the mouse with mouse-neuroadapted rabies viruses (Camelo et al. 2000; Galelli et al. 2000; Weiland et al. 1992; Lafon et al. 2008; Chopy et al. 2011a; Kojima et al. 2009; Koraka et al. 2012; Healy et al. 2013). A few analyses have been performed in monkeys, mostly experiments using rabies as a neurotracker exploiting the

rabies virus capacity to be transported by a retrograde pathway (Grantyn et al. 2002; Moschovakis et al. 2004; Ugolini et al. 2006; Kelly and Strick 2000). Rare immunopathological studies have been performed in bats (Davis et al. 2007; Aguilar-Setien et al. 2005; Jackson et al. 2008; McColl et al. 2002; Almeida et al. 2005).

Strains adapted to laboratory animals by prolonged serial passage, leading to death of the animal following a fixed 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 the Pasteur virus with further passage history in mice or guinea pigs. The strain Flurry was isolated from a human case. Fuenzalida, SAD (Street alabama Dufferin) and Kelev were isolated from dogs in Chile, USA and Israel, respectively. SHBRV-18 (Silver haired Bat rabies virus, strain 18) has been isolated from a silver haired bat and adapted to neuroblastoma cells.

Several strains of rabies virus with different levels of pathogenicity in mice have been selected. After intramuscular, intraplantar route, encephalitic rabies virus strains invade the spinal cord and brain regions and causes fatal encephalitis (Camelo et al. 2000; Xiang et al. 1995; Park et al. 2006). In contrast, injected by the same routes, the attenuated strains of rabies virus result in a non-fatal abortive disease characterized by a transient and restricted infection of the nervous system followed by irreversible paralysis of the inoculated limbs (Galelli et al. 2000; Weiland et al. 1992; Xiang et al. 1995; Hooper et al. 1998; Irwin et al. 1999).

Rabies virus may be cultivated in vitro in most types of mammalian cells including neuroblastoma and in chick fibroblasts. Virus directly isolated from animals requires several passages before it gets adapted to cell culture. Attenuation of pathogenicity has been obtained by further passages in non-neuronal 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. Primary sequences of ERA, PV and CVS are different by only a few amino acids (Tordo et al. 1986; Anilionis et al. 1982; Morimoto et al. 1998). The vaccine Ni-CE and RC-HL strains were obtained by passaging the Nishigahara strain in chick embryo or fibroblast. Recombinant rabies viruses could 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 (Schnell et al. 1994; Ito et al. 2001; Inoue et al. 2003) and for the bat virus strain SHRBV-18 (Faber et al. 2004) and Ni-CE (Masatani et al. 2013). These viruses with different pathogenicity, especially those engineered by reverse genetics, are powerful tools to understand the molecular basis of rabies virus pathogenicity (Faber et al. 2004, 2005a, 2007; Morimoto et al. 2000, 2005; Pulmanausahakul et al. 2001; Prehaud et al. 2010) or may constitute new promising vaccine candidates (Li et al. 2012; Faber et al. 2009).

Rabies Virus

Rabies Virus Structure

Rabies virus is an enveloped bullet-shaped virus belonging to the *Rhabdoviridae* family, genus *Lyssavirus*. It is a non-segmented negative-strand RNA virus 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 (M), surrounding a helical nucleocapsid (NC). The G protein is assembled in homotrimers and forms spikes of 6–10 nm long at the surface of the virion. NC is composed of a single non-segmented negative-strand RNA molecule protected by the nucleoprotein (N) and phosphoprotein (P) and the large RNA dependent RNA polymerase, L protein. The NC, and not the free RNA, is a template for viral gene expression and replication. The genome of rabies virus has a molecular weight of 4.6×10^6 Da and a sequence 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 in the order: N-P-M-G-L and results in monocistronic mRNAs production. Transcription of individual genes is differentially regulated by non-transcribed spacer regions (intergenic regions, IGR or pseudogenes) located between transcriptional start and stop signals of consecutive genes (Tordo et al. 1986; Conzelmann et al. 1990). Length of the IGR is 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 (Finke et al. 2000), IGR could contribute to rabies virus pathogenicity (Faber et al. 2004). Replication of full-length genomes is performed at the 3′ ends of genomic and anti-genomic 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 insure the production of appropriate amounts of viral proteins and viral genomes. The rate of viral replication has been proposed to contribute to virulence, with lower replication rate characterizing virulent strains compared to quicker rate of attenuated strains (Morimoto et al. 1999).

Properties of the Rabies Virus Proteins

The Nucleoprotein N

Rabies virus N protein is a 450 amino acid protein of 57,000 Da. It is the major constituent of the virus and of the NC and the most conserved antigen among genotypes (Conzelmann et al. 1990; Bourhy et al. 1999; Kissi et al. 1995; Yang et al. 1998). This sequence stability could be the result of the vital functions N performs such as protection of RNA template from ribonuclease activity and encapsidation of

genome RNA. Phosphorylation of N protein at serine 389 (Dietzschold et al. 1987; Prehaud et al. 1990) could control transcription and replication efficiency (Yang et al. 1999). N protein forms complexes with P and binds RNA (Kouznetzoff et al. 1998; Mavrakis et al. 2006). N protein and NC function as an exogenous superantigen (Lafon et al. 1992, 1994; Lafon 1993). This could explain its potent activation of peripheral blood lymphocytes in human vaccinees (Herzog et al. 1992) and its ability to increase and potentiate immune response to vaccination (Astoul et al. 1996; Martinez-Arends et al. 1995; Fu et al. 1991, 1994; Lodmell et al. 1993; Smith et al. 2006; Hooper et al. 1994). The N protein limits RIG-I-signalling (Masatani et al. 2010a, b). N protein of the virulent Nishigahara laboratory strain—residues 273 and 394 in particular—was found to favour viral spreading in the mouse brain and concomitantly dampen IFN production response, suggesting that N protein might play a role in controlling rabies virus pathogenicity (Masatani et al. 2013).

The Phosphoprotein P

The P protein (formerly termed M1 for membrane protein 1 or NS for non-structural) is a multifunctional 297 amino acid (40 kDa) phosphorylated protein. Post-translational phosphorylation is performed by two types of protein kinases: the rabies virus protein kinase and the gamma protein kinase C (Gupta et al. 2000). 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 non-specifically and irreversible binding to cellular RNA (Mavrakis et al. 2006). P is required for RNA encapsidation. P protein binds to N protein, L protein, the mitochondrial complex I (Kammouni et al. 2015), the Focal adhesion kinase FAK protein (Fouquet et al. 2015), the cellular dynein light chain 1 protein light chain 8, LC8 (Raux et al. 2000; Jacob et al. 2000), and STAT-1, 2 and 3 (Vidy et al. 2005; Chenik et al. 1994, 1998; Lieu et al. 2013). P protein mediates inhibition of the IFN system by different pathways: it inhibits IFN production by impairing the phosphorylation of IFN regulatory factor 3 (IRF3), IFN signalling by blocking nuclear import of STAT-1/2 and finally alters promyelocytic leukaemia (PML) nuclear bodies by retaining PML in the cytoplasm (Vidy et al. 2005; Blondel et al. 2002; Brzozka et al. 2005, 2006). By binding to STAT-3 P protein inhibits STAT3 nuclear accumulation and Gp130 dependent (IL-6 type cytokines) signalling (Lieu et al. 2013). Through its binding site to LC8, P could favour the transcriptional activity of viral polymerase (Tan et al. 2007). Binding to FAK was found to positively regulate rabies virus infection in cell cultures (Fouquet et al. 2015) whereas interaction of P (a peptide from residues 139 to 172) with mitochondrial complex I causes mitochondrial dysfunction and oxidative stress in rat dorsal root ganglion sensory neurons (Kammouni et al. 2015). The role P plays in controlling rabies virus multiplication and host innate immune response was confirmed by the decrease in pathogenicity induced by a recombinant virus expressing low level of P protein compared to those triggered by a wild-type virus (McGettigan et al. 2003).

The Matrix M protein

The 202 amino acid M protein (20 kDa) is located at the inner face 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 (Mebatsion et al. 1999) either in the plasma membranes for rabies virus (Finke et al. 2010) or at Endoplasmic reticulum in the case of European Bat lyssavirus infection (Pollin et al. 2013). M downregulates transcription (Finke and Conzelmann 2003; Finke et al. 2003) and by suppressing maximal viral gene expression, could promote cell survival. However, this M function is not conserved among Lyssavirus (Larrous et al. 2010) and M instead could play a role in controlling apoptosis in a mitochondrial or TRAIL-dependent pathway, at least in non-pathogenic strains of lyssavirus (Kassis et al. 2004; Gholami et al. 2008).

The Glycoprotein G

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 (19 amino acids) and a cytoplasmic domain of 44 amino acids. It adopts a homotrimeric form in the endoplasmic reticulum. The G is responsible for the attachment of rabies virus to target cells, transport to the central nervous system (Mazarakis et al. 2001; Etessami et al. 2000) and egress from the cell membranes. The rabies virus G induces virus-neutralizing antibodies and T cell responses (Celis et al. 1985, 1986; Cox et al. 1977). IgGs, but not IgM, confer passive protection against rabies virus (Turner 1978).

G protein, among other factors, contributes to pathogenicity of rabies virus. Reduced pathogenicity in lyssaviruses is generally associated with point mutations particularly in sites II and III of the ectodomain region of the G protein as shown in CVS (Ito et al. 2001; Tuffereau et al. 1998; Lafon et al. 1983; Dietzschold et al. 1983; Lafay et al. 1991; Coulon et al. 1998; Prehaud et al. 1988). Mutations in position 333 (antigenic site III) slow the virus uptake by the cell (Dietzschold et al. 1985) and in adult mice reduce the neuroinvasiveness (Coulon et al. 1989). In contrast, the mutation of N to K in position 194 was associated with increased pathogenicity (Faber et al. 2005b). G protein is a key player in the balance apoptosis/survival in neurons (Prehaud et al. 2003, 2010; Morimoto et al. 1999). The interaction of the cytoplasmic domain of the G protein with distinct cellular proteins controls the commitment of rabies virus infected neuronal cells towards neurosurvival or neurodeath (Prehaud et al. 2010; Caillet-Saguy et al. 2015; Terrien et al. 2012). Virulent laboratory strains which G protein cytoplasmic domain interact with a microtubule associated kinase have the capacity to enforce RABV-infected neurons to survive by stimulating neurosurvival pathway (Prehaud et al. 2010). A single amino acid change in the cytoplasmic domain of the G protein extending the recruitment of an additional cellular partner, a protein phosphatase, is sufficient to lose the virulence

property and trigger attenuation instead. Cytoplasmic tail of G could also play a role in the assembly of virions since production of recombinant viruses expressing G lacking the C terminal cytoplasmic tail was reduced compared to recombinant virus expressing full-length G cytoplasmic domain (Mebatsion et al. 1996; Morimoto et al. 2001). Thus the nature of the cytoplasmic tail of the G protein may be a critical factor in rabies virulence (Prehaud et al. 2010; Khan and Lafon 2014).

The Large RNA-Dependent RNA Polymerase L

The L protein with 2142 amino acids is the largest rabies virus protein (Tordo et al. 1986, 1988). 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. L and P protein have in common to interact with the dynein light chain suggesting that L contributes to the microtubule reorganization during infection (Bauer et al. 2015).

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 (Velandia-Romero et al. 2013; Bauer et al. 2014) or via the neuromuscular junctions (NMJs) where motor axons bifurcate in invaginations of the muscle surface (Watson et al. 1981; Lentz et al. 1982; Lewis et al. 2000; Burrage et al. 1985). Rabies virus infection propagates from one neuron to another one along the spinal cord to the brain before spreading to the salivary glands via cranial nerves. Virions are then excreted into saliva (Dierks et al. 1969) and transmitted to another host by bite.

RABV propagates in the host NS by transneural transfer to anatomically connected sites exclusively by the retrograde pathway exclusively in a retrograde direction (Kelly and Strick 2000; Gillet et al. 1986). The transport, estimated at 50–100 mm per day (Tsiang et al. 1991), is blocked by colchicine, which causes microtubule depolymerisation (Ceccaldi et al. 1989). The G protein homotrimer 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 (Mazarakis et al. 2001). Conversely, unlike G-expressing viruses, G-deficient rabies viruses are not transmitted trans-synaptically after being stereotaxically inoculated into the rat striatum (Etessami et al. 2000).

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 (Hotta et al. 2007; Lafon 2005a; Sissoeff et al. 2005; Lentz et al. 1988; Tuffereau et al. 2001; Langevin and Tuffereau 2002; Thoulouze et al. 1998; Langevin et al. 2002). Other components of the cell membrane, such as

gangliosides, may also participate in the entry of rabies virus (Superti et al. 1984). 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.

The scheme for virus entry and trafficking into the nervous system could be the following: Rabies virus particles, in saliva from rabid animals, is transferred by bites to the vicinity of NMJs and sensory termini. At the NMJs, free rabies virus particles bind to nAChR located on the top of junction folds, in area 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. 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 (Lafon 2005a).

After entry at the neuromuscular junction or passage through the synapse, via endocytosis in pits partially coated with clathrin 1 (Lewis et al. 1998, 2000) in an actin dependent manner (Piccinotti et al. 2013), RABV particles are transported retrogradely in axonal vesicles (Klingen et al. 2008) using the P75NTR transport machinery (Gluska et al. 2014). Once vesicles carrying viral particles have reached the cell body the NC is released into the cytoplasm by fusion of the virus envelope at low pH and the release of NC into the neuron cell body where replication can occur. It is not yet known whether NC release in the cytoplasm occurs directly from early endosome by fusion of the viral envelope with the membrane of the early endosome or from late endosomes by fusion of the vesicles containing NC with the membrane of the late endosome (Albertini et al. 2011). It is likely that RABV protein synthesis and viral particles assembly occur not only in cell bodies but also in dendrites, whereas axons are devoted to transport viral particles to the next order neuron.

Rabies virus infection forms cytoplasmic detergent resistant inclusions bodies located near the nucleus. These inclusions bodies contain RABV proteins (N and P, but no G) and viral RNAs and genome. The size of the inclusion bodies (3–5 μm), their composition, and the absence of surrounded membrane as shown by electronic microscopy suggest they correspond to the previously described Negri bodies, which appear in neurons in the course of RABV infection (Kristensson et al. 1996). They have been described as viral factories (Lahaye et al. 2009) and sites of viral replication which is consistent with the enrichment of rough endoplasmic reticulum, mitochondria (Menager et al. 2009) and sometimes the presence of viral particles (Miyamoto and Matsumoto 1965). Negri bodies structure is strictly organized with a nuclear core containing TLR3 surrounded by a corona composed with viral N and P proteins (Menager et al. 2009). In absence of TLR3, Negri bodies do not form, suggesting that Negri bodies and TLR3 entrapping have essential functions in rabies virus multiplication (Menager et al. 2009). Other cellular proteins, such as the cellular chaperonin CCT alpha and CCT gamma (Zhang et al. 2013, 2014) and HSP70 (Lahaye et al. 2012), are localized at the corona of the Negri body. Since the function of these chaperones is to protect the nascent polypeptide chain coming out from the ribosome and to facilitate protein folding, it is likely these cellular components facilitate rabies virus proteins production.

Rabies Virus Evasive Strategies

In the absence of post-exposure treatment, rabies is one of the very few human infections with nearly 100 % mortality rate (Warrell and Warrell 2004). 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 sneak into the nervous system despite the host immune responses and to preserve the integrity of neurons and those of the neuronal network. Protection of neuronal cell bodies from premature apoptosis and preservation of integrity of axons and dendrites at least during the period of time required for the virus to reach the brainstem and the salivary glands can be taken as a critical factor for rabies virulence (Lafon 2011). Rabies virus has evolved strategies to (1) avoid premature destruction of the infected neurons, (2) evade the innate immune response launched by the infected neurons, (3) minimize the neuroinflammatory reactions, (4) eliminate the protective T cells that migrate from the periphery into the infected nervous system and finally (4) trigger a centrally controlled immunosuppression (Fig. 2).

Rabies Virus Preserves the Integrity of the Infected Neurons

Apoptosis of infected neurons is not a prominent feature of natural rabies in humans (Juntrakul et al. 2005) nor in animal models infected peripherally with pathogenic strains of rabies virus (Guigoni and Coulon 2002; Yan et al. 2002; Baloul and Lafon

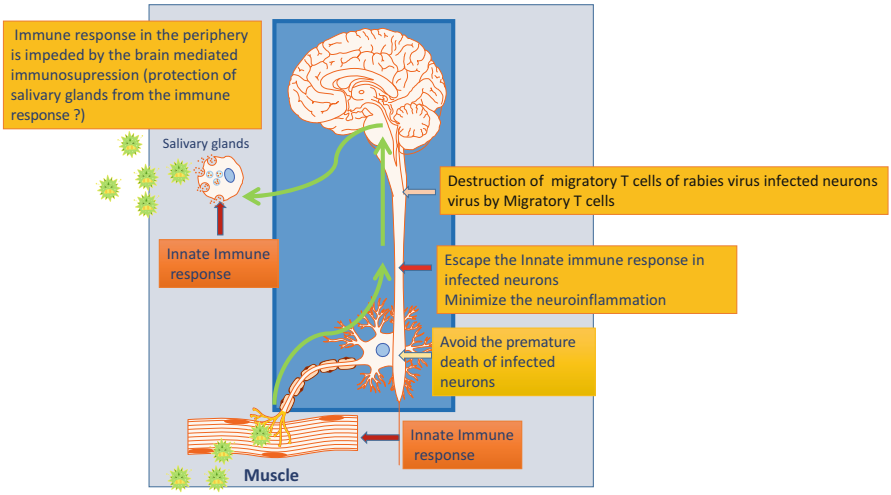

Fig. 2 Lafon rabies

2003). In a model of RABV in non-human primates 4 days after infection, infected motor neurons show no signs of degeneration with normal size, morphology and Nissl staining (Ugolini 2010). This feature is also observed in vitro, for example, in rat spinal motoneurons (Guigoni and Coulon 2002), gangliocytes of dorsal root ganglia (DRG) (Rossiter et al. 2009) or human neuroblastoma cells infected by neuronotropic virus strains (Prehaud et al. 2003). In contrast, attenuated rabies virus strains such as vaccine strains are strong inducer of apoptosis (Prehaud et al. 2003; Baloul and Lafon 2003; Thoulouze et al. 2003a). Induction of apoptosis by vaccine strain of rabies virus and release of immunogenic apoptotic bodies could contribute to the strong immunogenicity of live attenuated rabies virus vaccine (Megret et al. 2005). Rabies virus-induced apoptosis is inversely correlated with pathogenicity (Morimoto et al. 1999, 2000; Sarmento et al. 2005; Thoulouze et al. 2003b).

Protection against apoptosis could be determined by the level of expression of rabies virus G protein (Morimoto et al. 1999; Faber et al. 2002). The minimal G expression, the less apoptosis, and conversely, the more G expression, the more apoptosis. However the nature of the G-protein is also important since in a system of maximal expression of viral protein the replacement of a pro-apoptotic G gene by a non-apoptotic G gene was sufficient to prevent destruction of the infected cells by apoptosis (Prehaud et al. 2003). Moreover, the cytoplasmic domain of the protein is a critical factor in controlling apoptosis of the infected neurons or promoting prolonged survival instead, two features controlled by the amino acid sequence of the G protein cytoplasmic and the nature of the cellular partners the cytoplasmic domain interacts with (Prehaud et al. 2010). Other cellular factors could also control apoptosis induction upon rabies virus infection, since neurons of 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 (Guigoni and Coulon 2002; Reid and Jackson 2001; Theerasurakarn and Ubol 1998; Ubol et al. 2005, 2006). Late in the infection, neuronal destruction can be observed with degeneration, beadings, and demyelination of processes of infected neurons (Li et al. 2005) and peripheral nerve dysfunction occurs.

Rabies Virus Evades Interferon and Inflammatory Responses

Rabies virus triggers a RIG-I mediated innate immune response in infected cells (Hornung et al. 2006) by detecting the 5′ tri phosphate base pairing of the viral genome (Pichlmair et al. 2006). After infection, human neurons can mount a classical primary IFN response (activation of IRF3 and NF-kappa B), as well as a secondary IFN response (activation of STATs and IRF7), leading to the production of cytokines (IL-6, TNF-alpha) and chemokines (CXCL10 and CCL5) (Chopy et al. 2011a, b; Prehaud et al. 2005).

In vivo rabies virus infection within the spinal cord and the brain is accompanied by an innate immune response characterized by a Type I interferon response as well

as chemoattractive and inflammatory cytokine responses (Camelo et al. 2000; Baloul and Lafon 2003; Marquette et al. 1996; Wang et al. 2005). The intensity of the host response seems to be modulated according to the pathogenicity of the virus (Wang et al. 2005). The more pathogenic, the more limited the innate immune response (Masatani et al. 2013). Comparison of the inflammatory reaction triggered by RABV strains of various degree of pathogenicity indicates that the more pathogenic strains trigger weaker inflammatory responses (Baloul and Lafon 2003; Wang et al. 2005; Hicks et al. 2009; Laothamatas et al. 2008). Inflammation has been clearly shown to be beneficial for the clearance of the virus (Hooper et al. 1998; Niu et al. 2011). It is likely that this low inflammatory reaction in the infected NS contributes to keeping intact the BBB (Chai et al. 2014, 2015), a condition that correlates with RABV pathogenicity, with non-pathogenic RABV strains triggering a transient opening of the BBB, but not pathogenic strains (Phares et al. 2006; Roy et al. 2007). It has been shown in vitro that the P and the N protein of rabies virus interfere with type I IFN production (Masatani et al. 2010b, 2013; Vidy et al. 2005, 2007; Blondel et al. 2002; Brzozka et al. 2006), which has the capacity to decrease the number of virus particles at least in the muscle at the site of entry (Chopy et al. 2011b). Nevertheless, in vivo, during infection of the brain by rabies virus causing acute encephalitis sustained production of IFN-β mRNA could still be detected in the spinal cord and brain of mice (Lafon et al. 2008; Li et al. 2012; Chopy et al. 2011a, b). This may suggest that evasion of the IFN response by the infected neurons in vivo is not as strict as described in vitro. Alternatively, N and P proteins expressed by the infected neurons have indeed the capacity to counteract IFN production and IFN signalling, allowing the infection to proceed in the entire neuronal network, whereas the control exerted by the P and the N protein cannot function in non-infected glial cells, such as microglia and astrocytes which have the capacity to produce IFN, explaining why IFN can still be detected in the rabies virus infected brain (Chopy et al. 2011a). The production of IFN by the rabies virus infected brain parenchyma could stimulate the expression of IFN dependent molecules such as B7-H1, contributing to the evasive strategy of rabies (see below).

Rabies Virus Provokes the Killing of Migratory T Cells

In the periphery, injection of rabies virus into 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 are similar in mice infected either with pathogenic or attenuated strain of viruses (Camelo et al. 2000; Irwin et al. 1999; Roy et al. 2007; Roy and Hooper 2007). Rabies virus infection triggers the appearance of activated lymphocytes (CD69+) expressing Collapsin Response Mediator Protein 2 (CRMP2), a marker of cell polarization and migration (Vuaillat et al. 2008). Attracted by the gradient of chemokines and inflammatory mediators triggered by the infection of the nervous system (Faber et al. 2005a), lymphocytes migrate into the infected nervous system (Camelo et al. 2000; Baloul et al. 2004;

Lafon 2004). Migration of T cells is observed in mouse model and also in human rabies (Mitrabhakdi et al. 2005; Kojima et al. 2009; Baloul and Lafon 2003; Rossiter et al. 2009; Baloul et al. 2004; Hemachudha et al. 2005; Tobiume et al. 2009; Lafon 2005b) and the severity of rabies virus infection was inversely correlated with the number of CD3+ and CD8+ T cells in the central nervous system. Clearance of the infection by the migratory T cells was inefficient, because migratory T cells were destroyed by an apoptosis mechanism resulting of the upregulation of immunosubversive molecules, FasL and B7H1 by the infected neurons (Lafon et al. 2008; Baloul et al. 2004). In mice lacking a functional FasL or B7-H1, there was less T cell apoptosis in the NS than in control mice. Remarkably, RABV morbidity and mortality were reduced in these mice (Lafon et al. 2008; Baloul et al. 2004).

Thus, despite the triggering of a classical adaptive immune response in the periphery and the infiltration of the lymphocytes into the infected NS, the protection, which could have been conferred in the NS by this immune response, is drastically impeded by RABV infection.

Rabies Virus Infection Triggers a Neuro-Mediated Immune Unresponsiveness

The dampening of immune protection already triggered by RABV is completed by a neuro-mediated immune unresponsiveness (Camelo et al. 2001; Torres-Anjel et al. 1988; Wiktor et al. 1977a, b; Hirai et al. 1992; Kasempimolporn et al. 1997, 2001; Tshikuka et al. 1992; Perry et al. 1990) characterized by the impairment of T cells functions with an alteration of cytokine pattern, an inhibition of T cells proliferation and the destruction of immune cells without modifying immune cells proportion (CD4/CD8 ratio constant) in the lymphoid organs (Perry et al. 1990). This leads to the atrophy of the spleen and the thymus of RABV infected mammals. TNF-alpha receptor has been found to play a role in RABV immune-unresponsiveness, since immune cells lacking the TNF alpha p55 receptor were less immunosuppressed compared to the wild type (Camelo et al. 2000). Most importantly, infection of the brain is required since immune-unresponsiveness does not occur after the infection of the NS with an abortive RABV strain, which infects the spinal cord only (Camelo et al. 2001). This suggests that the property of the NS that centrally controls the immune response in the periphery might be triggered (Tracey 2002). NS modulates the immune functions through two main immune-neuroendocrine pathways: the hypothalamo-pituitary (HPA) axis and the autonomous NS (ANS) composed of sympathic and parasympathic nerves fibres (Johnston and Webster 2009). The homeostatic reflex is activated after the brain senses the presence of an excess of inflammatory cytokines such as TNF-alpha, IL-1β or IL-6 in the periphery, by neuronal (mainly through local afferent fibres of the vagus nerve) and by humoral pathway (Johnston and Webster 2009). This input is processed by the NS in frontal, hypothalamic and brainstem centres.

This general immune-unresponsiveness controlled by the NS may be advantageous for RABV propagation since a mouse strain having a less efficient HPA axis is less susceptible to rabies (Roy et al. 2007). This central immunosuppression may limit peripheral control of infection in the muscle or the salivary glands (see Fig. 2).

Thus, RABV infection not only actively inhibits the T cell response and inflammation in the NS by upregulating B7-H1 and FasL molecules, but also benefits from the intrinsic capacity of NS to trigger central immunosuppression in order to maintain whole body homeostasis. 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 combined sometimes with sterilization of feral dogs have been successful in countries with enzootic canine rabies (Dixon 2006). To be efficient, such preventive measures have to be constantly applied and repeated due both to the poor anamestic response of animal rabies vaccines and the turn-over of stray dog populations. Vaccines used in these campaigns are inactivated rabies vaccines injected by intramuscular or intradermal routes. Classical mass vaccination of dogs has been highly successful in Latin America (Schneider et al. 2011) indicating that eradication of dog rabies in Latin America could be a target at our reach.

Vaccines for oral administration have been engineered as an alternative to vaccine injection (Kieny et al. 1984; Wiktor et al. 1992; Schneider et al. 1988; Schumacher et al. 1993). Wide-scale rabies vaccination campaign of wildlife animals have been undertaken in Western Europe (Germany, Switzerland, France, Belgium, Italy) in the 1980s (Schneider et al. 1988; Wandeler et al. 1988; Brochier and Pastoret 1993; Cliquet et al. 2006; Pastoret et al. 1988). These campaigns consisted in baiting the red foxes, *Vulpes vulpes*. The vaccine were either recombinant vaccinia virus expressing rabies virus G protein (Kieny et al. 1984) or attenuated rabies virus variants, such as SADB19 (Conzelmann et al. 1990) or SAG-2 (Schumacher et al. 1993). The European rabies vaccination campaigns proved tremendously successful and constituted a paradigm for wildlife vaccination programs (Mahl et al. 2014). France was declared free of rabies in 2002. In North America, the success has been less dramatic, due to the prevalence of several species capable of transmitting rabies. Some of which, such as skunks (*Mephitis* sp.), were resistant to recombinant vaccines (Hanlon et al. 1998, 2002; Vos et al. 2002). Attempts in dogs have been so far disappointing (Frontini et al. 1992; Matter et al. 1995; Orciari et al. 2001; Rupprecht et al. 2005), however, new formula of bait formulas, adapted to stray dogs are currently in investigation (Darkaoui et al. 2014).

Post-exposure Vaccination

Treatment of rabies consists of series of injections of vaccine, combined with rabies immunoglobulin (RIG) given after the person has been exposed to rabies. The post-exposure treatment (PET) validated by the WHO Expert Committee uses four or five vaccine injections given either intramuscularly or intradermally altogether with instillation of RIG at the wound sites in case of severe exposure or when animal is not captured for rabies confirmation (category III exposure, WHO, 2005). PET against rabies could be considered like a race between the virus and the patient immunity against rabies. PET failures may occur when PET is begun late after the exposure.

Rabies vaccination triggers not only rabies virus neutralizing antibodies but also long lived antibody-secreting plasma cells, memory B and T cells. Comparison of the immunopathological events that participate in virus clearance from the central nervous system in animal models have identified the production of antibodies (Hooper et al. 1998; Miller et al. 1978) associated with a CD4+ T cell responses (Celis et al. 1990), 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 (Galelli et al. 2000; Perry and Lodmell 1991) and thus can initiate an immuno-pathological reaction. So far assays measuring rabies virus specific antibodies either by immunoassay (Feyssaguet et al. 2007) or by neutralization test with the RFFIT technique (Smith et al. 1973; Mannen et al. 1987) are the standard to assess immunoconversion after rabies vaccination. Test of cellular immunity measuring the T cell response could provide a more accurate knowledge of the immune status of the vaccinees (Moore et al. 2006).

Rabies disproportionally affects children in poor countries. Preventive childhood vaccination to rabies has been shown to be efficient and safe for both young children (12–18 month) (Malerczyk et al. 2013) and school children (Kamoltham et al. 2007). Rabies vaccine can be co-administrated with DT and inactivated polio vaccines without causing interferences (Pengsaa et al. 2009). So far Philippines is the only country that has introduced wide scale pre-exposure vaccination for children in highly endemic area (Dodet et al. 2014).

Conclusions

Rabies remains an important public health problem in the world due to uncontrolled enzootic rabies, lack of vaccines and information. Dogs are the main vector of rabies; efforts are currently made to make oral baiting vaccination of stray dogs. An important challenge would be the oral vaccination of stray dogs. Efficacy of PET requires population information, prompt vaccination and availability of RIG. Half of the victims are children; pre-exposure vaccination of young individuals should be considered in an attempt to improve the global health of mankind.

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Neurotropic Coronavirus Infections

Stanley Perlman and D. Lori Wheeler

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 lineage a betacoronavirus (de Groot et al. 2013) 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, HCoV-NL63, and HCoV-HKU1) to severe acute respiratory syndrome (SARS) and the Middle East Respiratory Syndrome (MERS) (Ksiazek et al. 2003; Peiris et al. 2003a; Zaki et al. 2012), 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 (Compton et al. 1993; Homberger et al. 1992). 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 concomitant development of demyelination to rapidly fatal encephalitis. Thus, the neurotropic strains of MHV have proved to be useful systems in which to

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study processes of virus- and immune-mediated demyelination, virus clearance, and/or persistence in the CNS and the mechanisms of virus evasion of the immune system.

Structure

Coronaviruses are large (80–120 nM) pseudospherical particles that contain a long, helical nucleocapsid surrounded by an envelope bearing both virus- and host-derived glycoproteins (reviewed in Masters and Perlman 2013). The largest among known RNA viruses, the genome of coronaviruses consists of a single-stranded, positive-sense, 5′-capped, and polyadenylated RNA of 30–32 kilobases. 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 emagluttinin-esterase protein (HE), spike glycoprotein (S), envelope protein (E), transmembrane glycoprotein (M), and nucleocapsid protein (N), as well as additional, group-specific accessory proteins (reviewed in Masters 2006). The three MHV ORFs encoding accessory proteins are interspersed within the structural genes and include ORF2a, ORF4, and ORF5a (encode ns2, ns4, and ns5a respectively) (Lai and Cavanagh 1997; Masters and Perlman 2013) (Fig. 1b). 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 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 ORF3a, 7a, 7b proteins, have turned out to be structural (Huang et al. 2006a; Ito et al. 2005; Schaecher et al. 2007).

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 (Fig. 1a). 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. 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 (Masters 2006; Masters and Perlman 2013; Shi and Lai 2005). As the most abundant of all structural proteins in the virion, the M protein is known to play a key role in assembly and particle formation through specific interaction with S (Godeke et al. 2000), N (Hurst et al. 2005), and possibly (Baudoux et al. 1998) E proteins. Even though it is relatively underrepresented in the mature virus particle (Yu et al. 1994), the E protein is also believed to play a role in virus assembly, but is not absolutely required for this process (Kuo and Masters 2003). In addition, the E protein forms pentameric bundles within lipid bi-layers that have cation-selective ion channel activity (Wilson et al. 2006). While the function of the HE is not fully understood, this protein possesses esterase activity (Vlasak et al. 1988a), and is not required

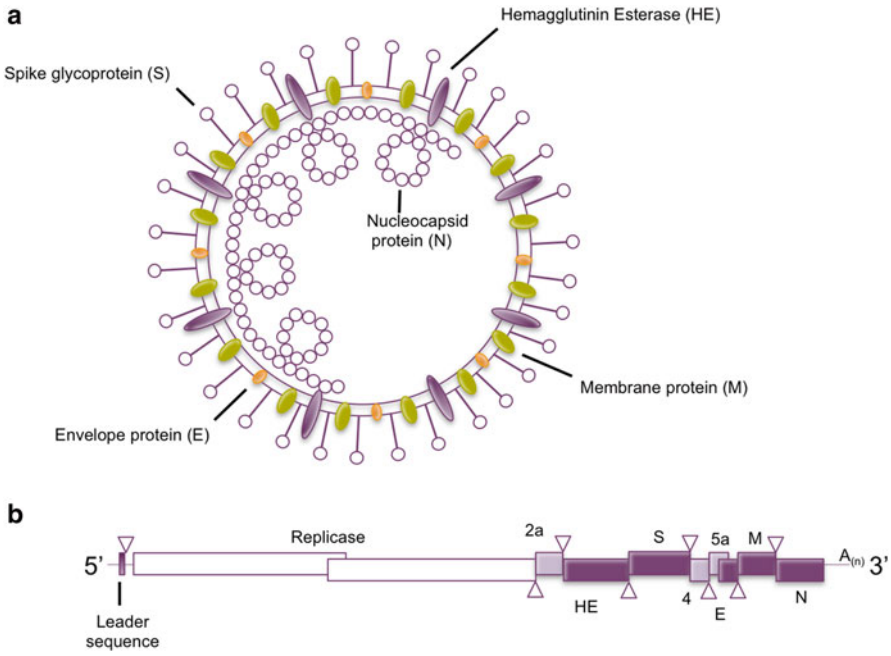

Fig. 1 (**a**) Structure of the MHV coronavirus virion depicting structural proteins. (**b**) Schematic representation of the genomic organization of mouse hepatitis virus. Replicase genes, accessory genes, and structural genes are indicated by *open*, *shaded,* and *solid rectangles*, respectively. *Open triangles* depict the location of transcription-regulating sequences (TRS)

for virus replication in tissue culture cells (de Haan et al. 2002; Schwarz et al. 1990). However, recent evidence suggests that the HE may enhance infectivity and spread of coronaviruses within certain tissues, perhaps by serving as a second receptor-binding protein or by modulating virus release (Kazi et al. 2005).

The coronavirus accessory proteins (Fig. 1b) are not individually required for growth in tissue culture cells (Vlasak et al. 1988a). However, deletion of all accessory genes from MHV attenuates the virus in vitro and in vivo (de Haan et al. 2002). Although deletion of individual 3′ accessory proteins has little effect on virus replication in tissue culture, their conservation within the species suggests that the accessory proteins play important roles in modulating the host immune response or general host cellular processes in vivo. With regard to pathogenesis, some accessory proteins appear dispensable while others (alone or in combination) are critical for replication in the intact animal. For example, deletion of the ORF4 gene in MHV–JHM does not affect lethality (Ontiveros et al. 2003), while mutation or deletion of the ORF2a gene did not affect growth in tissue culture cells but attenuated replication in mice (Schwarz et al. 1990; Sperry et al. 2005). A recent study has shown that ORF2a has 2′,5′-phosphodiesterase activity which prevents the activation of cellular RNase L, thereby preventing the degradation of viral RNA (Zhao et al. 2012). This antagonism of the

interferon response promotes hepatitis in MHV-A59 infected mice, but does not seem to affect brain pathology (Zhao et al. 2012). As the protein ns4 of MHV–JHM has not been detected in infected cells, its function remains unknown. The ns5 protein also has unknown functions but may antagonize the interferon response (Koetzner et al. 2010).

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 (reviewed in Masters 2006). MHV infection of host cells involves specific interaction of the S glycoprotein with a proteinaceous host cell receptor, carcinoembryonic antigen cell adhesion molecule (CEACAM-1a) (Williams et al. 1991); 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 (Chen et al. 1995), only CEACAM-1a has been definitely proven to be the receptor used in mice (Hemmila et al. 2004). The S protein consists of two functional domains: 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 (Kubo et al. 1994; Suzuki and Taguchi 1996; Taguchi et al. 1995), but the precise location of the fusion domain within the S protein remains controversial. 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 (Frana et al. 1985). However, for some coronaviruses, including MHV-2 and SARS-CoV, virion S protein is not cleaved during virus egress, but is cleaved in low pH endosomes by cathepsin (Huang et al. 2006b). 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. 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. Alternatively, virus can be taken up into endocytic vesicles where S protein is cleaved, 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 (Kooi et al. 1991; Nash and Buchmeier 1997). The replication life cycle of MHV, like all coronaviruses, is believed to take place entirely within the host cell cytoplasm (reviewed in Masters 2006).

For HCoV-OC43, cellular binding and entry involves ligation of the S protein to sialidated carbohydrate moieties on the surface of cells (Vlasak et al. 1988b), while for SARS-CoV, entry requires binding to the angiotensin-converting enzyme (ACE2) (Li

et al. 2003). As with MHV, entry of SARS-CoV, MERS, or HCoV-OC43 involves conformational changes in the S protein, cleavage by one of several identified proteases and functional activation of the fusogenic S2 domain. Fusion of SARS-CoV and MERS has been more characterized than fusion of MHV (reviewed in Millet and Whittaker 2014).

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 (Fig. 2). The translation of the viral RNA genome results in the generation of

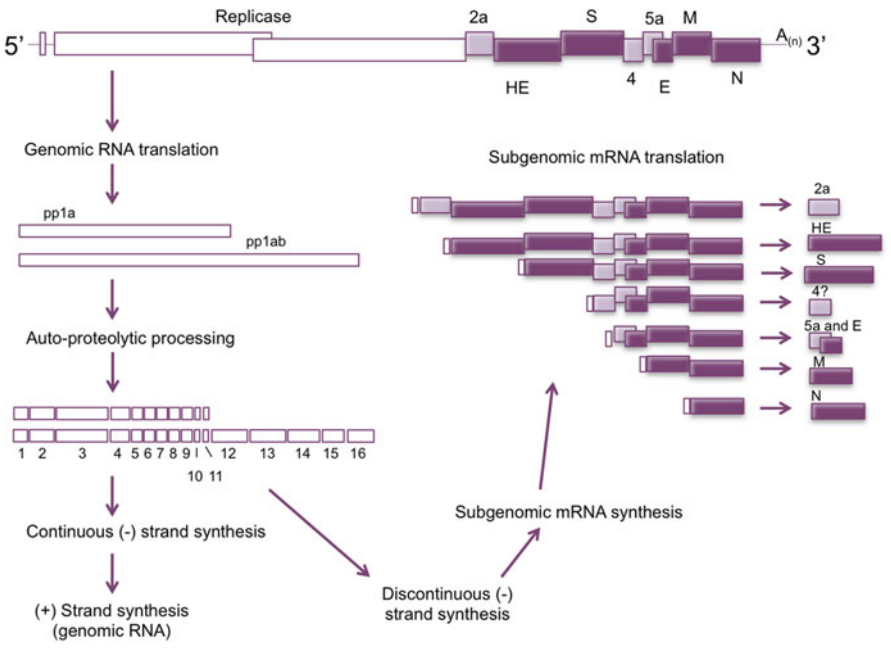

Fig. 2 Overview of coronavirus replication. Upon uncoating, virus RNA is directly translated via host cell machinery into two large polyproteins, pp1a and pp1ab. Both polyproteins undergo autoproteolytic processing to generate nonstructural proteins (nsp) of the replicase gene complex (nsp1–nsp16). The replicase proteins mediate continuous or discontinuous replication of negative-strand RNA templates. Genome-length negative strands serve as template for the replication of the 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. E, M, and S proteins 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

two large polyproteins, 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 (Brierley et al. 1989). 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 (Ziebuhr et al. 2000). Sixteen total proteins are generated from the two large polyproteins (nsp1-16), eight of which are predicted to have enzymatic activity (reviewed in Weiss and Leibowitz 2011) Interestingly, while many of the described functions of 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 (Snijder et al. 2003). For example, the viral 2′-*O*-ribose methyltransferase is important in immune evasion (Daffis et al. 2010).

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, and within the genome (Lai and Cavanagh 1997; Masters 2006; Sawicki et al. 2007) (see below). 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, Fig. 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, *cis*-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 (Fig. 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 (Sawicki et al. 2007). 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 subgenomic-length mRNA synthesis. The subgenomic mRNAs are subsequently translated via host cell machinery into structural and nonstructural 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 (Vennema et al. 1996). The S protein also co-localizes at these sites of E and M accumulation and has a more disperse distribution throughout the cell. Full-length genomic RNA is encapsidated by the N protein via specific binding between N or M and a packaging signal present on viral genomic, but not subgenomic RNA, located in gene 1 (Molenkamp and Spaan 1997; Narayanan et al. 2003). Deletion of this packaging signal results in subgenomic RNA incorporation into the virion, and loss of selective advantage for the virus (Kuo and Masters 2013). Virus assembly, thought to be driven by both host- and virus-specific factors, occurs in the ERGIC, 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.

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 (Compton et al. 1993). 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. Neurotropic strains of MHV, such as MHV–JHM, do not spread to uninfected animals, even those within the same cage, even though these strains are highly virulent. For respiratory coronaviruses, including HCoV-OC43, HCoV-229E, MERS-CoV, and SARS-CoV, virus is spread via large droplets and contact. Additionally, SARs-CoV is detected in the feces and may have spread via the fecal-oral route during the 2002–2003 epidemic (Peiris et al. 2003b). 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, but is most important during superspreading events and when patients are undergoing aerosol-generating procedures. SARS-CoV was almost always transmitted from patients after they developed clinical signs (Lipsitch et al. 2003; Peiris et al. 2004).

Interspecies Transmission

Like all RNA viruses, the coronavirus RNA-dependent RNA polymerases lack proofreading activity; therefore, these viruses exist as a quasi-species, 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 (Baric et al. 1997, 1999; Schickli et al. 1997, 2004; Thackray and Holmes 2004). 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 (Guan et al. 2003; Lau et al. 2005; Li et al. 2005). Similarly, MERS-CoV is thought to have originated in bats (Wang et al. 2014) with camels as an intermediate host (Hemida et al. 2014). In fact, MERS-CoV can infect cells from a wide array of animal species (Barlan et al. 2014; Raj et al. 2013). 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 (Vijgen et al. 2005). 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 have 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 (Bailey et al. 1949; Cheever et al. 1949). Serial passage through suckling mouse brains resulted in the selection of viruses that caused rapid and fatal encephalitis in adult mice (Weiner 1973; Weiner et al. 1973). However, this pool also contained less virulent viruses, that were isolated as described below. MHV strain A59 is a naturally occurring variant of MHV that was isolated from a mouse with severe hepatitis (Manaker et al. 1961). MHV–JHM and A59 are very distinct from one another in their relative infectivity, spread, cell tropism, and neurovirulence. While A59 is generally hepatotropic, intracranial, 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 (Lavi et al. 1984b, 1986). On the other hand, intracranial or intranasal inoculation of mice with the most neurovirulent strains of MHV–JHM generally results in rapid and fatal encephalitis. Attenuated variants of MHV–JHM have been selected from these initial pools by limiting dilution assays (with identification by plaque

size), by chemical mutagenesis, by exposure to neutralizing antibodies and by cold sensitivity. These attenuated variants are commonly used to study mechanisms of virus persistence and virus- and immune-mediated demyelination. Treatment of viral stocks with the anti-S protein neutralizing monoclonal antibody, J2.2, resulted in one of the most commonly studied attenuated variants, termed 2.2-V-1 (alternatively, rJ2.2) (Fleming et al. 1986). Unlike the parental strain of MHV–JHM, 2.2-V-1 preferentially infects oligodendrocytes and minimally infects neurons. Because this variant is relatively neuroattenuated, infected mice uniformly survive the acute infection but remain persistently infected. The disease course and the nature of the host immune response make infection with 2.2-V-1 particularly useful for examining the host response to persistent virus infection of the CNS, and for studying virus-induced immune-mediated pathology.

Initial studies with MHV–JHM suggested that demyelination was largely virus-mediated (Lampert et al. 1973; Weiner 1973). However, subsequent studies showed 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 (Houtman and Fleming 1996a; Wang et al. 1990; Wu and Perlman 1999). Moreover, during the course of viral infection, demyelination occurs in immunocompetent mice and in SCID or $RAG^{-/-}$ mice reconstituted with immune cells. 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 surface expression levels of CEACAM-1a (Morales et al. 2001). In addition, MHV replicates efficiently in the CNS of mice despite extremely low levels of CEACAM-1a mRNA and protein expression in this tissue (Godfraind et al. 1995, 1997; Nakagaki et al. 2005). These observations suggest that virus or host cellular factors other than CEACAM-1a also contribute to productive infection, and other data indicates that MHV can spread in CNS-derived cells independent of CEACAM-1a expression (Gallagher et al. 1992; Nash and Buchmeier 1996). 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. In fact, virus spreads in a receptor-independent manner in the CNS of CEACAM1a$^{-/-}$ knockout mice (Miura et al. 2008).

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 (Fleming et al. 1986). As discussed below, the infection of neurons and astrocytes may directly contribute to virus persistence in the CNS, as these cell types do not express similar levels of major histocompatibility complex (MHC) class I or class II antigen as compared to other tissues (Aloisi et al. 2000; Fabry et al. 1994; Hickey 2001).

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 (Wang et al. 1992b). 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) (Barnett et al. 1993; Lavi et al. 1988). The virus disseminates via retrograde spread along axonal tracts to the spinal cord (Barnett et al. 1993). 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 (Sun and Perlman 1995). Astrocytes are intimately associated with neurons in the gray matter and with oligodendrocytes and neurons in the white matter. 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 (Lampert et al. 1973; Weiner 1973). 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. Widespread apoptosis in CNS-resident cells is not generally observed after acute MHV–JHM-induced encephalitis (Phillips et al. 2002; Wu and Perlman 1999). However, the extent to which direct virus destruction of infected cells contributes to the death of the mouse is unknown, and recent data suggest that this disease, like the chronic demyelinating disease, may also be partly immune-mediated.

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 the 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 (Fleming et al. 1986). The virus replicates at high titers during the acute phase with replication peaking at approximately 5 days post-infection (p.i.). In mice that survive the acute disease, the virus cannot be recovered from mice beyond approximately 2 weeks p.i., but virus antigen and RNA can be identified in the CNS out to 1 year post infection (Adami et al. 1995; de Aquino et al. 2014; Lavi et al. 1984a; Rowe et al. 1997; Zhao et al. 2009). 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 (Zhou et al. 2003). Interestingly, the infiltration of inflammatory cells coincides with the onset of clinical disease. Chronic inflammatory changes in the spinal cord of mice likely result in ongoing clinical disease and the progression of demyelination.

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, oligodendroglia, and neurons) do not express high levels of MHC class I or MHC class II antigen in the absence of injury (Aloisi et al. 2000; Fabry et al. 1994; Hickey 2001; Malone et al. 2008). 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 the analyses of MHV-infected, antibody-protected suckling mice (model described below) (Perlman and Pewe 1998). Second, the brain is a tissue subject to minimal immune surveillance (Hickey 2001; Ransohoff and Engelhardt 2012; Ransohoff et al. 2003), 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 of these cells results in diminished ability to activate virus-specific CD8 T-cells (Turner et al. 2004; Zhou et al. 2003). Interestingly, CNS infection results in downregulation of CEACAM-1a receptor expression on macrophages and microglia (Ramakrishna et al. 2004). 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 (Bergmann et al. 1999).

Other Experimental Models of MHV Infection

In addition to infecting mice, MHV is also capable of infecting and replicating in the CNS of rats (Barac-Latas et al. 1997; Nagashima et al. 1978; Sorensen et al. 1980; Watanabe et al. 1987), hamsters (Cheever et al. 1949) and nonhuman primates (Murray et al. 1992). 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 in rats. Infection generally results in fatal encephalitis in both suckling Lewis rats and suckling outbred animals; however, a percentage of rats 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, brainstem, 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 post-infection. 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 (Watanabe et al. 1983). 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 (Schwender et al. 1991) and little evidence for virus persistence or continued replication (Watanabe et al. 1987). 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 MHV S Protein in Pathogenesis

It is well established that the S protein of coronaviruses dictates species specificity and cell tropism. Additionally, a large body of evidence also suggests that the S protein influences pathogenesis and neurovirulence of MHV, presumably by altering cellular tropism (Fleming et al. 1986; Parker et al. 1989; Phillips et al. 2002) or efficiency of spread (Ontiveros et al. 2003; Phillips et al. 2002) 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 (MacNamara et al. 2005; Phillips et al. 2002; Rempel et al. 2004). 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 mutation in the S glycoprotein (Fleming et al. 1986). A recombinant variant of MHV-A59 was engineered to express the MHV–JHM S glycoprotein (Navas and Weiss 2003; Phillips et al. 1999). This recombinant virus was nearly as virulent as parental MHV–JHM, manifested by increased infection of neurons, 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 Bergmann et al. 2006). 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 (Lane et al. 2000; Trifilo et al. 2003; Zhou et al. 2002).

Important pro-inflammatory cytokines that are detected early in the infected CNS include IL-1α, IL-1β, IL-6, and TNF (Rempel et al. 2004). Although IL-1α, IL-1β, and 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 in modulating infection remains uncertain (Pewe et al. 2002; Stohlman et al. 1995). Depletion of TNF with neutralizing antibody does not change the inflammatory response, diminish virus clearance, or affect the demyelinating process (Stohlman et al. 1995). In addition, viral replication kinetics, expression of pro-inflammatory cytokines, and histopathological changes were similar in TNF-deficient mice and wild-type mice (Shirato et al. 2008).

The type I interferons, IFN-alpha and IFN-beta, are known to be critically important for establishing an antiviral state in virus-infected tissues. IFN-α/β has been shown to modestly inhibit MHV replication and infectivity in vitro (Pewe et al. 2002; Taguchi et al. 1985; Ye et al. 2007). However, several studies demonstrate that MHV infection does not trigger production of IFN-β from the most infected cells (Garlinghouse et al. 1984; Pewe et al. 2005; Versteeg et al. 2007; Zhou and Perlman 2007). However, MHV-A59 induces IFN-α at high levels in plasmacytoid-dendritic cells after infection with MHV-A59 (Cervantes-Barragan et al. 2007) and MHV–JHM induces IFN-β by an MDA5-dependent pathway in macrophages and microglia in the brain of infected animals (Roth-Cross et al. 2008). Expression of type I interferon by plasmacytoid-dendritic cells (pDC) is likely important for innate control of coronavirus infection. In a recent study, mice genetically engineered to lack pDCs, had increased viral load and viral spread in MHV-A59 infected mice (Cervantes-Barragan et al. 2012). Ifit2, an IFN-stimulated gene, seems to be important as a regulator of IFN-α/β expression (Butchi et al. 2014). 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 in the CNS for prolonged periods of time, but express 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 (Iacono et al. 2006; Phillips et al. 2002; Rempel et al. 2004). These differences are also consistent with the notion that an effective adaptive immune response is critical for downregulating a prolonged and eventually deleterious innate immune response.

Important chemokines detected early in the CNS include MIP-2, CCL2, CCL3, CCL4, and CXCL10 (Lane et al. 1998; Rempel et al. 2004; Trifilo et al. 2004). 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 microglia and triggering the proliferation of astrocytes within the brain parenchyma. Levels of chemokines must be carefully balanced to effect optimal virus clearance and survival. In one study, constitutive transgenic expression of CCL2 in the CNS resulted in macrophage accumulation adjacent to the brain parenchyma. The presence of these partially primed cells might be expected to be protective upon subsequent infection with MHV. On the contrary, upon infection, CCL2 overexpression induced the accumulation of large numbers of alternatively activated macrophages, which were characterized by elevated YM-1 expression, and were immunosuppressive. The net result was decreased virus clearance and diminished survival (Trujillo et al. 2013).

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 (Liu et al. 2000, 2001). Moreover, infection of $RAG1^{-\backslash-}$ mice (which lack B- and T-cells) with a recombinant MHV engineered to express CXCL10 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 (Trifilo et al. 2004). In addition to the chemokines mentioned above, ELR^+ chemokines, CXCL1, CXCL2, and CXCL5 have a protective role within the acutely infected brain. Blocking with CXCR2 antibody (the receptor for ELR^+ cytokines), reduced the infiltration of neutrophils and virus-specific T-cells into the brain resulting in death from uncontrolled viral infection (Hosking et al. 2009). 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 3–5 days post-infection, there is a massive infiltration of macrophages, neutrophils, and NK cells (Bergmann et al. 1999; Zhou et al. 2003). Depletion of neutrophils with anti-Ly6C/G (Gr-1) antibody results in diminished BBB breakdown and enhanced virus replication (Zhou et al. 2003). These results are not completely straightforward, because 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 (Xue et al. 1999). 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 (Fig. 3). NK cells are detected at early times after infection as part of the initial response (Bukowski et al. 1983; Williamson and Stohlman 1990). While NK cells are known to secrete significant amounts of IFN-γ in response to virus infection (Biron and Brossay 2001), there is little evidence that their presence is important in the host response to MHV in immunocompetent mice (Bergmann et al. 1999; Daniels et al. 2001; Marten et al. 2000b; Williamson et al. 1991). The possible exception to this may be the protective role of NK cells 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). Both inflammatory cells, such as neutrophils, and CNS-resident cells secrete MMPs. 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 (Goetzl et al. 1996; Yong et al. 2001). 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 (Zhou et al. 2002, 2005). This is similar to the array of MMPs that are expressed during autoimmune and autoinflammatory processes such as experimental autoimmune encephalomyelitis (EAE) (Yong et al. 2001). 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 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 (Zhou et al. 2005). 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 overexuberant inflammation. Future studies are required to precisely define the roles of these pro- and anti-inflammatory mediators in the MHV-infected CNS.

Innate Immune 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 RAG1-deficient mice. 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 (Houtman and Fleming 1996a; Wang et al. 1990; Wu and Perlman 1999). 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. Transferred hyperimmune MHV–JHM-specific serum is able to mediate demyelination in MHV–JHM-infected RAG1$^{-/-}$ mice although not as robustly as virus-specific T-cells. 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 (Houtman and Fleming 1996a). 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. For example, in one study, Kim (Kim and Perlman 2005a) used targeted recombination to generate a virus recombinant version of 2.2-V-1 that expressed the macrophage chemoattractant MCP1/CCL2. Virus-derived CCL2, in the absence of any antiviral T-cells or antibody was sufficient to induce demyelination in the spinal cord. 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 (Dandekar et al. 2001). Of note, similar findings are observed in the CNS of MS patients and contribute to long-term, irreversible disability (Trapp et al. 1998). The cells and effector molecules that have been identified as playing a critical role in virus-induced demyelination are summarized in Fig. 3. Activated macrophages/microglia are a common feature of MHV-induced, immune-mediated demyelination as well as active lesions in patients with MS, suggesting that these cells may actually serve as the final effectors of this process. Macrophages enter the CNS as inflammatory monocytes and mature in situ. Maturation is characterized by the downregulation of Ly6C and the upregulation of CD11c, a molecule usually used to define dendritic cells. In this instance, it is not clear whether CD11c expression is involved in antigen presentation or if CD11c is only a maturation marker. In support of the latter, both CD11c^{+} and CD11c^{-} cells were found in proximity to areas of demyelination (Templeton et al. 2008).

As mentioned above, CXCL10 is another important chemokine in MHV-infected animals. 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 (Liu et al. 2001). The ELR^{+} chemokines are protective in both the acute and chronic phase. Through signaling of CXCR2, these cytokines protect oligodendrocytes from apoptosis and decrease demyelination as shown via CXCR2 neutralization (Hosking et al. 2010).

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 overexpression 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. A future research goal will be to determine how to maximize virus clearance without also causing myelin/oligodendrocyte destruction.

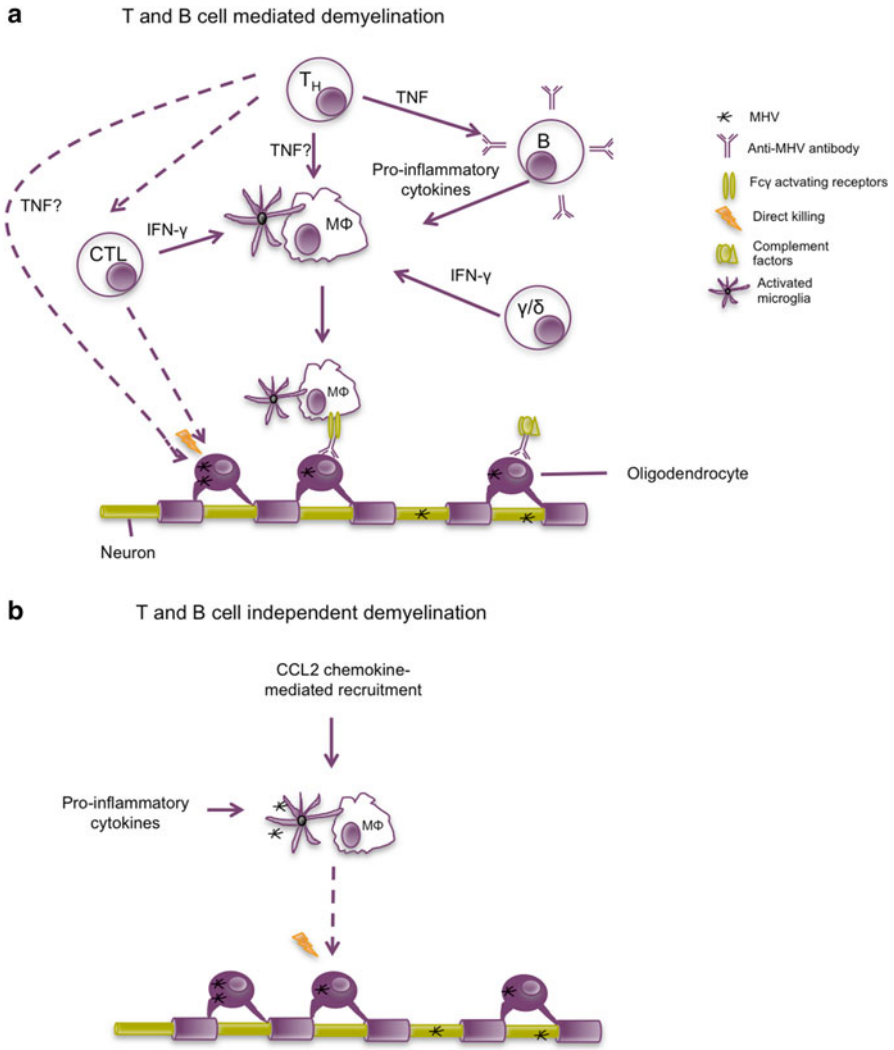

Fig. 3 Schematic representation of the host-specific factors and cell types that contribute to demyelination in the infected CNS. (**a**) Intranasal or intracranial MHV infection initiates an inflammatory cascade that results in the recruitment of CD8 (CTL) and CD4 (T_H) T-cells, B-cells, γ/δ T-cells, and macrophages (MΦ) to the CNS. CTL and T_H cells may kill infected oligodendrocytes directly (*dashed lines*), but it more likely that they secrete pro-inflammatory 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. The common feature of macrophage/microglia activation in each scenario underscores the critical role of these two cell types in MHV-induced demyelination

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 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 (Haring et al. 2001; Williamson and Stohlman 1990). 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 (Phares et al. 2012a, b). 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 (Matthews et al. 2001; Ramakrishna et al. 2002). 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 (Marten et al. 2000b; Zhao et al. 2009).

Infiltrating T-cells are largely MHV–JHM-specific, but it is now clear that infection also results in the recruitment and activation of virus-nonspecific, bystander T-cells (Chen et al. 2005; Haring and Perlman 2003; Haring et al. 2002). Several lines of evidence suggest that efficient virus clearance is critically dependent on both CD8 and CD4 T-cells. Depletion of CD4 or CD8 T-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 (Houtman and Fleming 1996a; Williamson and Stohlman 1990). While clearly important for virus clearance, infiltrating CD8 and CD4 T-cells also appear 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

Several CD8 T-cell epitopes have been discovered in MHV. One dominant CD8 T-cell epitope, located in a conserved region of the N protein, has been identified in BALB/c mice. This epitope, N318, is H-2L^{d}-restricted and encompasses residues N318-326 (Table 1). In C57BL/6 (B6) mice, at least two immunodominant CD8 T-cell epitopes are recognized. Approximately 30–50 % of CD8 T-cells that infiltrate the B6 CNS at the peak of the T-cell response specifically recognize the dominant epitope, S510 (H-2D^{b}-restricted, S510-518) when measured by staining with MHC class I tetramer. A second population of infiltrating CD8 T-cells is specific for a subdominant epitope S598 (598-605; H-2K^{b}-restricted). Both epitopes are derived from the hypervariable region of the S protein. This region tolerates both deletions

Table 1 CD8 T-cell epitopess of MHV recognized in MHV-infected mice

Mouse strain	MHV protein	Amino acids
C57BL/6	S	510–518
C57BL/6	S	598–605
BALB/c	N	318–326

and mutations, although deleted virus is usually attenuated (Dalziel et al. 1986; Fleming et al. 1987; Lavi et al. 1990; Parker et al. 1989; Wang et al. 1992a).

CD8 T-cells mediate virus clearance from astrocytes and microglia/macrophages largely by perforin-mediated cytolysis, whereas clearance of virus from oligodendrocytes is primarily dependent upon IFN-γ expression (Lin et al. 1997; Parra et al. 1999). CD8 T-cells are also capable of eliminating virus-infected cells via the FasL/Fas pathway, but this mode of clearance does not play a prominent role in clearance of MHV in vivo (Parra et al. 2000). 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 (Bergmann et al. 1999; Marten et al. 2000a, b).

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, administration of antivirus antibody protects mice from MHV–JHM but virus persists (Buchmeier et al. 1984). In the suckling mouse experimental system, suckling mice are infected at 10 days post-natal and are nursed by dams that were previously immunized with MHV–JHM (Perlman et al. 1987). Maternal antibodies protect the mice from acute encephalitis and death; however, a variable percentage of survivors develop clinical signs of chronic disease (hind limb paralysis) by 3–8 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 a vigorous CD8 T-cell response on MHV–JHM-infected cells results in the selection of variant viruses that have undergone mutation in the immunodominant CD8 T-cell epitope (Pewe et al. 1996). Generally, a single mutant is isolated from each animal, with mutation detected in position 2–7 of the CD8 T-cell epitope that abrogates either binding to the MHC class I molecule or T-cell receptor 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 (Pewe et al. 1998). 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 Goulder and Watkins 2004). 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 antiviral antibody (Dandekar et al. 2003), epitope immunodominance (Kim and Perlman 2003), and virus fitness and T-cell functional avidity (Butler et al. 2008a, b). For example, the anti-MHV antibody response at the site of infection 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 are detected in the infected CNS (Dandekar et al. 2003).

During the persistent infection, MHV-specific CD8 T-cells are retained in the CNS at low levels and can be detected out to greater than 70 days p.i. (Bergmann et al. 1999; Marten et al. 2000a, b; Zhao et al. 2009). These virus-specific T-cells are recruited from peripheral antigen-experienced and naive cells and retain the ability to degranulate and produce cytokines (Zhao et al. 2009). 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 2). 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) (Xue and Perlman 1997). 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 (Haring et al. 2001; Zhao et al. 2009). 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), although neither elicits as strong a CD4 T-cell response as the M133 epitope (van der Veen 1996).

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 (Houtman and Fleming 1996a; Pearce et al. 1994; Sutherland et al. 1997; Williamson and Stohlman 1990). Also, adoptive transfer of MHV-specific CD4 T-cell lines into infected mice or rats revealed that CD4 T-cells of multiple virus specificities could confer protection. 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 (Erlich et al. 1989; Korner et al. 1991; Stohlman et al. 1986, 1988; Yamaguchi et al. 1991). 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

Table 2 CD4 T-cell epitopes of MHV recognized in MHV-infected mice

Mouse strain	MHV protein	Amino acids
C57BL/6	M	133–147
C57BL/6	S	333–347
C57BL/6	S	358–372
BALB/c	S	333–347
BALB/c	N	266–279

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 pro-inflammatory cytokines, most importantly IFN-γ, which may promote antigen presentation by blood-borne and CNS-resident cells (Bergmann et al. 2006). Furthermore, depletion of CD4 T-cells correlated with decreased numbers of virus-specific CD8 T-cells infiltrating the brain parenchyma (Stohlman et al. 1998). Thus, secretion of cytokines that serve as survival or accumulation 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, one study (Heemskerk et al. 1995) 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 (Heemskerk et al. 1995; Wijburg et al. 1996). Further analyses on MHV-induced CNS disease in CD4-deficient mice revealed a role for CD4 T-cells in sustaining recruitment of macrophages and lymphocytes to the MHV-infected CNS. This lack of CD4 T-cells correlated with a decrease in the release of RANTES, a chemokine that has been shown to be critical for recruitment of leukocytes (Lane et al. 2000).

Similar to CD8 T-cells, 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 lacking 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 (Anghelina et al. 2006). Introduction of a novel CD4 T-cell epitope into the variant virus reversed the phenotype, resulting in 50 % mortality. The absence of this viral epitope had minimal effects on virulence in BALB/c mice, in which the M133 epitope is not recognized. Together, these results showed that the antivirus 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 as transfer of Tregs decreased clinical disease and demyelination (Trandem et al. 2010). Interestingly, virus-specific Tregs exist and are even more suppressive compared to bulk Tregs, and inhibit pathogenic CD4

T-cell responses (Zhao et al. 2014). These virus-specific Tregs function in both the draining lymph nodes and site of infection, the brain, to reduce the M133-specific CD4 T-cell response and to a lesser extent, the total anti-MHV CD4 T-cell response. Strikingly, these cells express IFN-γ and TNF, cytokines usually associated with a pro-inflammatory immune response.

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 and will be discussed below.

T-Cell Responses Important for Demyelination

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 pro-inflammatory molecules such as TNF, MCP-1, CCL2, and IP-10/CXCL10 (Haring et al. 2001; Houtman and Fleming 1996b; Wang et al. 1990; Wu et al. 2000a, b; Wu and Perlman 1999). 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 (Wang et al. 1990). Later, similar results were obtained after transfer of splenocytes into infected SCID or RAG1$^{-/-}$ mice: demyelination occurred with only modest reductions in virus titers (Dandekar et al. 2003; Pewe et al. 2002; Pewe and Perlman 2002; Wu et al. 2000a; Wu and Perlman 1999). Both primary effector cells (Wu and Perlman 1999) and memory T-cells (Bergmann et al. 2004) are able to mediate demyelination.

Subsequent analyses revealed that both MHV–JHM-experienced 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 transfer of CD4 T-cell-enriched fractions resulted in severe clinical disease, with mice presenting as moribund by 7 days post-transfer (Pewe et al. 2002), sooner than is observed after transfer of undepleted splenocytes (Wu and Perlman 1999). 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 (Pewe and Perlman 2002). In addition, experiments using splenocytes isolated from mice deficient in IFN-γ, TNF, or perforin reveal several interesting features (Pewe et al. 2002; Pewe and Perlman 2002). Adoptive transfer of unfractionated splenocytes from IFN-γ$^{-/-}$, perforin$^{-/-}$, or TNF$^{-/-}$ 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-γ$^{-/-}$ mice nearly completely abrogated demyelination (Pewe and Perlman 2002), similar to the effect observed in mice with CD8 T cell-mediated EAE (Huseby et al. 2001). The transfer of IFN-γ$^{-/-}$ CD4 T-cell-enriched fractions exacerbated demyelination and clinical disease (Pewe et al. 2002). 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 (Tran et al. 2000). In contrast to IFN-γ, there were only modest reductions in demyelination after transfer of perforin$^{-/-}$ or TNF$^{-/-}$ CD8 cells (Pewe and Perlman 2002). However, transfer of CD4 T-cells from TNF$^{-/-}$ resulted in milder disease with prolonged survival and only modest amounts of demyelination (S. Perlman, unpublished observations), suggesting that TNF 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 CD4 or CD8 T-cells express it.

In addition to conventional α/β T-cells, γ/δ T-cells are also able to mediate demyelination (Dandekar and Perlman 2002). 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. In these animals, myelin destruction is mediated by γ/δ T-cells, since depletion of these cells abrogates myelin destruction. γ/δ T-cell-mediated demyelination, like that mediated by α/β CD8 T-cells, is dependent upon the expression of IFN-γ.

Antibody Responses

The critical role of antiviral antibody responses is best illustrated in 2.2-V-1-infected mice that lack either functional antibody (μ chain (IgM)-deficient, μMT mice) (Lin et al. 1999) or in mice that lack mature B-cells (Jh locus-deficient, JhD mice) (Ramakrishna et al. 2002). Initial virus clearance was not significantly impaired in these mice; however, several weeks p.i., virus recrudescence 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 until antivirus antibody was cleared. 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 of antivirus antibodies 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. (Watanabe et al. 1987). 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 (Burrer et al. 2007).

Whether autoantibody production occurs to a significant extent in MHV–infected nontransgenic mice remains unknown.

Anti-MHV Antibody Responses in Demyelination

As discussed above, 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 FcRγ$^{-/-}$ mice and after depletion of complement with cobra venom factor (Kim and Perlman 2005b).

Conclusions

Due in part to the emergence of SARS in 2002 and MERS in 2012, and the continued potential for novel coronavirus emergence from zoonotic sources, new emphasis has been placed on understanding both coronavirus-induced pathology and the host immunological response to coronavirus infections. 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. 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. How macrophages cause demyelination and what T-cells or antibody do to assist macrophage recognition of damaged myelin and what cytokines may be involved are other questions to be answered. Virus spread within this CNS is also of interest. How does virus cross synapses and does this require receptor expression? Also of interest are the differential effects of infection on astrocytes, oligodendrocytes, and neurons.

Developing ways to combat virus replication during the acute phase of CNS infection while simultaneously minimizing damage to the CNS is an important and clinically relevant avenue of research. It is clear that the cells of the immune system that function to clear virus also contribute to morbidity of coronavirus-infected mice. One surprising observation has been that memory CD4 T-cells play an important role in modulating disease outcome during the acute infection. Thus, understanding the difference between effector and memory CD4 T-cells and the mechanisms of memory CD4 T-cell function in the acutely infected CNS will be of particular interest, as memory CD4 T-cells may also have broadly protective roles in encephalitides including human encephalitis.

In addition, continued work on how virus-specific factors contribute to acute and chronic encephalitis and on the curious predilection of coronaviruses to infect the CNS will enable development of therapeutic and prophylactic interventions. These

strategies will likely provide novel strategies and new tools to modulate virus infection within the acutely infected CNS while minimizing damage to host tissue.

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The Arenaviruses

Daniel J. Bonthius

Introduction

The arenaviruses are an important family of viruses for both clinical and scientific reasons. They are important clinically because many of them can induce substantial disease in humans (Bonthius et al. 2008). They are important scientifically because of the great insights that the study of these viruses—especially in experimental animals—has provided regarding immunology and immunopathology (Zhou et al. 2012).

While the arenaviruses are diverse in their virulence for humans, they all share several important characteristics regarding natural history, morphology, and genomic structure. The arenaviruses are maintained in nature through the persistent infection of rodent hosts (although a recent report suggests that some divergent arenaviruses may infect snakes) (Bodewes et al. 2013). Each of these rodent host species is relatively specific for each of the arenaviruses. The infected rodents remain essentially symptom-free, despite harboring high viral titers. The rodents shed the virus in their urine, saliva, and other secretions, and the principal targets of these shed viruses are other members of the same rodent species. Humans are incidental hosts and can become infected when they contact fomites or inhale aerosols containing the secreted virus.

The arenaviruses share a morphology whose image is the source of the family name. The viruses are enveloped, pleomorphic particles that mature by budding from the surface of infected cells. The viral particles are covered with 8–10-nm-long club-shaped projections and contain granular ribosomes. In the electron microscope, these characteristics endow the viral particles with a sandy appearance. Hence, the name *arenaviruses*, from the Latin term *arenosus*, for "sandy" (Buchmeier et al. 2001).

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The arenaviruses are similar not only in their morphology, but also in their genomic organization (Wilson and Peters 2014). All contain a linear, bi-segmented, single-stranded, negative-sense RNA genome. Thus, each infectious particle contains two nucleocapsids. One of these contains the large (L) RNA, consisting of 7200 basepairs, and the other contains the small (S) RNA, consisting of 3400 base pairs. Each of the nucleocapsids is organized as a closed circle. The S strand codes for the viral nucleoprotein and glycoprotein precursor, while the L strand encodes the viral polymerase and the small RING finger protein. The arenavirus genomic organization is uniquely bidirectional, as a single RNA can direct the synthesis of two polypeptides in opposite orientation. As will be shown below, despite having only four genes, the viruses can induce complex patterns of infection and disease that are highly specific.

Thirty-four arenavirus species have been identified, but only nine are human pathogens (see Table 1). Among the arenaviruses that cause disease in humans, lymphocytic choriomeningitis virus (LCMV) is the most prevalent, infects the most people, and covers the largest geographic range. It is also the best studied, in both humans and experimental animals. For these reasons, LCMV is the prototype arenavirus and will be the focus of this chapter. Other arenaviruses and the diseases they induce will be discussed briefly at the chapter's end.

Lymphocytic Choriomeningitis Virus

Historical Note

In 1933, Armstrong and Lillie were the first to isolate LCMV, which was obtained from the cerebrospinal fluid of a woman with meningoencephalitis (Armstrong and Lillie 1934). Initially, this patient was thought to have St. Louis encephalitis, as the isolated virus produced symptoms identical to those of St. Louis encephalitis virus in monkeys. However, when the virus was injected into a monkey known to be immune to St. Louis encephalitis virus, this animal, also, became ill, thus demonstrating that the pathogen must be a separate entity from St. Louis encephalitis virus. This new infectious agent was given the burdensome, but accurate, name of lymphocytic choriomeningitis virus (LCMV) for the striking lymphocytic infiltration that the infection produced in the choroid plexus and meninges of infected monkeys and mice.

During the immediate decades that followed its discovery, this pathogen was identified as one of the most common causes of aseptic meningitis in humans (Meyer et al. 1960). In more recent decades, the proportion of meningitis cases attributed to LCMV has declined. Nevertheless, LCMV remains an important cause of meningitis in humans (Foster et al. 2006; Asnis et al. 2010).

Congenital infection with LCMV was first noted in England in 1955 (Komrower et al. 1955). Over the course of the next several decades, many cases of congenital

Table 1 Arenaviruses that are human pathogens

Virus	Geographic distribution	Disease in humans	Host in nature	Listed by NIAID and CDC as Category A pathogen?	Ribavirin-responsive?
Lymphocytic choriomeningitis virus (LCMV)	All continental land masses (except Antarctica)	Acquired prenatally: congenital LCMV Acquired postnatally: lymphocytic choriomeningitis Acquired via organ transplant: multi-organ-system failure	*Mus musculus* (house mouse)	No	Equivocal
Lassa fever virus	Western Africa	Lassa fever	*Mastomys* sp. (multiple mouse species of west Africa)	Yes	Yes
Chapare virus	Bolivia	Chapare hemorrhagic fever	Unknown	Yes	No
Lujo virus	Southern Africa (Zambia and South Africa)	Lujo hemorrhagic fever	Unknown	Yes	No
Junin virus	Argentine pampas	Argentine hemorrhagic fever	Calomys musculinis (Drylands vesper mouse)	Yes	No
Machupo virus	Bolivia	Bolivian hemorrhagic fever	Calomys callosus (large vesper mouse)	Yes	No
Guanarito virus	Venezuela	Venezuelan hemorrhagic fever	*Sigmodon alstoni* (cotton rat)	Yes	No
Sabia virus	Brazil	Brazilian hemorrhagic fever	Unknown	No	Yes
Whitewater Arroyo virus	Western United States	Whitewater Arroyo hemorrhagic fever	*Neotoma albigula* (white-throated woodrat)	No	No

LCMV infection were detected in Europe. However, it wasn't until 1993 that the first case of congenital LCMV infection was reported in the United States (Larsen et al. 1993). Since then, dozens of cases of congenital LCMV infection have been reported in America (Bonthius et al. 2007a, b; Wright et al. 1997; Barton et al. 1993). Nevertheless, LCMV remains relatively unknown to the pediatricians and neurologists who would be in a position to diagnose it in children. As a result, congenital LCMV infection is almost certainly an under-recognized condition (Bonthius 2012; Jahrling and Peters 1992).

Natural History

The common house mouse, *Mus musculus*, is the natural host and reservoir for LCMV. Through intrauterine infection, the virus is transmitted vertically from one generation to the next, thus sustaining the virus within the mouse population. After acquiring LCMV transplacentally, mice harbor high viral titers, yet remain asymptomatic, because the virus is not cytolytic and because congenital infection with LCMV in mice renders them immunologically tolerant for the virus for life. Infected mice shed large quantities of LCMV in their saliva, semen, urine, feces, and nasal secretions (Buchmeier and Zajac 1999).

Humans can become infected with LCMV in three ways (Bonthius 2009). First and most commonly, postnatal humans can acquire LCMV by inhaling aerosolized virus or by direct contact with fomites contaminated with infectious virus (Foster et al. 2006). Secondly, prenatal humans can acquire LCMV by vertical transmission, as the virus can cross the placenta from mother to fetus during maternal viremia. This is the basis for congenital LCMV infection (Barton et al. 1995). A third, rare, but still important mechanism is human-to-human transmission via transplantation of infected organs. This occurs when an infected individual with an unrecognized infection serves as an organ donor, thus transmitting the virus to the organ recipient (Fischer et al. 2006).

Epidemiology

Throughout all temperate regions of the world, LCMV is endemic and probably exists as an infectious pathogen wherever the genus *Mus* resides, which is every continent except Antarctica. Epidemiological studies have shown that substantial proportions (~10 %) of wild mice are infected with LCMV, in both urban and rural settings (Childs et al. 1992; Lledo et al. 2003). These studies have further shown that significant clustering occurs, where the proportion of infected mice is much higher. This clustering reflects the behavioral territoriality of mice, in the sense that mice tend to stay near their places of birth. As a result, when a mouse "neighborhood" is infected with LCMV, the limited propensity of mice to wander far afield tends to keep the virus relatively confined to that neighborhood, thus creating a clustering effect.

Epidemiologic studies in humans have shown that about 5 % of people possess antibodies against LCMV, reflecting previous exposure and infection (Ambrosio et al. 1994). People can acquire LCMV infections year-round, but most occur during late autumn and early winter, reflecting seasonal differences in the interaction of humans with mice. In late autumn and early winter, cold weather tends to drive mice into the warmth of human dwellings. The mice carry LCMV with them and share it with their ungrateful hosts, as the two species cohabitate.

The incidence of congenital LCMV infection is not known. No epidemiological studies examining the frequency of LCMV infection during pregnancy have been conducted. Most case reports of congenital LCMV infection report children who are very severely affected neurologically by the viral infection. However, this may reflect an ascertainment bias. It is possible that LCMV infection during pregnancy induces a wide range of outcomes—determined by such factors as age at infection, viral strain, and maternal and fetal immune responses—and that the case reports reflect only the most severely affected children in which a viral cause was suspected and sought. The high prevalence of LCMV in the environment and of seropositive humans suggest that LCMV is responsible for far more disease among newborns and infants than has previously been recognized (Enders et al. 1999). For this reason, LCMV is considered an emerging obstetric infection (Jamieson et al. 2006).

Pathogenesis

The pathogenesis of LCMV is not fully understood for acquired or for congenital infections. Nevertheless, much information is known regarding pathogenesis in both scenarios. For both acquired and congenital LCMV infection, disease is caused by a combination of the virus itself and by the host immune response to it (Bonthius 2015).

Acquired (Postnatal) LCMV Infection

In acquired LCMV, infectious viral particles typically enter the human lung via aerosol or mouth via contact with infected fomites (Danes et al. 1963). The virus is deposited in the lung, where viral replication first occurs. Both the lung parenchyma and adjacent hilar lymph nodes are important early sites of viral growth. Often, interstitial lung infiltrates and edema will reflect this early lung parenchymal involvement. Via the blood stream, the virus then travels to other organs, where further infection and replication occur. Eventually, the virus reaches some select tissues of the brain, including the choroid plexus, ventricular ependymal linings, and leptomeninges, where the virus replicates to high titers. The presence of the virus within these tissues triggers an inflammatory response. This inflammatory response, principally consisting of lymphocytic infiltration, especially within the choroid plexus and meninges, produces the characteristic pathology and symptoms of meningitis that underlie the virus's name *lymphocytic choriomeningitis*.

In acquired LCMV infection, the immune response is a double-edged sword. On the one hand, it is protective, as it plays an indispensable role in clearing the virus and preventing repeat infections. On the other hand, the heavy infiltration of lymphocytes into the meninges underlies the symptoms of meningitis, and the inflammation-induced ventriculitis can sometimes result in the serious complication of noncommunicating hydrocephalus.

Congenital (Prenatal) LCMV Infection

In most cases of congenital LCMV infection, the virus reaches the fetus via the transplacental route (Plume and Bonthius 2014). In a smaller proportion of cases, the fetus acquires the virus by exposure to vaginal secretions or maternal blood during the birthing process. In either case, the virus infects the fetus during a maternal primary infection with LCMV. If a woman has had a previous infection with LCMV, then her immunity will protect her and her fetus against infection.

In congenital infections with LCMV, the brain is the principal target of infection and pathology (Bonthius et al. 2007a). This distinguishes LCMV from the other pathogens that commonly infect the fetus and that go by the acronym *TORCH* infections (toxoplasmosis, rubella, cytomegalovirus, and herpes). These other infections, while they often infect the brain, also tend to heavily infect other fetal organs and cause a host of characteristic symptoms in the newborn. In contradistinction, congenital LCMV infection is often principally or solely a brain infection with symptoms restricted to the nervous system.

Within the developing brain, neuronal populations that are mitotically active are particularly vulnerable to LCMV infection (Bonthius et al. 2002). This propensity of LCMV to infect mitotically active brain regions probably reflects the use of neuronal mitotic machinery for viral replication. As a result of LCMV targeting of the fetal brain, microencephaly, periventricular calcifications, cerebellar hypoplasia, focal cerebral destruction, and neuronal migration defects are common pathologic effects of congenital LCMV infection (Bonthius et al. 2007a) (Fig. 1). The retina, an extension of the brain, is likewise prone to infection and pathology (Mets et al. 2000). As a result, chorioretinitis is an additional hallmark of congenital LCMV infection. The pathologic changes within the brain and retina reflect the strong tropism of LCMV for replicating neuroblasts and the injury induced by the virus and the immune response to it.

The precise mechanisms by which LCMV damages the human fetal brain are unknown, but it is likely that several mechanisms are at work, producing different forms of pathology. The hydrocephalus, which is commonly observed in congenital LCMV infection, is probably caused by ependymal inflammation within the ventricular system, especially at the cerebral aquaduct, where the debris produced by inflammation can block CSF egress from the ventricles and induce noncommunicating hydrocephalus. The periventricular calcifications are almost certainly due to infection and death of periventricular neuroblasts, which are mitotically active and

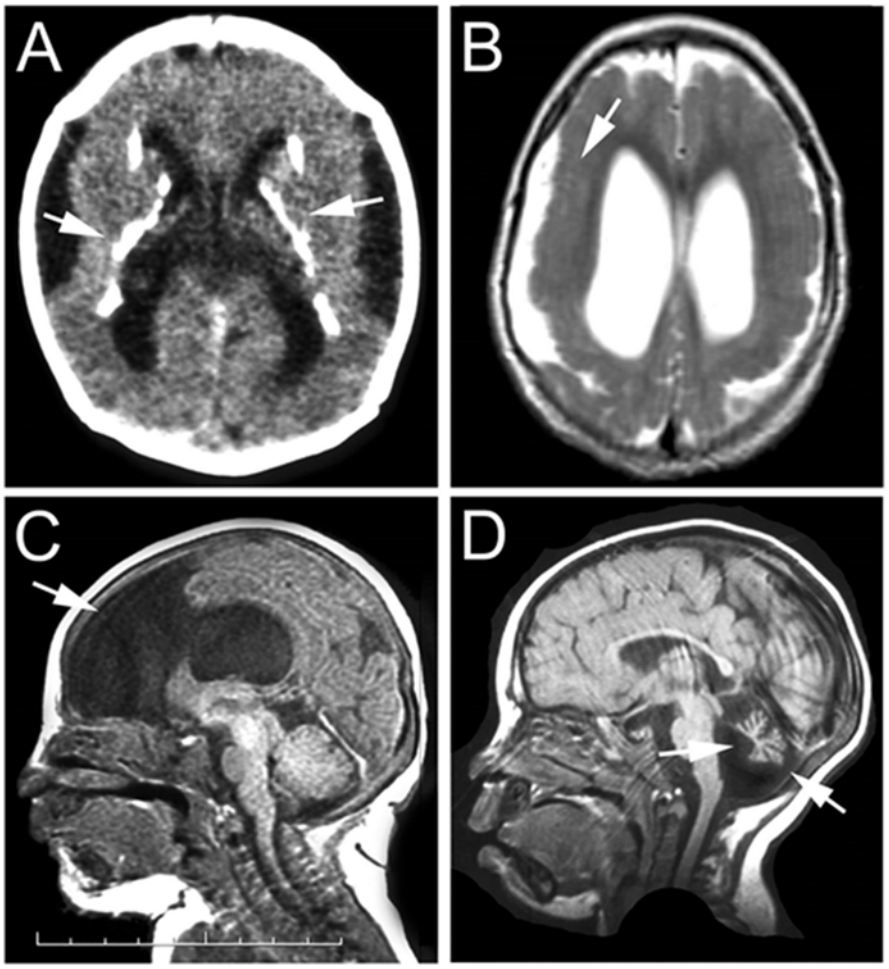

Fig. 1 Neuroimaging abnormalities commonly observed in congenital LCMV infection. (**a**) The most common set of abnormalities observed by CT scan in congenital LCMV infection is the combination of microencephaly and periventricular calcifications (*arrows*). (**b**) MRI scan frequently reveals gyral malformations (*arrow*), suggestive of a neuronal migration abnormality. (**c**) Focal regions of cerebral destruction often produce porencephalic cysts (*arrow*). (**d**) In some children with congenital LCMV infection, the only neuroimaging abnormality is isolated cerebellar hypoplasia (*arrows*)

normally give rise to neurons of the cerebral cortex. The microencephaly likewise reflects this loss of neuroblasts and the subsequent deficits in neuronal number. The sites of focal cerebral destruction probably reflect focal inflammatory lesions, and the gyral malformations are almost certainly due to virus-induced disruptions in neuronal migration (Bonthius and Perlman 2007).

Clinical Effects

The clinical manifestations of LCMV infection are determined by the developmental stage of the patient at the time of infection. In particular, effects of infection during postnatal life are very different from those that occur during the prenatal period (Bonthius 2012, 2015).

Acquired (Postnatal) Infection

LCMV infection during postnatal life (childhood or adulthood) is usually a relatively mild disease, from which the patient fully recovers (Asnis et al. 2010). In most cases, LCMV is a biphasic disease in which the initial symptoms are "flu-like" and include fever, myalgia, anorexia, nausea, and vomiting. The patient often begins to improve from the symptoms of this first phase before a second phase of disease begins, whose symptoms are those of aseptic meningitis. This central nervous system phase has symptoms that include headache, nuchal rigidity, fever, vomiting, and photophobia. Rarely is there evidence of brain dysfunction, such as seizures or altered mental status. The entire course of acquired LCMV disease usually lasts only 1–3 weeks and is followed by a rapid and full recovery (Bonthius 2009).

During the initial febrile phase of LCMV infection, laboratory findings are abnormal in ways that are similar to those of other nonspecific "flu-like" illnesses. These abnormalities often include thrombocytopenia, leukopenia, mild elevations of liver enzymes, and occasional infiltrates on chest radiographs. However, during the second (CNS) phase of the illness, a marked CSF pleocytosis occurs, which is the hallmark laboratory abnormality of LCMV infection. The CSF may contain hundreds or thousands of leukocytes per microliter. Most of these infiltrating white blood cells are lymphocytes, although CSF eosinophilia can also occur (Chesney et al. 1979). Other CSF abnormalities may include hypoglycorrhachia and mild elevations of CSF protein.

For most immunocompetent people, LCMV infections are mild. In fact, in one-third of infected people, the infection is asymptomatic. However, for some people, LCMV infections are clinically severe and may include symptoms that extend beyond the nervous system (Lewis and Utz 1961). Orchitis, pneumonitis, myocarditis, parotitis, dermatitis, and pharyngitis have all been described in patients with LCMV. In addition, the neurologic symptoms may be more severe than just those of aseptic meningitis and may include encephalitis and hydrocephalus (Larsen et al. 1993). In some cases, LCMV may induce Guillain–Barre syndrome and transverse myelitis. Most immunocompetent people recover fully from acquired LCMV infections, but some do not, and, in rare instances, LCMV infections are fatal.

While most postnatal LCMV infections in humans are acquired via contact with rodents, some are acquired via transplantation of infected organs (Schafer et al. 2014). Five clusters of organ transplant-associated LCMV infections have been reported in the United States within the last decade. The donors in these cases were

not known in advance to have LCMV, but some did have signs of central nervous system infection and a history of rodent contact. Groups of solid organ transplant recipients, each group having received organs from a common donor, have developed signs and symptoms of infection within days to weeks of transplantation. In most cases, the transplant recipients have developed severe disease, with fever, encephalopathy, abdominal pain, coagulopathy, thrombocytopenia, leukocytosis, and diarrhea among the most prominent symptoms (MacNeil et al. 2012; Fischer et al. 2006). The great majority of infected organ recipients have died. Some have survived, however, especially those that received only corneal transplants. A few recipients of other infected organs have also survived after receiving ribavirin and reduced levels of immunosuppressive therapy.

It is likely that the severe outcome in most LCMV cases in transplant recipients is due to their immunosuppressed state. The key role played by T-lymphocytes in the control and clearance of LCMV suggests that the T-cell depletion, in particular, is an important factor underlying the severe disease of LCMV in organ transplant recipients. Because immunosuppression plays such a central role in LCMV disease in organ recipients, immunosuppressive therapy should be reduced in these patients. Treatment with ribavirin may also be useful (Schafer et al. 2014).

Congenital (Prenatal) Infection

While most *postnatal* cases of LCMV infection in humans are ultimately benign, this is not the case for most *prenatal* infections (Plume and Bonthius 2014). Human fetal infection with LCMV often results in spontaneous abortion and fetal death. Those fetuses that do survive typically have severe retinal and brain injuries. As a result, children with congenital LCMV often have vision disturbance and brain dysfunction that are typically permanent and severe (Bonthius et al. 2007a) (Fig. 2).

The retinas are injured because prenatal LCMV infections lead to chorioretinitis and the formation of chorioretinal scars (Mets et al. 2000). This scarring most commonly occurs in the periphery of the fundus, but the macula may also be involved (Barton and Mets 2001). The chorioretinal scarring alone can lead to substantial vision impairment, but additional pathologic processes often worsen the visual deficits even further. These include optic atrophy, vitritis, microphthalmos, nystagmus, and cataracts. Furthermore, LCMV infection often affects the cerebral hemispheres by inducing focal injuries and neuronal migration disturbances (Bonthius et al. 2007a). These cerebral injuries further impair vision. Thus, children with congenital LCMV infection often have severe vision deficits as a result of both ocular injuries and cortical vision impairment.

While the vision disturbances in congenital LCMV infection are often severe, it is the effect of LCMV on overall brain function that is most disabling. The fact that LCMV has affected the fetal brain is often evident by changes in the size of an infant's head, which may be either macrocephalic or microcephalic, depending on the nature of the injury. Large heads (macrocephaly) in congenital LCMV infection are virtually always due to noncommunicating hydrocephalus, in which the lateral

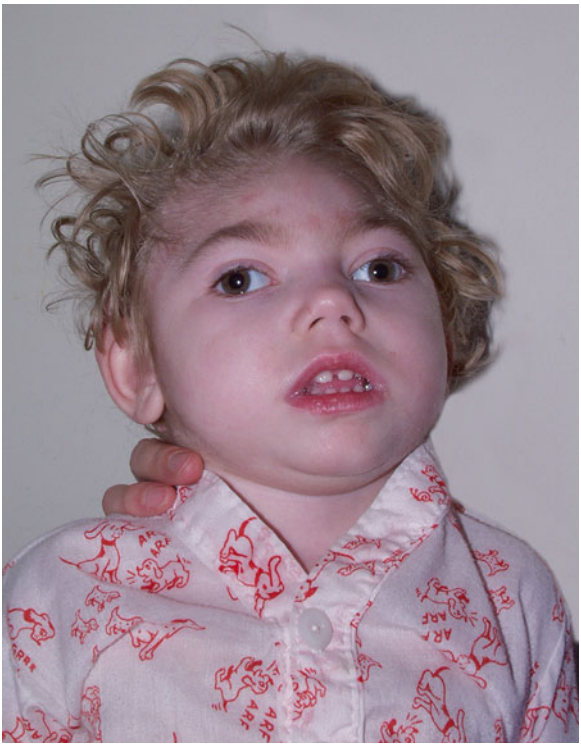

Fig. 2 A child with congenital LCMV infection. This 3-year-old child has microencephaly and obvious strabismus. Note, also, that the pupils are large, reflecting ocular blindness, due to chorioretinitis, and that the child has a stiff, erect posture, reflecting the spasticity of cerebral palsy

ventricles expand due to obstruction of the ventricular system at the level of the cerebral aquaduct (Larsen et al. 1993). This hydrocephalus is often detectable by prenatal ultrasound and may be the first clue that a prenatal infection with LCMV has occurred.

Small heads in congenital LCMV infection are usually due to failure of brain growth, probably reflecting virus-induced interference with neuronal generation from neuroblasts (Fig. 2). Sites of focal brain destruction also contribute to small brains and heads.

Periventricular calcifications are very common in congenital LCMV infection (Wright et al. 1997). These are often detectable by prenatal ultrasound or postnatal CT scan. The calcifications are often restricted to the periventricular region and probably reflect the specific infection and death of mitotically active neuroblasts present at that site (Bonthius and Perlman 2007). It is the loss of these neuroblasts, which normally give rise to the neuronal populations of the cerebral hemispheres, that underlies the microencephaly of congenital LCMV infection.

The combination of microencephaly and periventricular calcifications is the most common abnormality evident in congenital LCMV infection (Fig. 1). However, a wide range of abnormalities have been reported, including isolated cerebellar hypoplasia, focal cerebral destruction with porencephalic cysts, and gyral malformations reflecting neuronal migration disturbances (Bonthius et al. 2007a) (Fig. 1).

Why human fetuses infected with LCMV have such a wide range of pathologic effects is unknown, but may be due to differences in the gestational age at the time of infection. Indeed, animal models have shown that the effect of LCMV on the developing brain depends crucially on the developmental stage of the animal at the time of infection. Differences in as little as 1 day of developmental age can alter the cellular targets and pathologic effects of the infection (Bonthius et al. 2007b).

The neurologic outcomes of children with congenital LCMV infection are typically poor. Virtually all have significant vision loss. Most have substantial cognitive deficiencies and cerebral palsy. Epilepsy is very common. Those children with isolated cerebellar hypoplasia are jittery at birth and ataxic in childhood, but their cognitive impairments are generally less severe than those with cerebral hemispheric involvement (Bonthius et al. 2007a).

Prenatal LCMV infection tends to affect mostly or exclusively the central nervous system. Unlike many other congenital infections, in which babies are born small for gestational age and have multi-organ system dysfunction, congenital LCMV infection often induces no fetal growth disturbances or systemic organ dysfunction. Thus, rashes, thrombocytopenia, hepatosplenomegaly, and auditory deficits that are common in other congenital infections tend to be absent in congenital LCMV (Plume and Bonthius 2014).

Differential Diagnosis

Because prenatal and postnatal LCMV infections induce such different diseases, the differential diagnoses differ as well. For postnatal LCMV infections, the differential diagnosis mainly centers on other causes of aseptic meningitis (Bonthius and Karacay 2002). Most of these causes are other viruses (Han et al. 2015). In the United States, the most common are the non-polio enteroviruses. Others include St. Louis encephalitis virus, mumps, herpes, influenza, West Nile virus, and the arboviruses (Bonthius and Bale 2015). Bacterial infections, such as Brucellosis, can also induce meningitis and closely mimic the signs and symptoms of LCMV. Noninfectious causes include drug-induced aseptic meningitis (which is most commonly caused by antibiotics, NSAIDs, vaccines, and radiographic agents) and meningitis of systemic disease, as is commonly observed in systemic lupus erythematosus and Behcet syndrome.

The principal considerations in the differential diagnosis of congenital LCMV infection are other infectious pathogens that can cross the placenta and injure the fetus. These infectious agents are often referred to as the "TORCH" infections, an acronym referring to toxoplasmosis, rubella, cytomegalovirus, and herpes. All of these infections can cause injury to the fetal brain and cause long-lasting and severe neurologic dysfunction in postnatal life. Among the TORCH infections, cytomegalovirus and toxoplasmosis may be particularly difficult to distinguish from LCMV, because all three infections induce microencephaly, hydrocephalus, chorioretinitis, and cerebral calcifications (Wright et al. 1997; Brezin et al. 2000). However, both CMV and toxoplasmosis typically have prominent systemic signs, including hepatosplenomegaly,

Table 2 Diagnostic tests for LCMV infection

Method	Advantage	Disadvantage
1. Virologic (culture)	High specificity	Low sensitivity
2. Molecular (PCR)	Fast and accurate	Viral clearance may precede the test in congenital infections
3. Serologic		
(a) Complement fixation	Widely available	Low sensitivity
(b) Immunofluorescent antibody	Moderate sensitive	Only moderate sensitivity
(c) ELISA	Detects IgM and IgG with high sensitivity	Available only at CDC

bone marrow suppression, and rash, all of which are typically absent in LCMV (Bonthius et al. 2007a). Confidently differentiating among these infections, however, can be done only with laboratory testing.

Diagnosis

Diagnostic tests for LCMV infection are listed in Table 2. Because most patients with postnatally acquired LCMV infection still harbor the virus at the time that they are symptomatic and presenting to a physician's office, the infection can be diagnosed by isolation of the virus. Sites from which LCMV can often be isolated include blood, CSF, urine, and nasopharyngeal secretions. While the diagnosis can be made by viral isolation, it is much more commonly made serologically by assessing acute and convalescent anti-LCMV antibody titers. Examination of the CSF, showing a marked lymphocytic pleocytosis along with normal or near-normal protein and glucose levels, further supports the diagnosis. A finding of elevated protein and moderately low glucose in the CSF is also consistent with LCMV (Bonthius 2015).

For congenital LCMV infections, the situation is more complicated. A minority of infants born with congenital LCMV infection still harbor infectious virus at the time of birth. In these cases, the infection can be definitively diagnosed by isolating the virus from CSF (Schulte et al. 2006). However, by the time of birth, many babies who were prenatally infected with LCMV no longer harbor the virus, as it was cleared by the mother's or fetus's immune system earlier in pregnancy. Thus, in the majority of cases, congenital LCMV infection cannot be diagnosed virologically, and must be diagnosed serologically. A further complication, however, is the fact that maternal antibodies, especially IgG antibodies, can cross the placenta. Thus, a previous maternal infection with LCMV may yield positive anti-LCMV antibodies in an infant's blood—even if that maternal infection occurred and was cleared long before the pregnancy occurred. For this reason, the serologic tests for LCMV should include both IgM and IgG titers on both maternal and infant serum (Wright et al. 1997).

The complement fixation (CF) test is the most widely available serologic test for LCMV, but has a lower sensitivity than the immunofluorescent antibody (IFA) test, which detects both IgM and IgG antibodies (Lehmann-Grube et al. 1979). The IFA test is commercially available and is sufficiently sensitive to make it an acceptable test for congenital LCMV. However, an even more sensitive test for congenital LCMV is the enzyme-linked immunosorbent assay (ELISA), which also detects both IgG and IgM antibodies, but is available only at the Centers for Disease Control and Prevention (CDC).

In addition to virologic and serologic methods, molecular methods may also be used to diagnose LCMV infections (Cordey et al. 2011). Polymerase chain reaction (PCR) has been used to detect LCMV RNA in an infected infant (Enders et al. 1999). PCR could theoretically be an effective way of diagnosing both prenatal and postnatal LCMV infections. However, LCMV is not known to induce persistent infections in humans, and the time course of viral clearance from an infected human fetus is unknown. A fetus may sustain substantial brain damage from LCMV infection, but clear all virus prior to birth. In this case, the infant would have congenital LCMV infection, but have no LCMV RNA to be detected in the postnatal period, thus rendering the PCR assay ineffective.

Treatment

No antiviral therapy is definitively effective against LCMV in infected patients. Ribavirin slows LCMV replication in vitro and appears to have helped several patients who acquired the infection via transplantation (Schafer et al. 2014). However, its effectiveness is unproven. As a result, treatment for LCMV infection is essentially supportive.

Luckily, the vast majority of patients who acquire LCMV postnatally have a self-limited disease and require only symptomatic care until the infection spontaneously resolves. Those who acquire the virus via transplantation of an infected organ are at considerable risk for severe disease because of their immunosuppression. These patients should be treated with reductions in their immunosuppressive therapy and with ribavirin (Schafer et al. 2014).

Children with congenital LCMV infection typically have many secondary neurologic complications that require treatment. Many are born with hydrocephalus that requires placement of a ventriculo-peritoneal shunt. Seizures often have their onset during infancy and require aggressive, life-long pharmacologic interventions to control them. Cerebral palsy, often taking the form of spastic quadriplegia, is often severe and requires physical therapy, along with oral baclofen or a baclofen pump. Cognitive deficiencies in congenital LCMV infection are almost universal and require educational interventions throughout childhood (Plume and Bonthius 2014).

Prognosis

As is true of all aspects of LCMV infection, the prognosis depends strongly on whether the infection was acquired postnatally or prenatally (Bonthius 2015). The great majority of postnatally acquired infections resolve spontaneously with no long-term sequelae. Deaths are rare. In contrast, the prognosis for prenatal LCMV infections is generally poor. All have some degree of vision disturbance and cognitive impairment (Bonthius et al. 2007a). In most cases, these vision and cognitive deficits are severe. Furthermore, most children with congenital LCMV infection have additional neurological problems, which often include epilepsy, cerebral palsy, and hydrocephalus. The severity of these neurological problems often substantially shortens the lives of children with congenital LCMV.

Prevention

No vaccine is available to prevent LCMV infection. However, preventive measures can be taken to reduce the risk of infection. Because humans acquire LCMV via rodent vectors, risk of infection can be greatly reduced by eliminating access to rodents in human houses and by limiting rodent infestation in animal and food storage areas.

Congenital LCMV occurs only when a woman contracts a primary LCMV infection during pregnancy. Whereas rodents are the primary reservoir of LCMV in the environment, women can minimize their risk of infection by limiting their exposures to rodents. To the extent possible, cohabitation with mice during pregnancy should be eliminated. If a mouse is captured alive or dead in a trap, pregnant women should not empty the trap. Contact with pet rodents should also be avoided by pregnant women and new pet rodents, especially mice and hamsters, should not be purchased or brought into the home during pregnancy.

Outbreaks of LCMV have occurred among laboratory personnel who work with rodents (Centers for Disease Control and Prevention (CDC) 2012; Knust et al. 2014). Pregnant women who work with rodents in research laboratories or animal care facilities should wear protective gloves, gowns, and face masks to avoid potential aerosolized or secreted LCMV. Rodent colonies in animal care facilities should be tested periodically for LCMV, because the virus may be widely present and at high titers, even if the animals are virtually asymptomatic.

Basic Research with LCMV

LCMV has contributed mightily to mankind's knowledge of virology, immunology, and immunopathology. It could be argued (and has been) that the study of no other virus has added so much to the fundamental concepts of modern biology. The

characteristics of LCMV that allowed it to be such a useful tool are the dual facts that it (a) triggers an intense immune response, while it (b) is a non-cytolytic virus. As a result of this combination, scientists have had the opportunity to isolate and study in detail the effects of the immune response from the effects of the virus itself.

The contributions of LCMV to viral immunology have been reviewed in detail elsewhere (Zhou et al. 2012). They will be reviewed only briefly here.

The central observation regarding LCMV that gave rise to so many new ideas and discoveries was made by Rowe in the 1950s that depletion of the immune response allowed mice with acute LCMV to survive an infection that would otherwise be fatal (reviewed in Buchmeier et al. 1980). This observation showed that mortality of LCMV in mice was due to the immune response and opened the door wide to the study of viral immunopathology. Subsequent studies stemming directly from this observation gave rise to the critically important concepts of Major Histocompatibility Complex (MHC) restriction, T cell memory and exhaustion, cytotoxic T lymphocytes, immunologic tolerance, persistent infections, and virus-antibody immune complex disease.

Persistent infections in LCMV further showed that cells' physiology can be disrupted without changing their survival rate. In particular, LCMV can impair the specialized functions of cells by blocking their ability to exercise their specialized functions, without simultaneously blocking the cells' vital functions (de la Torre et al. 1991). This observation led to the novel concept that viral infections could cause disease through cell dysfunction without cell destruction—a notion that remains hotly pursued in many areas of modern medicine, especially psychiatry, neurology, and endocrinology.

The basic mechanisms underlying acquired (postnatal) LCMV infection have been known for decades and were elucidated principally through the use of an adult mouse model of the disease. The virus infects the choroid plexus and meningeal coverings of the brain, thus leading to an intense lymphocytic meningitis (Gilden et al. 1972). The immune response, driven by CD8+ lymphocytes, produces the symptoms of meningitis, but eventually clears the virus, thus allowing all symptoms to resolve.

In humans, congenital (prenatal) LCMV infection differs substantially from the postnatal infection in its effects and outcomes. In particular, congenital infection leads to substantial brain and retinal injury, while the postnatal infection leads only to meningitis. This suggests that the brain parenchyma is infected in the prenatal case, while only the meninges are infected in the postnatal case. The adult mouse model—in which only the meninges are infected and the brain is spared—is not an effective model for the study of congenital LCMV. However, the neonatal rat inoculated with LCMV is an excellent model system of human congenital LCMV infection, as it can recapitulate all effects seen in humans (Monjan et al. 1971; Bonthius and Perlman 2007). Thus, my laboratory has utilized the neonatal rat model to shed light on the effects and mechanisms of congenital LCMV infection.

Utilizing the neonatal rat model, our group has shown that LCMV heavily infects the brain parenchyma in the developing animal. In the initial phase of infection, however, not all brain cells are equally vulnerable to infection. On the contrary, in the developing brain, LCMV initially specifically targets astrocytes (Bonthius et al.

2002) (Fig. 3). Astrocytes are the portal through which LCMV enters the brain, the principal site of LCMV replication, and the conduit through which the virus spreads throughout the central nervous system (Fig. 4). This finding may have clinical implications, as therapies directed toward blockade of viral entry or spread through astrocytes could effectively interrupt the infection.

After infecting astrocytes, LCMV spreads into neurons (Fig. 3). However, not all neurons are infectable. LCMV infects neurons only in those brain regions that contain mitotically active neuroblasts (Monjan et al. 1975). Thus, in the neonatal rat inoculated on postnatal day 4, LCMV selectively infects neurons in only four brain regions—the cerebellum, olfactory bulb, periventricular region, and dentate gyrus. Neuropathology, then ensues in these brain regions. However, the nature and progression of disease differ markedly among brain regions (Bonthius et al. 2002). This observation may, again, have clinical relevance, humans with congenital LCMV infection tend to have focal brain pathology.

The cellular targets of infection and the nature and severity of the pathology depend strongly on the age of the animal at the time of infection (Bonthius et al. 2007b). For example, inoculation of the neonatal rat on postnatal day 1 results in a widespread infection of cortical neurons and astrocytes (Fig. 5). However, inoculation just 3 days later, on postnatal day 4, yields infection only of astrocytes, and no neocortical neurons are infected. Likewise, the nature and severity of pathology change substantially, depending on age at infection. For example, inoculation on postnatal day 1 leads to cerebellar hypoplasia, in which the cerebellum is small but has normal cytoarchitecture, while inoculation on postnatal day 4 leads to cerebellar destruction (Fig. 6). These differences in pathologic outcome reflect, at least in part, developmental stage-specific and brain region-specific differences in immune response to the virus. As shown in Fig. 7, infection with LCMV on postnatal day 1 elicits minimal lymphocytic infiltration into the cerebellum or olfactory bulb, while infection just several days later elicits a robust infiltration into both brain regions. However, while infection as late as postnatal day 21 continues to induce a lymphocytic infiltration into the olfactory bulb, no such infection or infiltration occurs in the cerebellum. These findings again mimic the human condition, in which infants prenatally infected with LCMV have a wide range of neuropathology. In fact, all of the various pathologic changes observed in children with congenital LCMV infection can be recapitulated in the rat model by inoculating the rat pups at slightly different ages (Bonthius et al. 2007b). This finding suggests that the variation in outcome among children with congenital LCMV infection is due to differences in the gestational age of infection.

Additional Arenavirus Infections of Humans

Besides LCMV, several additional arenaviruses can infect humans and cause disease. All of these pathogenic arenaviruses share a common virionic structure and are endemic in rodent hosts. In addition, they all have the propensity to produce acute hemorrhagic fevers in humans (Shao et al. 2015). Each of these arenaviruses can induce a spectrum of disease that ranges from mild to life threatening, in which

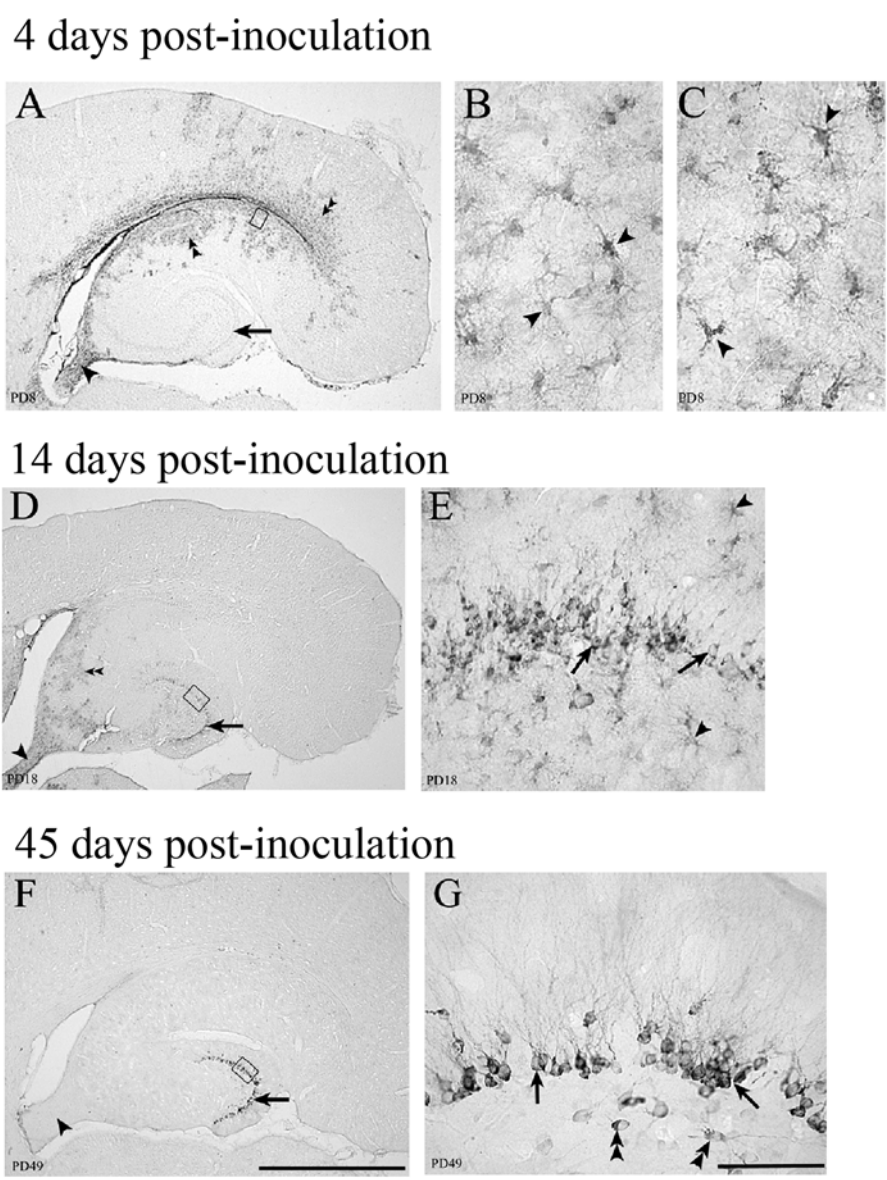

Fig. 3 The sequential spread of LCMV through the developing brain. Shown here are 40-μmicron-thick sections through the hippocampal formation immunohistochemically stained for LCMV antigens with a polyclonal antibody. The rat pups were inoculated with LCMV intracerebrally at postnatal day 4 and killed at a series of times postinoculation. (**a**) On postnatal day 8 (4 days post-inoculation), viral antigen is restricted to patches throughout the brain (*double arrowheads*). The neurons of the dentate gyrus are not infected at this early stage (*arrow*). (**b**) Higher power view of the *small box* shown in (**a**) reveals that the infected cells have the size and shape of astrocytes. (**c**) An adjacent section stained for glial fibrillary acidic protein (GFAP) shows that the cells have an identical shape and distribution as astrocytes. (**d**) On postnatal day 18 (14 days postinoculation), the virus is still present in patches (*double arrowheads*) and has moved into the dentate gyrus (*arrow*). (**e**) Higher power view of the box in (**d**) reveals that the infected cells of the dentate gyrus are neurons. (**f**) On postinoculation day 49 (45 days postinoculation), the virus has been cleared from astrocytes, but persists in the dentate gyrus. (**g**) Higher power view of the box in (**f**) reveals that the infected cells of the dentate gyrus are clearly neurons

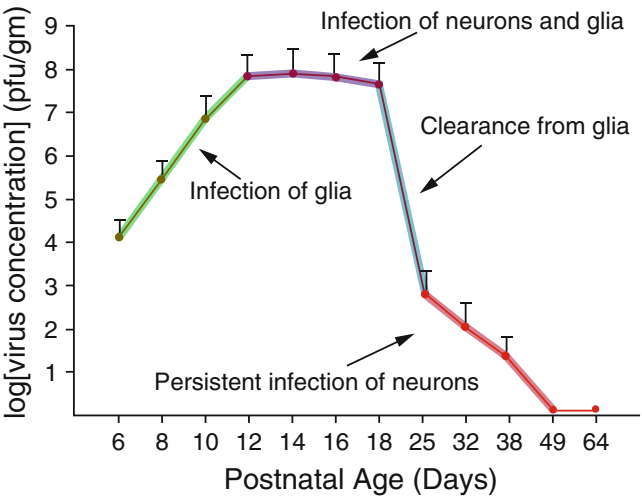

Fig. 4 LCMV titers within the developing hippocampal formation and the corresponding cellular targets of infection. LCMV (1000 plaque forming units) was inoculated into the forebrain on postnatal day (PD) 4, and viral titers were measured by plaque assay at a series of postnatal ages. Following inoculation on PD 4, viral titers rise rapidly and progressively. This rising phase reflects the progressive infection of astrocytes. By PD 12, viral titers have risen to high levels reflecting a dual infection of astrocytes and neurons. On PD 18, viral titers begin to plummet. This reflects the clearance of virus from astrocytes. After PD 25, the virus persists for weeks in neurons, until it is eventually cleared completely

shock is a frequent feature. Early common signs of all of these infections include fever, headache, lethargy, myalgia, abdominal pain, and conjunctivitis. Signs of brain dysfunction, including mental status changes, seizures, and tremor, may occur in severe Lassa fever and in the South American hemorrhagic fevers.

In this age of international and domestic terrorism, the specter has been raised of unleashing infectious agents for the purpose of bioterrorism. Because many arenaviruses can produce acute hemorrhagic fevers, a subset of them has been listed by the Centers for Disease Control and Prevention (CDC) and by the National Institute of Allergy and Infectious Diseases (NIAID) as Category A Priority Pathogens (Table 1). In fact, in the past year, two additional arenaviruses have been added to this list of particularly threatening pathogens. The viruses are considered high-priority risks to national security because they are easily disseminated, have the potential for major public health impact, and could induce social disruption and public panic (http://www.niaid.nih.gov/topics/biodefenserelated/biodefense/pages/cata.aspx).

Lassa Fever Virus

The Lassa Fever virus is endemic in western Africa and is named after the Nigerian town where the first cases were identified (Frame et al. 1970). The natural reservoirs of the Lassa fever virus are rat-like rodents of the genus *Mastomys*, which are

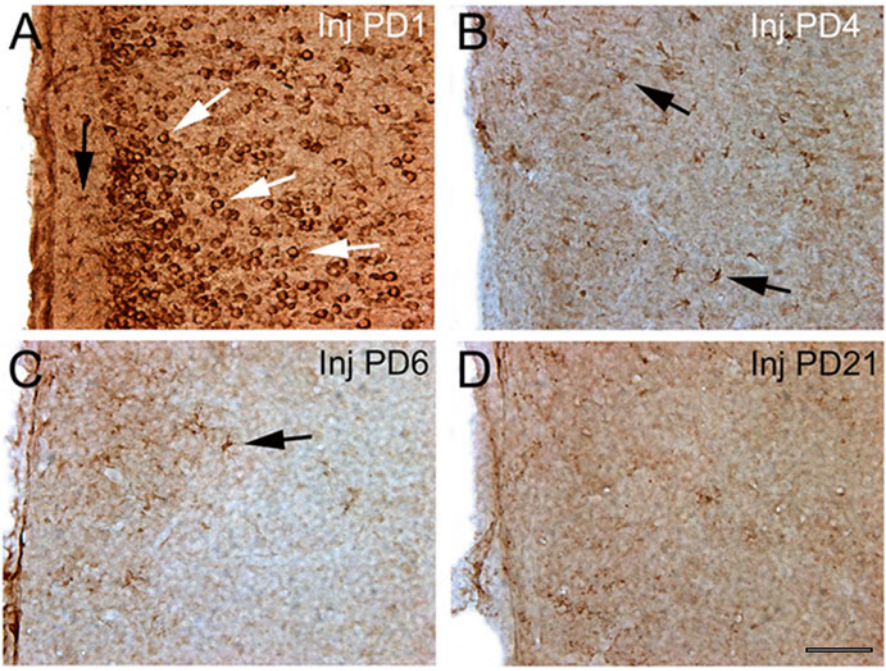

Fig. 5 The cellular targets of infection within the developing brain depend on the age of the animal at the time of infection. Shown here are 40-micron-thick sections through rat cerebral cortex immunohistochemically stained for LCMV 10 days postinoculation. (**a**) Inoculation on postnatal day (PD) 1 results in a robust infection of cerebral cortical cells, including neurons (*white arrows*) and astrocytes (*black arrow*). (**b**) Inoculation just 3 days later, on postnatal day 4, results in an infection restricted to astrocytes alone (*black arrows*). No neurons are infected. (**c**) Inoculation on PD 6 results in even fewer astrocytes infected and, again, no neurons. (**d**) By PD 21, no cells of the cerebral cortex—neither astrocytes nor neurons—are infectable

numerous in western Africa and often invade human dwellings, thus spreading the virus from infected rodents to humans. Most people who contract the virus have no or minimal symptoms from it. In a minority of infected people, however, the virus induces severe multi-system disease. Like most of the hemorrhagic fever viruses, Lassa fever virus often induces fever, chest pain, headache, gastrointestinal symptoms, and conjunctivitis. In addition, Lassa fever virus often causes an exudative pharyngitis. Neurological symptoms are often prominent and include encephalitis, hearing loss, and tremor.

Lassa fever is a relatively common disease in western Africa, where several hundred thousand cases occur yearly, of which at least 5000 are fatal (Birmingham and Kenyon 2001). The infection is especially dangerous to pregnant women and their fetuses. When a mother is infected, the vast majority of fetuses die in utero.

Distinct among the arenaviruses, Lassa fever can be treated effectively with intravenous ribavirin. This anti-viral agent is most effective against Lassa fever if it is administered during the first week of illness. No effective vaccine for Lassa fever exists, but development of a Lassa fever vaccine is considered a research priority among international health agencies (Fisher-Hoch and McCormick 2001).

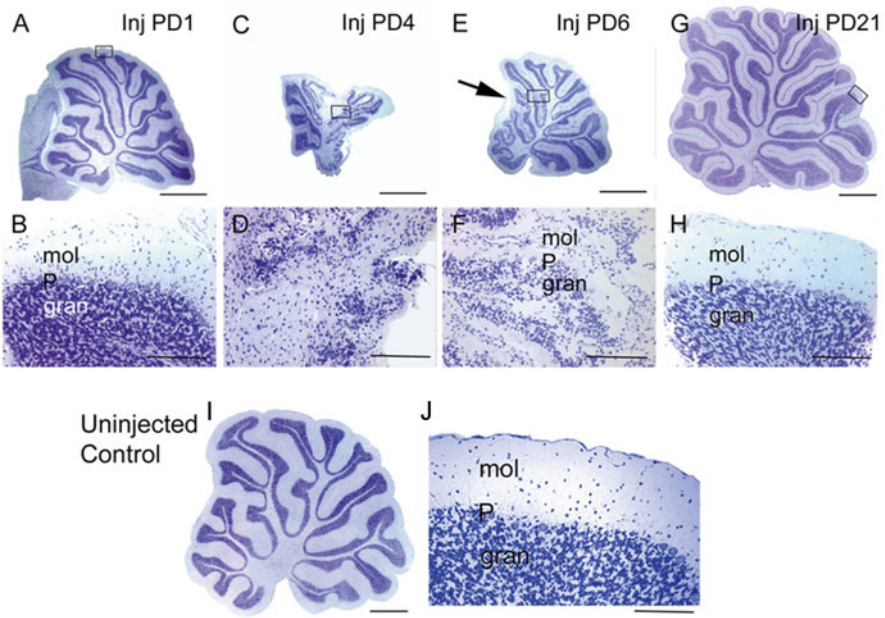

Fig. 6 The nature and severity of pathology from LCMV infection depends on the age of the animal at the time of infection. Shown here are 40-micron-thick sections of cerebellar vermis stained with a Nissl stain. The rat pups were injected with LCMV at the ages indicated and killed as adults. Injection on PD 1 (**a**, **b**) results in cerebellar hypoplasia, in which the cerebellum is abnormally small, but histologically normal. In contrast, inoculation just 3 days later (on PD 4) leads to cerebellar destruction (**c**) and a complete disintegration of cerebellar architecture (**d**). Inoculation on PD 6 leads to some cerebellar hypoplasia and destruction (**e**, **f**), but not as severe as that seen on PD 4. Inoculation with LCMV on PD 21 has no effect on the size (**g**) or histology (**h**) of the cerebellum, and (**i**, **j**) are uninfected controls

Chapare Virus

Chapare virus was discovered in 2004, when an outbreak of hemorrhagic fever occurred in the eastern foothills of the Andes Mountains of Bolivia (Delgado et al. 2008). During this outbreak, one young man, who had been previously healthy, died of the disease, and a viral specimen was isolated from him. IFA staining of infected cultured cells identified the virus as a member of South American Arenaviruses. However, sequence analysis of the viral genes revealed that the virus was novel. Thus, the new pathogen was named Chapare Virus, after the Chapare River, near which the outbreak occurred. Since the initial outbreak, no further outbreaks have been reported. Because the cluster of Chapare virus infections occurred in such a remote and resource poor region, little additional information has been learned about the virus or the disease it induces. Even the reservoir for Chapare virus remains unknown, but is presumed to be a rodent. What is known, however, is that

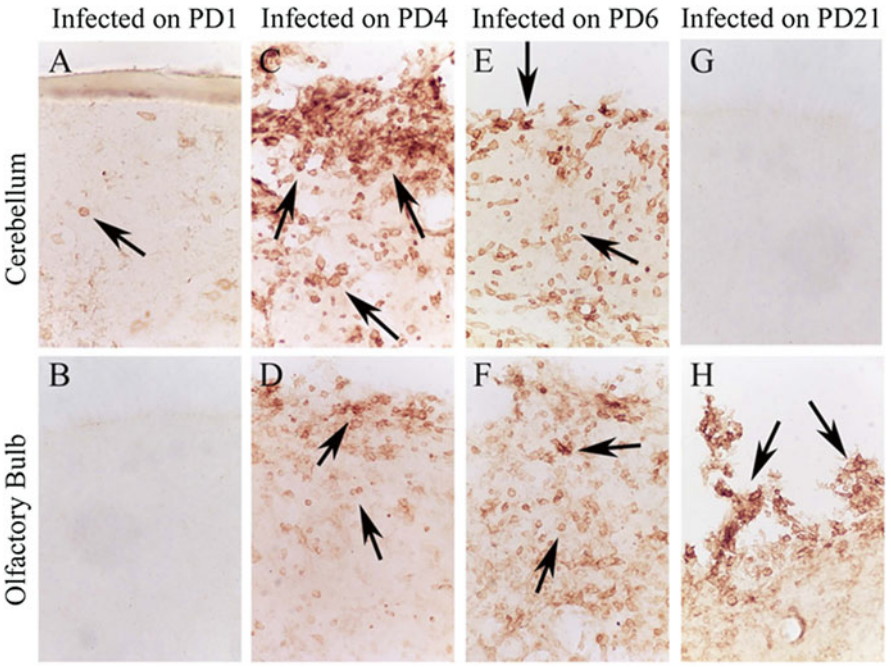

Fig. 7 The immune response to LCMV within the developing brain depends on the age of the animal at the time of infection and is region-dependent. Shown here are 40-μmicron-thick sections through the cerebellum and olfactory bulb of rats immunohistochemically stained for CD8 antigen (a lymphocyte marker). The rat pups were injected with LCMV at the ages indicated and killed 10 days postinoculation. Inoculation on PD 1 results in minimal lymphocytic infiltration into the cerebellum (**a**) (*arrow*) and no lymphocytic infiltration into the olfactory bulb (**b**). In contrast, inoculation 3 days later (on PD 4) results in a robust lymphocytic infiltration into the cerebellum (**c**) and olfactory bulb (**d**). The same basic patterns are seen following inoculation on PD 6 (**e, f**). In further contrast, however, inoculation on PD 21 results in no lymphocytic infiltration into the cerebellum (**g**) but a substantial infiltration into the olfactory bulb (**h**). This difference between cerebellum and olfactory bulb on PD 21 reflects the fact that the cerebellum contains no mitotically active neuroblasts and is thus uninfectable on PD 21, while the olfactory bulb still has many mitotically active neuroblasts and remains infectable at that age

Chapare virus is an arenavirus that can induce hemorrhagic fever, that the infection can be fatal, and that the disease can occur in outbreaks. Thus, last year (2014), Chapare virus was added to the list of Category A Priority Pathogens.

Lujo Virus

Lujo virus is the latest discovered arenavirus that is pathogenic in humans. This virus was discovered in 2008 in Africa and gains its name from the combination of the two cities in which the 2008 outbreak occurred—Lusaka (in Zambia) and Johannesburg

(in South Africa). The first patient in the 2008 outbreak developed a fever, followed eventually by multiple organ dysfunction (Briese et al. 2009). In the course of treating this patient, the viral infection was transmitted to three healthcare workers, one of whom transmitted the virus to a fourth healthcare worker. This outbreak of Lujo hemorrhagic fever had a mortality rate of 80 %, as the first four patients all died, while the fifth patient received ribavirin and survived. The horizontal spread of infection demonstrates that, unlike most arenaviruses that require exposure to rodents, Lujo can be transmitted from human to human. (The only other arenaviruses that can be transmitted this way are Lassa fever and Machupo). The clinical signs of Lujo virus infection begin after an incubation period of 7–13 days. Fever, headache, muscle pain, and pharyngitis are the first symptoms. A period of transient improvement is followed by rapid deterioration with respiratory distress, encephalopathy, and circulatory collapse (Sewlall et al. 2014). Death typically occurs 10–13 days after onset. Along with Chapare virus, Lujo virus was added last year to the list of Category A Priority Pathogens.

Junin Virus

Carried by the corn mouse (*Calomys musculinis*), Junin virus causes Argentine hemorrhagic fever (AHF), which is endemic in the central pampas region of Argentina. Infection with the Junin virus causes classic hemorrhagic fever, with signs and symptoms similar to Lassa fever. In addition, Junin induces thrombocytopenia, which makes florid hemorrhaging even more common in AHF than in Lassa fever (Harrison et al. 1999). Several hundred cases of AHF occur annually in Argentina, where agricultural workers are its most common victims, probably because of their contact with the corn mouse. The death rate of AHF is as high as 33 %, but this can be substantially reduced by treating patients during the early phase of the illness with plasma from convalescent patients. AHF can be prevented by inoculation with a live attenuated Junin vaccine (Maiztegui et al. 1998).

Machupo Virus

The rodent *Calomys callosus* carries Machupo virus, which is the cause of Bolivian hemorrhagic fever, a disease that is endemic in the El Beni region of Bolivia. Bolivian hemorrhagic fever closely resembles AHF in its clinical signs and course and has a mortality of 25–35 %. The most recent outbreak of Bolivian hemorrhagic fever occurred in 1994, with seven family members infected, of whom six died of the disease (Centers for Disease Control and Prevention (CDC) 1994).

Guanarito Virus

Infection with Guanarito virus causes Venezuelan hemorrhagic fever (VHF), a disease very similar to AHF. Prominent signs of VHF include thrombocytopenia, bleeding, and encephalopathy. The disease has a reported fatality rate of 34 %. VHF was first reported as an outbreak in the Portuguesa state of Venezuela in 1989. That outbreak included 15 cases, of which 9 were fatal. Since then, VHF has been limited to Venezuela, where it continues to induce outbreaks in isolated villages. The common rat, *Sigmodon alstoni*, is the principal reservoir for Guanarito virus in nature and is the source of infection for humans (Tesh et al. 1993). In Venezuela, recent land use changes, especially the conversion of deciduous forests to agricultural lands, has promoted the growth of rat populations and rat–human interactions (Pan American Health Organization 1995).

Sabia Virus

Of all of the arenaviruses pathogenic for man, Sabia virus is the one about which the least is known. Sabia virus is the cause of Brazilian hemorrhagic fever, the first case of which occurred in 1990 when a female agricultural engineer contracted the disease in the region of Jardin Sabia, Brazil. She presented with a rapidly progressive hemorrhagic fever and died of the disease. Her autopsy revealed liver necrosis (Lisieux et al. 1994). Since then, there have been only two additional cases of Brazilian hemorrhagic fever, both of which occurred following accidental exposures to aerosols in virology research labs. One of these virologists was treated with intravenous ribavirin and had a shorter and less severe clinical course, suggesting that ribavirin is effective against Sabia virus (Armstrong et al. 1999). The reservoir for Sabia virus is unknown, but is assumed to be a rodent endemic to the region of Sabia, Brazil.

Whitewater Arroyo Virus

In 1996, it was discovered that white-throated woodrats in New Mexico are persistently infected with an arenavirus (Fulhorst et al. 1996). This virus was later identified as Whitewater Arroyo virus. This virus was initially thought to cause a zoonosis only. However, in the year 2000, the virus was identified as a human pathogen, when three fatal cases of acute hemorrhagic fever occurred, and the Whitewater Arroyo virus was found to be the cause (Byrd et al. 2000). The signs and symptoms of Whitewater Arroyo virus infection are those of classic hemorrhagic fever, with fever, thrombocytopenia, bleeding, liver dysfunction, and respiratory distress. The geographic range of woodrats extends from Canada to Central America. Thus, most of the contiguous United States is within range of the rat and of the virus.

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Neurotropic Alphaviruses

Diane E. Griffin

Introduction

Alphaviruses are members of the *Togaviridae* family of icosahedral, enveloped, single-strand, message-sense RNA viruses. Eastern equine encephalitis (EEE), western equine encephalitis (WEE), and Venezuelan equine encephalitis (VEE; "Venezuelan Equine Encephalitis" chapter) 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. Alphaviruses primarily associated with rash and arthritis can also cause neurologic disease. Infection with Chikungunya virus (CHIKV) has caused encephalitis in humans and Semliki Forest virus and Sindbis virus (SINV) provide important mouse models for alphavirus encephalomyelitis. This chapter will cover infection with EEEV, WEEV, CHIKV, and SINV.

EEEV was first isolated in 1933 from the brains of horses during an epizootic of equine encephalitis in Virginia and New Jersey and was demonstrated to cause human encephalitis in 1938 (Ten Broeck and Merrill 1933; Webster and Wright 1938). 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, followed in 1938 by recovery of the same virus from the brain of a child with fatal encephalitis (Meyer et al. 1931; Howitt 1938). A related WEEV complex virus, Highlands J virus (HJV), was isolated in the eastern part of the USA in 1952 (Hayes and Wallis 1977).

SINV was first isolated in 1952 from mosquitoes collected near Sindbis, Egypt. Humans living in the Nile Delta at that time had a SINV seroprevalence of 27 %, but no disease was associated with infection (Taylor et al. 1955). The first human

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isolates of SINV were in 1961 from the blood of febrile patients in Uganda and in 1963 SINV was recognized as a cause of rash and arthritis in South Africa (Malherbe et al. 1963) and was developed as a mouse model of encephalomyelitis in 1965 (Johnson 1965). However, the first clear association of an alphavirus with arthritic disease was in 1953 when CHIKV was isolated from the sera of individuals in Tanzania with severe arthritis (Ross 1956). CHIKV neurologic disease began to be reported soon thereafter during outbreaks in India and Thailand (Nimmannitya et al. 1969; Thiruvengadam et al. 1965).

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. 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. Mosquito virus titers in saliva are highest early after the mosquito is infected and decline, along with transmission rates, after 1–2 weeks. 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.

Eastern equine encephalitis—EEEV is enzootic in the Americas along the Atlantic and Gulf coasts from eastern Canada to northern Argentina, in the Caribbean and in Central America (Gibney et al. 2011; Weaver et al. 2012; Carrera et al. 2013; Lubelczyk et al. 2013). Inland foci exist around the Great Lakes extending to South Dakota and Quebec (Chenier et al. 2010). Birds are the primary reservoir host and many avian species are susceptible to infection (Kissling et al. 1954). In North America the primary enzootic cycle is maintained in shaded swamps with the ornithophilic mosquito *Culiseta melanura* as the vector (Cupp et al. 2003; Scott and Weaver 1989). The amplifying species are wading birds, passerine birds, and starlings (Dalrymple et al. 1972; Estep et al. 2013; Molaei et al. 2013) and reptiles may be over-wintering hosts (Bingham et al. 2012; White et al. 2011). Outbreaks of equine and human encephalitis are facilitated when the virus spreads from the enzootic cycle into mosquito populations that feed on a variety of hosts (Mitchell et al. 1992; Cupp et al. 2003; Scott and Weaver 1989).

Small numbers of human cases of EEE are diagnosed in the USA each year (Fig. 1). There is no evidence that either horses or humans are important in the transmission cycle during epizootics. In South America, EEEV is enzootic along the north and east coasts and in the Amazon Basin. Sequence comparisons indicate that EEEV has evolved independently in North and South America with one subtype in North America and the Caribbean and three subtypes in South America (Brault et al. 1999). North American isolates are highly conserved, differing by less than 3 % in nucleotide sequence. The three South American subtypes diverged

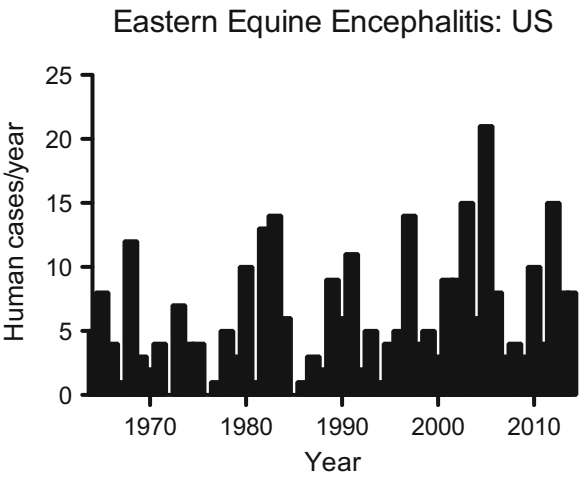

Fig. 1 Numbers of human cases of eastern equine encephalitis reported annually in the United States since 1964. Data are from the Centers for Disease Control and Prevention

1000–2000 years ago, are evolving locally with small mammals as reservoir hosts, and differ by up to 25 % in nucleotide sequence (Arrigo et al. 2010).

Western equine encephalitis—WEEV is endemic in the western portions of the United States and Canada and in 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 (Calisher 1994). 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 (Karabatsos et al. 1988) and is a recognized pathogen for turkeys, pheasants, partridges, ducks, emus, and whooping cranes (Ficken et al. 1993; Weaver et al. 1999).

WEEV in North America has caused seasonal epidemics of encephalitis in humans, horses, and emus. Major epizootics occurred every 2–3 years from 1931 to 1952 with attack rates up to 167/100,000 population (Calisher 1994). An average of 34 human cases of WEE occurred per year in the USA from 1955 to 1984, but numbers of cases have steadily declined with the last documented case in 1998 (Fig. 2). In rural areas of California endemic for WEEV seroprevalence in humans was 34 % in 1960 and 1.3–2.6 % in 1993–1995 (Froeschle 1964; Reisen and Chiles 1997). The reasons for this decline are not clear, but there is no evidence that it is due to a change in virus virulence or vector competence (Zhang et al. 2011; Reisen et al. 2008; Forrester et al. 2008).

WEEV is the result of a recombination between EEEV and a Sindbis-like virus (Hahn et al. 1988). There are four major lineages; two in South America and two widely distributed in the Americas and the Caribbean (Weaver et al. 1997; Bergren et al. 2014). The WEEV complex also includes HJV, Fort Morgan virus (FMV), and Aura virus (Calisher et al. 1988). HJV and FMV belong to lineages that diverged since recombination while Aura is a "pre-recombinant" virus (Allison et al. 2015;

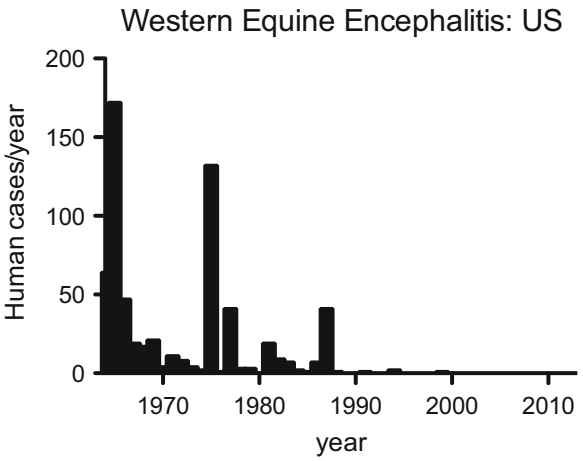

Fig. 2 Numbers of human cases of western equine encephalitis reported annually in the United States since 1964. Data are from the Centers for Disease Control and Prevention

Cilnis et al. 1996). 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 to cause the epizootic (Bianchi et al. 1993). 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 (Sabattini et al. 1985).

Chikungunya—In addition to Sub-Saharan Africa, CHIKV has caused large outbreaks of disease in India, Asia, and most recently on islands in the Indian Ocean and Caribbean and in South America (Carey et al. 1969; Halstead et al. 1969; Powers 2015; Renault et al. 2007). In Africa, the virus is maintained in a sylvatic cycle involving *Aedes africanus* and *Ae. furcifer* and nonhuman primates as well as an urban mosquito–human–mosquito cycle involving *Ae. aegypti* or *Ae. albopictus*. In India and Southeast Asia there is no evidence for a sylvatic cycle; transmission to humans in urban areas is by *Ae. aegypti* and in rural areas by *Ae. albopictus* (Ho et al. 2011; Powers et al. 2000). Three distinct lineages of CHIKV have been identified: West African; East, Central, and South African (ECSA); and Asian (Powers et al. 2000; Powers 2015). A mutation that results in an amino acid A226V substitution in the E1 protein of the ECSA genotype increased the efficiency of *Ae. albopictus* infection and likely facilitated the recent widespread outbreaks of disease in regions where *Ae. aegypti* is not prevalent (Vazeille et al. 2007; Hapuarachchi et al. 2010; Tsetsarkin et al. 2007).

Sindbis virus—SINV is a widespread alphavirus that has been isolated in Europe, India, Asia, Australia, and the Philippines from a variety of mosquito and vertebrate species (Lundstrom 1999; Lundstrom and Pfeffer 2010). The virus is maintained in a cycle between *Culex* spp. or *Culiseta* spp. mosquitoes and wild birds (Mackenzie et al. 1994). Five genotypes have been identified (Lundstrom and Pfeffer 2010) with clinical disease primarily in South Africa and northern Europe where it is known as Ockelbo disease, Pogosta disease, or Karelian fever (Espmark and Niklasson 1984; Jupp et al. 1986; Laine et al. 2004).

The Virus, Its Life Cycle, and Cellular Effects

Alphavirus virions are 60–70 nm in diameter and the RNA genome is composed of approximately 11,700 nucleotides, capped and polyadenylated. Multimers of a single capsid (C) protein arranged as an icosahedron with $T=4$ symmetry are enclosed in a lipid envelope that is derived from the host cell plasma membrane and contains the viral-encoded glycoproteins, E1 and E2. E1-E2 heterodimers are grouped as trimers to form 80 spikes on the virion surface. E2 is involved in attachment to cellular receptors, while 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 (Zhang et al. 2002; Li et al. 2010).

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 alphaviruses infect a wide range of hosts, often including birds, reptiles, 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 many receptors identified to date appears to be used exclusively, suggesting the possibility of several receptors. Alternatively, alphaviruses may use receptor–coreceptor combinations to achieve 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 glycosaminoglycans (GAGs), negatively charged unbranched carbohydrates of repeating disaccharides found on the surface of most cells (Silva et al. 2014). In cell culture, basic amino acids in the E2 glycoprotein that increase the efficiency of virus attachment to cells through GAGs are rapidly selected (Klimstra et al. 1998). Improved GAG binding generally increases clearance from the blood and decreases virulence for mice after peripheral inoculation (Byrnes and Griffin 2000). However, natural isolates of EEEV are dependent on GAGs for infection of cells that correlate with neurovirulence (Gardner et al. 2011).

Entry requires endocytosis followed by a conformational change in the trimer of E1-E2 heterodimers induced by exposure to low pH (Helenius et al. 1980; White and Helenius 1980; Sanchez-San Martin et al. 2009). This conformational change results in dissociation of E2 from E1, formation of E1 trimers, fusion of the viral envelope with the endosomal membrane, and delivery of the nucleocapsid into the cytoplasm (Wahlberg et al. 1992; Wahlberg and Garoff 1992). Cholesterol and sphingomyelin are required for E1 binding and membrane fusion. Amino acid changes in E1 can affect the fusion capacity, the lipid requirements for the target cell membrane, and the optimal pH for fusion (Boggs et al. 1989; Chatterjee et al. 2002).

The genome is released from the nucleocapsid by ribosomal removal of C and then translated (Singh and Helenius 1992). 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 (Strauss and Strauss 1994).

nsPs are translated from genomic RNA as two polyproteins (P123 and P1234) that form replication complexes tethered to cytoplasmic vacuoles that are formed from modified endosomal membranes. The polyproteins are processed into individual proteins by a papain-like protease in the C-terminal portion of nsP2. NsP1 has methyl transferase and guanylyltransferase activities, is palmitoylated and binds the replication complex to membranes. The N-terminal domain of nsP2 has helicase, ATPase, GTPase, methyl transferase, and 5′-triphosphatase activity. nsP3 is a phosphoprotein that induces membrane remodeling necessary for the formation of cytoplasmic vacuoles. nsP4 is the RNA-dependent-RNA polymerase and in vivo viral fitness is dependent on its error-prone properties (Coffey et al. 2011).

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. Replication complexes are formed as spherules at the plasma membrane and are then internalized to form a stable cytoplasmic compartment (Frolova et al. 2010; Spuul et al. 2010). RNA synthesis is regulated by sequential processing of the nsPs (Frolov et al. 2001). Early in infection, nsP2 cleaves P1234 into the minus-strand replicase, P123 plus nsP4. Later, P123 is cleaved into nsP1, nsP2, and nsP3 that changes the template specificity of the replicase to increase synthesis of plus strands and to shut off synthesis of minus strands. Only fully cleaved nsP1 + nsP2 + nsP3 + nsP4 complexes are functional in 26S RNA synthesis (Hardy and Strauss 1989; Shirako and Strauss 1994; Lemm et al. 1994).

The 26S subgenomic RNA is the mRNA for translation of the structural proteins (Strauss and Strauss 1994). Five potential structural proteins (C, E3, E2, 6K, and E1) are encoded in the subgenomic RNA as a polyprotein and an additional transframe protein (TF) is produced by −1 ribosomal frameshifting within the 6K coding region (Firth et al. 2008). 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 cysteine-rich 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 and TF is an 8K protein important for assembly that is incorporated into the virion in small amounts (Snyder et al. 2013).

PE2 and E1 are transported 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, which is shed from the cell surface. The N-terminal portion of C is conserved, basic, and 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. At the plasma membrane, the specific association of E2 tails with nucleocapsids initiates a budding process that leads to the release of mature virions (Jose et al. 2009).

Alphaviruses replicate rapidly in most vertebrate cell lines with the release of progeny virus within 4–6 h after infection. Infection causes extensive cytopathic effect characterized by cell rounding, shrinkage, and cytoplasmic blebbing with apoptotic death of infected cells within 24–48 h. Viral proteins are concentrated in the surface blebs from which budding continues to occur. This process does not hamper, and may enhance, virus replication because inhibition of apoptosis usually decreases virus yield (Levine et al. 1993, 1996; Dhanwani et al. 2012).

Apoptosis of cultured cells can be initiated during SINV fusion when membrane-bound sphingomyelinases are activated releasing ceramide, an efficient inducer of cellular apoptosis (Jan and Griffin 1999; Jan et al. 2000). Subsequent early events include activation of poly(ADP ribose) polymerase and cellular caspases, cleavage of caspase-3 substrates and fragmentation of chromosomal DNA (Nargi-Aizenman et al. 2002; Ubol et al. 1996). Alphavirus-induced apoptosis can be slowed or prevented, often in virus strain- and cell type-dependent ways, by expression of ceramidase, expression of a dominant inhibitory form of Ras, inhibition of constitutive expression of NFkB, overexpression of Bcl-2 family member and interacting proteins (Liang et al. 1998; Lewis et al. 1999), phosphorylation of PKCδ, and inhibition of caspase activity (Jan et al. 2000; Joe et al. 1996).

Alphavirus-induced vertebrate cell death can also occur by nonapoptotic mechanisms. Alphaviruses efficiently shut down host protein and mRNA synthesis (Gorchakov et al. 2005), deplete NAD and energy stores (El-Bacha et al. 2004; Ubol et al. 1996), and induce dysfunction of Na^+K^+ATPase causing loss of membrane potential and altered intracellular cation concentrations (Bashford et al. 1985; Ulug et al. 1989). In vivo, immature neurons die by apoptosis, but mature neurons are more resistant to apoptotic cell death. In particular, mature motor neurons become pale and swollen and are not protected from death by Bcl-2 family member proteins (Havert et al. 2000; Kerr et al. 2002).

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 (Calisher 1994). South American strains usually cause only mild human disease, but encephalitis has recently been documented (Causey et al. 1961; Carrera et al. 2013). A prodromal illness consisting of 1–2 weeks of fever, chills, malaise, and myalgias begins days after the bite of an infected mosquito. 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. Children are most susceptible with 1 in 8 infections resulting in encephalitis compared with 1 in 23 infections in adults (Goldfield et al. 1968). Meningismus is frequent as are focal signs including cranial nerve palsies and paralysis. Hyponatremia due to inappropriate secretion of antidiuretic hormone is a common complication. The case-fatality rate is 30–40 %, with the highest rates in children and the elderly.

Death typically occurs within 2–10 days after onset of encephalitis (Silverman et al. 2013; Deresiewicz et al. 1997; Feemster 1957).

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. Polymorphonuclear leukocytes may be abundant early with a shift to mononuclear cells over the first few days. Electroencephalograph (EEG) abnormalities are relatively nonspecific, usually showing slowing. Computed tomographic (CT) scans may be normal or show only edema. Magnetic resonance imaging (MRI) scans are more often abnormal with focal lesions most commonly observed in the thalamus, basal ganglia, and brain stem (Silverman et al. 2013; Deresiewicz et al. 1997).

Poor outcome is predicted by high CSF white cell count or severe hyponatremia, not by the size of the radiographic lesions. Recovery is more likely in individuals who have a long (5–7 day) prodrome and do not develop coma. Sequelae, including paralysis, seizures, and cognitive impairment, are common and 35–80 % of survivors, particularly children, have significant long-term neurological problems (Silverman et al. 2013; Przelomski et al. 1988; Deresiewicz et al. 1997; Feemster 1957).

Western equine encephalitis—WEEV causes encephalitis with signs and symptoms similar to those of EEEV, but with a lower case fatality rate of 3 %. There is a 3–5 day prodrome of fever and headache that may progress to restlessness, tremor, irritability, nuchal rigidity, photophobia, altered mental status, and paralysis. CSF pleocytosis is typical with 100–1500 cells/μL. Neutrophils are present early in disease and mononuclear cells later. Infants often present with rigidity, seizures, and a bulging fontanel. Transplacental transmission results in perinatal infection manifesting within the first week of life as fever, failure to feed, and seizures (Finley et al. 1955; Longshore et al. 1956; Kokernot et al. 1953).

Clinically apparent disease is most common in the very young and those over 50. The estimated case to infection ratio is 1:58 in children under 5 years and 1:1150 in adults. In older children and adults, males are 2–3 times more likely to develop disease than females. Infants and young children are more likely to develop seizures, fatal encephalitis, and significant sequelae. In infants less than 1 year approximately 60 % of survivors have brain damage, and in some the disease is progressive. In older individuals recovery is typically rapid with remission of signs and symptoms within 5–10 days, and sequelae are less common (Longshore et al. 1956; Calisher 1994; Kokernot et al. 1953; Finley et al. 1955; Noran 1944).

Chikungunya—Arthralgia with or without rash occurs in approximately 85 % of those infected (Gerardin et al. 2008) and CNS disease occurs in 16 % (Chandak et al. 2009). Neurologic manifestations are most common in children and the elderly. Encephalitis, meningitis, seizures, encephalopathy, myelopathy, and peripheral neuropathy have been observed. There is a case-fatality rate of up to 5 % and neurologic sequelae are common in those that survive (Robin et al. 2008; Renault et al. 2007). MRI abnormalities may or may not be present and the EEG abnormalities are not specific. Cerebrospinal fluid is usually unremarkable, but viral RNA can often be detected by RT-PCR (Arpino et al. 2009; Tandale et al. 2009; Chandak et al. 2009; Robin et al. 2008).

Pathogenesis and Determinants of Outcome

The initial sites of virus replication probably vary with the virus and host. Mice have received most extensive study (Taylor et al. 2015). After subcutaneous inoculation alphaviruses may infect skeletal muscle, fibroblasts or macrophages at the local site or enter Langerhans cells in the skin (Gardner et al. 2010; Morrison et al. 2011; Couderc et al. 2008; Johnson 1965). Infection of antigen-presenting cells in the skin may be the most common mode of initiating natural mosquito-transmitted infection followed by transport of virus to the draining lymph node.

Initial replication is followed by a substantial plasma viremia in amplifying hosts and 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 (Aguilar 1970; Liu et al. 1970; Monath et al. 1978). 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 (Lustig et al. 1999).

The mechanism by which encephalitic alphaviruses enter the CNS is not entirely clear. Murine studies have shown infection or transport by cerebrovascular endothelial cells, infection of choroid plexus epithelial cells, infection of olfactory neurons, and transport by peripheral nerves. Once within the CNS virus can spread cell to cell or through the CSF (Paessler et al. 2004; Vogel et al. 2005; Phillips et al. 2013; Jackson et al. 1987, 1988; Thach et al. 2000; Cook and Griffin 2003). For most encephalitic alphaviruses targeted cells within the CNS are neurons and damage to this cell can be severe and irreversible (Liu et al. 1970; Jackson et al. 1988; Phillips et al. 2013).

Outcome is influenced by characteristics of both the host and the virus. Most alphaviruses show an age-dependent susceptibility to disease (Johnson et al. 1972; Couderc et al. 2008; Aguilar 1970; Morgan 1941). Resistance to fatal disease develops between 1 and 3 weeks of age in mice 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 an innate or virus-specific adaptive immune response (Johnson et al. 1972; Oliver et al. 1997; Griffin 1976). Maturation-dependent restriction of virus replication is also seen during differentiation of neurons in culture (Schultz et al. 2015; Vernon and Griffin 2005; Castorena et al. 2008). Genetic background of the host is an additional determinant of severity of encephalitis, but only a few specific susceptibility genes have been identified (Thach et al. 2000; Tucker et al. 1996). In mice, defects in acid sphingomyelinase and innate immune responses (e.g., IFN pathway genes) increase susceptibility to fatal disease (Ng and Griffin 2006; Byrnes et al. 2000; Ryman et al. 2000, 2007; Lenschow et al. 2007; Couderc et al. 2008). For SINV, C57BL/6 mice are more susceptible to fatal encephalomyelitis than BALB/c mice and this is determined in part by an unidentified gene on chromosome 2 (Thach et al. 2001).

Virus strains differ in virulence. 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 influenced by different molecular characteristics of the virus (Dubuisson et al. 1997). Many alphavirus strains lack neuroinvasiveness in that they can cause fatal encephalitis after intracerebral or intranasal inoculation, but not after subcutaneous or intraperitoneal inoculation. Induction of IFN and susceptibility to IFN-mediated inhibition of replication also affect virulence (Gardner et al. 2009; Cruz et al. 2010; Frolova et al. 2002; Aguilar et al. 2008).

Natural isolates vary in virulence. For instance, human encephalitis due to EEEV has only rarely been recognized in South America, but occurs regularly in North America (Causey et al. 1961; Carrera et al. 2013; Goldfield and Sussman 1968). South American strains are also less virulent in experimental animals (Gardner et al. 2011). 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 (Forrester et al. 2008; Logue et al. 2009; Bianchi et al. 1993; Hardy et al. 1997). Viruses with altered virulence have also been selected after chemical mutagenesis, passage in tissue culture, passage in mice, isolation of plaque variants, and sequence manipulation of cDNA virus clones (Brown et al. 1975; Griffin and Johnson 1977; Tucker et al. 1993). Nucleotide and amino acid changes affecting virulence have been mapped to multiple regions of the genome, including the 5′ NTR, nsP1, nsP2, nsP3, E1 and E2 (Tucker et al. 1993; Dubuisson et al. 1997; Lustig et al. 1988; Tucker and Griffin 1991; Mcknight et al. 1996; Suthar et al. 2005).

Eastern equine encephalitis—EEEV is neurovirulent for marmosets, macaques, mice, guinea pigs, and hamsters and can initiate infection in the CNS by spread from the periphery to the choroid plexus (Liu et al. 1970; Paessler et al. 2004; Vogel et al. 2005). 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 (Vogel et al. 2005; Liu et al. 1970). Strain-dependent inhibition of the IFN response is postulated to facilitate CNS infection (Gardner et al. 2008). Hamsters develop fatal encephalitis, hepatitis, and lymphadenitis characterized by extensive vasculitis and hemorrhage (Paessler et al. 2004) and marmosets and macaques develop encephalitis characterized by neuronal loss, perivascular cuffing, leptomeningitis, and neutrophil-rich inflammation similar to the changes seen in humans (Adams et al. 2008).

Western equine encephalitis—Newborn mice experimentally infected with WEEV die within 48 h 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 (Aguilar 1970). After intracerebral inoculation there is infection of the choroid plexus and ependyma with subsequent spread to neurons and glial cells in the cortex, cerebellum, and brain stem and to motor neurons in the spinal cord (Liu et al. 1970). After peripheral inoculation WEEV replicates in skeletal and cardiac

muscle and occasionally spreads to the CNS (Liu et al. 1970; Monath et al. 1978). Infection of hamsters with relatively avirulent WEEV strains leads to progressive neuropathological changes consisting of perivascular inflammation, microcavitation, and astrocytic hypertrophy. Macaques develop fever and encephalitis with infection of neurons and mononuclear inflammation (Reed et al. 2005).

Chikungunya virus—Most studies have focused on arthritis and have shown that viral RNA persists in joint tissues (Hawman et al. 2013), but a few have explored the pathogenesis of neurologic disease in mice and macaques. In vitro, both neurons and astrocytes are susceptible to infection and recent strains with the A226V mutation in E1 replicate more efficiently in neuroblastoma cells (Priya et al. 2013; Das et al. 2015). Young mice are susceptible to CHIKV infection of the CNS after peripheral inoculation (Fraisier et al. 2014; Dhanwani et al. 2011; Ziegler et al. 2008; Priya et al. 2014). Older mice infected intranasally with the original Ross strain also develop neuronal infection (Powers and Logue 2007). Macaques develop neurological complications similar to those described in humans (Labadie et al. 2010).

Sindbis virus—In young mice SINV 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 (Johnson et al. 1972). 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 (Jackson et al. 1987).

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 (Lustig et al. 1992; Griffin and Johnson 1977). Virulence is determined primarily by sequences in the 5′ NTR and the E2 glycoprotein, but is influenced by changes in the E1 glycoprotein and the nsPs (Davis et al. 1986; Lustig et al. 1988; Mcknight et al. 1996; Suthar et al. 2005; Schoepp and Johnston 1993). In the 5′ NTR, a change in nucleotide 5 or 8 from A to G increases neurovirulence by unknown mechanisms (Dubuisson et al. 1997; Mcknight et al. 1996). 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 (Bear et al. 2006; Davis et al. 1986; Tucker et al. 1993; Lee et al. 2002). Neuroinvasion is affected by changes at residues 55 and 190 of E2 (Dubuisson et al. 1997).

The neuroadapted strain, NSV, was derived from AR339 (Taylor et al. 1955) by passage through mouse brain and causes fatal encephalomyelitis in 4–12-week-old mice that is immune-mediated (Griffin and Johnson 1977; Kulcsar et al. 2014). NSV has the same cellular tropism (i.e., neurons) as AR339, but replicates to higher titers in the CNS (Jackson et al. 1988). 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 (Davis et al. 1986; Tucker and Griffin 1991; Tucker et al. 1993, 1997; Lee et al. 2002; Dropulic et al. 1997). Motor neurons in the brain and spinal cord are particularly susceptible to infection and paralysis is a frequent manifestation of disease (Jackson et al. 1987, 1988). C57BL/6 mice are more susceptible to fatal disease than BALB/c mice (Thach et al. 2000; Tucker et al. 1996).

In vitro studies of cultured cortical neurons showed that SINV infection induces both apoptotic and lytic neuronal cell death and that treatment with antagonists of glutamate excitotoxicity protects from early lytic death (Nargi-Aizenman and Griffin 2001). However, in vivo neuronal death in mature animals is not due to virus replication, but rather to the immune response and AMPA glutamate receptor antagonists protect from fatal paralysis by inhibiting the inflammatory response (Kulcsar et al. 2014; Nargi-Aizenman et al. 2004; Greene et al. 2008).

Immune Responses. Virus Clearance and Immune-Mediated Damage

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 infectious virus clearance, but can also lead to immune-mediated neuronal damage.

Innate Responses

IFN is an important part of the host response to alphavirus infection. Type I (α/β) IFN is abundantly induced after alphavirus infection of experimental animals and humans (Schilte et al. 2010; Sherman and Griffin 1990; Trgovcich et al. 1999; Vilcek 1964). Animals can be protected from disease if treated with IFN or IFN-inducers before or soon after infection (Gardner et al. 2010). 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 (e.g., STAT-1) develop fatal infections even when infected with normally avirulent strains of virus (Schilte et al. 2010; Couderc et al. 2008; Byrnes et al. 2000). In the absence of IFN signaling, virus replication may occur in cells normally resistant to infection (Ryman et al. 2000).

Strains of alphaviruses vary in their ability to induce IFN and the amount of IFN produced by infected cells is usually linked to the level of virus replication (Schilte et al. 2010; Schleupner et al. 1969; Postic et al. 1969; Sherman and Griffin 1990; Hackbarth et al. 1973; Gardner et al. 2009). In vitro, induction of IFN requires viral entry and RNA synthesis and results in activation of IFN regulatory factor (IRF)-3 (Schilte et al. 2010; Behr et al. 2001). Study of temperature sensitive (*ts*) mutants suggests that formation of dsRNA is necessary for IFN induction because viruses that cannot initiate plus strand RNA synthesis do not induce IFN (Hahn et al. 1989; Marcus and Fuller 1979). SINV stimulates formation of the IRF-3/CBP/p300 transcriptional activation complex for immediate early IFNs (Behr et al. 2001) but production of IFN is cell type-dependent (Ho and Breinig 1962; Burke et al. 2009).

Treatment of cells with IFN inhibits alphavirus replication (Ryman et al. 2002; Despres et al. 1995a), but the mechanism by which this occurs, and therefore the IFN-induced host responses important for control of replication, are only partially understood. IFN-induced proteins shown to have an effect on alphavirus replication include IFN-stimulated gene (ISG)-15 (Werneke et al. 2011; Lenschow et al. 2005, 2007), 2′3′-oligoadenylate synthetase (Brehin et al. 2009), and zinc finger antiviral protein (ZAP/PARP-13) that blocks translation of incoming viral genomic RNA (Guo et al. 2007; Zhang et al. 2007; Macdonald et al. 2007). Well-studied antiviral proteins PKR and RNase L have limited roles in the IFN-induced antiviral response in vitro or in vivo (Ryman et al. 2002). 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 (Sawicki et al. 2003; Silverman 2007). Virus strains vary in their sensitivity to the antiviral activities of IFN and this may or may not correlate with virulence. Mutations associated with altered sensitivity to IFN have been mapped to the 5′ NTR, nsP1, and nsP2 (Simmons et al. 2010; Fros et al. 2010; Bordi et al. 2011).

In the CNS, neuronal infection leads to production of IFN-β by both neurons and glial cells that plays a role, independent of IFN-α, in early control of virus replication (Burdeinick-Kerr et al. 2007). Injured neurons can also produce IFN-γ, interleukin (IL)-6, and chemokines. Macrophages and glial cells become activated in response to neuronal infection and rapidly produce an additional array of cytokines and chemokines. Production of these factors results in the upregulation of MHC molecules on microglial cells and increased adhesion molecule expression on capillary endothelial cells which facilitate subsequent entry of inflammatory cells into the CNS (Metcalf et al. 2013; Lee et al. 2013).

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 and it has been postulated that the rapidly fatal disease in newborn mice is due to the production of large amounts of IFN and other cytokines (Trgovcich et al. 1999). Acute phase responses induced by alphaviruses prior to the virus-specific adaptive 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 (Klimstra et al. 1999; Wesselingh et al. 1994). 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 (Liang et al. 1999).

Virus-Specific Adaptive Responses

Both humoral and cellular immune responses are induced by infection. In experimentally infected adult mice antiviral IgM antibody is usually detected in serum 3–4 days after infection (Griffin 1976; Paessler et al. 2004). 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 begins 3–4 days after infection (Griffin and Johnson 1973; Mcfarland et al. 1972; Metcalf and Griffin 2011). Both appear to play a role in recovery from infection and protection against reinfection.

Humoral immunity—Virus-specific IgM is produced very early in human disease and often provides a means for rapid diagnosis of infection (Calisher et al. 1985, 1986). Likewise, in experimentally infected mice, IgM-secreting cells enter the CNS coincidentally with the clearance of infectious virus (Metcalf and Griffin 2011). IgG antibody appears in serum and IgG-secreting B cells enter the CNS within 7–14 days and are maintained for years (Calisher et al. 1983; Metcalf et al. 2013). 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 (Griffin and Johnson 1977; Olitsky et al. 1943; Zichis and Shaughnessy 1945). 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 (Calisher et al. 1986).

Extensive experimental studies to define the antibody specificity and the mechanisms of antibody-mediated recovery and protection have been done using VEEV (see chapter "Venezuelan Equine Encephalitis"), WEEV and SINV infections of mice. Passive transfer of antibody before or after infection can provide protection. Both neutralizing antibodies, mostly anti-E2, and non-neutralizing antibodies, both anti-E1 and anti-E2, can protect against challenge and promote recovery suggesting that virus neutralization per se is not the only mechanism of protection (Stanley et al. 1986; Hunt and Roehrig 1985; Mendoza et al. 1988; Wust et al. 1989).

Antiviral antibody can inhibit alphavirus replication intracellularly and therefore promote clearance and recovery. Treatment of immunodeficient mice that are persistently infected with SINV with immune serum clears infectious virus from the CNS and antibodies specific for either of the neutralizing epitopes on SINV E2 can downregulate intracellular virus replication in vivo and in vitro by a noncytolytic mechanism (Levine et al. 1991). Anti-E1 MAbs may also be able to alter intracellular virus replication, but this has been less extensively studied (Chanas et al. 1982).

Antibody-mediated inhibition of intracellular virus replication requires bivalent antibody, but does not require the Fc portion of the antibody, complement, or other cells (Hirsch et al. 1979; Levine et al. 1991; Ubol et al. 1995). 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 (Despres et al. 1995b; Byrnes et al. 2000). IFN and antibody act synergistically to promote recovery from alphavirus infection both in vivo and in vitro, but the mechanisms by which these systems interact have not been identified. Mechanisms of virus clearance that depend on antibody may be particularly important in the CNS where limited expression of major histocompatibility complex antigens may restrict the role of T lymphocytes (Kimura and Griffin 2000).

Antibody is also important for protection from infection (Morgan et al. 1942). Delivered before or shortly after infection, passive transfer of antibody can protect

from acute fatal disease, but may predispose to late disease associated with persistent infection, inflammation, and neuronal degeneration (Griffin and Johnson 1977; Kimura and Griffin 2003).

Cellular Immunity—Alphavirus infection induces virus-specific lymphoproliferative, cytokine and cytotoxic T lymphocyte responses (Griffin and Johnson 1973; Mokhtarian et al. 1982). Antigen-presenting cells travel to local lymph nodes where immune responses are induced and activated T cells enter the circulation and home to sites of virus replication. Activated T cells routinely cross the blood–brain barrier as part of normal immunologic surveillance of the CNS and with infection the entry of activated cells is enhanced (Irani and Griffin 1996). Infiltration of mononuclear cells into the CNS can be detected within 3–4 days after infection and includes natural killer cells, $CD4^+$ and $CD8^+$ T lymphocytes, B cells and macrophages. Proportions of different mononuclear cell populations in the CNS vary with time after infection (Moench and Griffin 1984; Rowell and Griffin 1999; Metcalf and Griffin 2011; Griffin 1976; Mcfarland et al. 1972; Irani and Griffin 1991).

Retention of T cells is immunologically specific and they have many roles during alphavirus infection (Irani and Griffin 1996). Viral RNA levels in the CNS of SINV-infected mice decrease more rapidly when $CD8^+$ T cells are present (Kimura and Griffin 2000). IFN-γ is an important effector cytokine produced by T cells and mice lacking the ability to produce antibody can clear infectious virus from some populations of neurons through production of IFN-γ (Binder and Griffin 2001). IFN-γ treatment of infected mature neurons improves cellular protein synthesis and decreases viral RNA and protein synthesis, but the intracellular effectors of IFN-γ action are not known (Burdeinick-Kerr and Griffin 2005).

Mice deficient in production of both IFN-γ and antibody do not clear infectious virus, but titers of virus during persistence are lower than in mice with severe combined immunodeficiency, suggesting that additional factors contribute to virus clearance (Burdeinick-Kerr et al. 2007).

Immunopathology—Although in young animals, virus infection of neurons can lead to neuronal death, data from several model systems of alphavirus encephalomyelitis suggest that the virus-specific cellular immune response is the major contributor to neuronal damage in older animals (Kimura and Griffin 2003; Nargi-Aizenman et al. 2004; Rowell and Griffin 2002). Mice infected with NSV become paralyzed and die during the process of viral clearance. Outcome is improved in mice deficient in β2 microglobulin, 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, suggesting a role for T cells in immunopathology (Rowell and Griffin 2002; Kimura and Griffin 2000). Furthermore, protection of NSV-infected mice from paralysis and death by AMPA glutamate receptor antagonists despite delayed virus clearance is associated with a decrease in the inflammatory response (Nargi-Aizenman et al. 2004; Greene et al. 2008).

A detrimental role for T cells in outcome of NSV infection is also suggested by study of C57BL/6 mice protected from fatal disease by passive transfer of immune serum after infection. These mice clear infectious virus, but viral RNA and antigen persist. Progressive loss of parenchyma and development of terminal deoxynucleotidyl

transferase-mediated dUTP nick end-labeling (TUNEL)-positive pyramidal neurons is correlated with infiltration of $CD4^+$ T cells and macrophage/microglial cells (Kimura and Griffin 2003). $CD4^+$ T cells have been further implicated in immunopathology by association of fatal disease with infiltration into the CNS of pathogenic Th17 cells that produce GM-CSF and IFN-γ, a process that is accelerated in the absence of the immunomodulatory cytokine IL-10 (Kulcsar et al. 2014).

Pathology

Much of the pathology observed during alphavirus encephalomyelitis is associated with the inflammatory response to infection and damage to infected neurons. Neuronal cell death may be apoptotic, particularly in young animals (Lewis et al. 1996), or may be characterized by cytoplasmic swelling, vacuolation, membrane breakdown, and cellular degeneration suggesting necrosis (Havert et al. 2000; Garen et al. 1999; Murphy and Whitfield 1970). Demyelination has occasionally been described as a consequence of EEEV and WEEV infection in humans and WEEV infection of mice, probably as a result of infection of oligodendrocytes (Bastian et al. 1975; Noran 1944).

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 cells into perivascular regions (Mcfarland et al. 1972; Moench and Griffin 1984). This phase can include perivascular extravasation of red blood cells and endothelial cell swelling and hyperplasia. 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 (Garen et al. 1999).

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 brainstem. Virus antigen is localized to neurons and neuronal death is marked by cytoplasmic swelling and nuclear pyknosis. Apoptotic glial and inflammatory cells are frequently found in the regions of affected neurons (Garen et al. 1999; Silverman et al. 2013). 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.

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. In addition, there are areas of focal necrosis and demyelination, particularly in the subcortical white matter and basal ganglia (Finley et al. 1955).

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 (Frolova et al. 2002; Dryga et al. 1997) and mouse fibroblasts producing IFN can be persistently infected (Inglot et al. 1973; Weiss et al. 1980). Persistent infection can also be established if the cell infected, such as a mature neuron, is resistant to virus-induced apoptosis (Burdeinick-Kerr and Griffin 2005; Levine et al. 1993; Ubol et al. 1995; Vernon and Griffin 2005; Schultz et al. 2015).

In vivo, there is substantial evidence that alphaviruses can persist after appearance of an immune response and clearance of infectious virus from the circulation and from tissue (Levine and Griffin 1992; Tyor et al. 1992; Metcalf and Griffin 2011). Infants and children surviving months to years after onset of WEE (often with progressive disease) may have cystic lesions, gliosis, and demyelination with areas of active mononuclear inflammation (Herzon et al. 1957; Noran 1944). Chronic joint inflammation after CHIKV infection is associated with the persistent presence of viral RNA and antigen (Hawman et al. 2013; Labadie et al. 2010).

Mice clear infectious SINV from the CNS within 7–8 days but the decline in viral RNA occurs slowly (Griffin and Metcalf 2011). Viral RNA and proteins can be detected in the nervous system and memory deficits persist long after apparent recovery (Potter et al. 2015; Burdeinick-Kerr et al. 2007). It is postulated that persistence is due to failure 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. This leads to a need for long-term control of virus replication to prevent reactivation of infection by continued presence of virus-specific T lymphocytes and antibody-secreting B cells within the CNS (Metcalf et al. 2013; Metcalf and Griffin 2011; Tyor et al. 1992).

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 (Strizki and Repik 1995). An inactivated WEEV vaccine is available for horses and as an experimental preparation for laboratory workers (Randall et al. 1947). Yearly booster doses are required for both. A live attenuated vaccine has been developed for CHIKV, but has not been licensed (Levitt et al. 1986). Many new potential vaccines for these alphaviruses are currently in preclinical development (Carossino et al. 2014).

Protection of human populations relies primarily on personal protection from mosquito bites. Mosquito populations can be monitored for infection 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.

Diagnosis is based on virus isolation or detection of viral RNA or antiviral antibody. Virus can be isolated from CSF, blood, or CNS tissue by inoculation into newborn mice or onto a variety of tissue culture cells. Direct virus detection and identification in field and clinical samples can be accomplished through various nucleic acid amplification assays (Lambert et al. 2003). Antibody is usually measured by enzyme immunoassay with detection of IgM in serum and CSF particularly useful (Calisher et al. 1986).

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.

Summary and Future Directions

Alphaviruses are mosquito-borne causes of acute encephalomyelitis 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. 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, vaccines and treatments that interfere with virus replication and protect neurons from immune-mediated damage are needed.

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Venezuelan Equine Encephalitis

Robert Seymour and Scott C. Weaver

Introduction

Venezuelan equine encephalitis virus (VEEV) is a member of the genus *Alphavirus*, family *Togaviridae*, which includes 31 species grouped into 11 complexes based on antigenic and/or genetic similarities (Weaver and Powers 2014). Like many other alphaviruses, VEEV is a mosquito-borne arbovirus that can cause disease in both humans and domesticated animals. First described in 1938 during a Venezuelan outbreak of equine encephalitis (Beck and Wyckoff 1938; Kubes and Rios 1939), VEEV has been responsible for periodic equine epizootics and human epidemics, along with an estimated tens of thousands of endemic cases annually that result from spillover from the enzootic cycles throughout much of Latin America (Aguilar et al. 2011) (Fig. 1). Like several other neurotropic alphaviruses, VEEV was also

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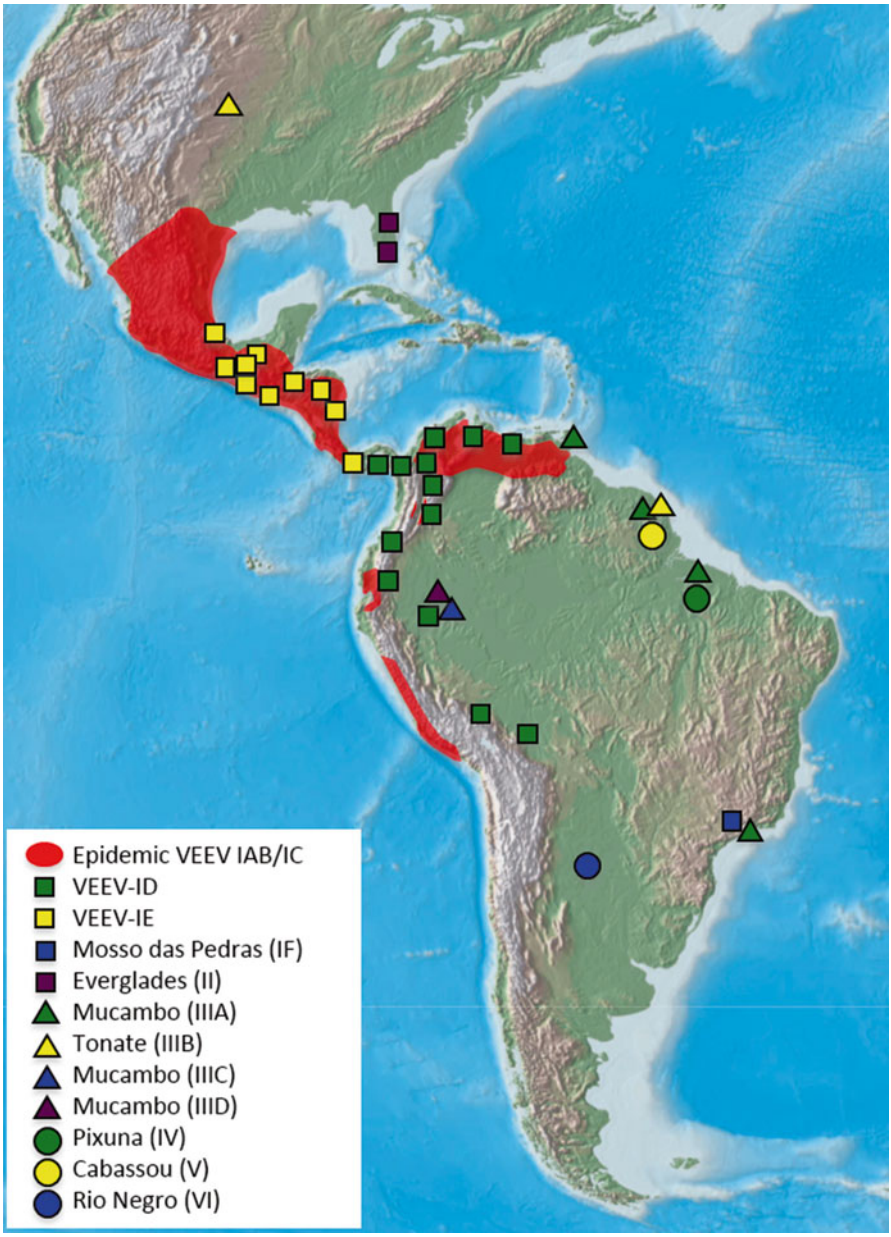

Fig. 1 Known distribution of VEE complex alphaviruses, including enzootic strains and historic epizootics/epidemics dating to the 1920s. Adapted from (Aguilar et al. 2011) with permission

highly developed as a biological weapon during the cold war and remains of concern for its potential use in biological terrorism or warfare (Sidwell and Smee 2003).

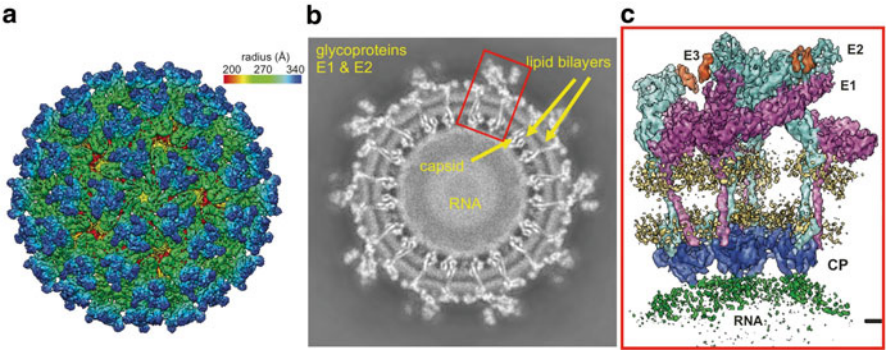

Fig. 2 Structure of VEEV including the ca. 70 nm diameter virion as determined by high resolution cryoelectron microscopy. (**a**) Single particle reconstruction of the virion showing surface spikes. (**b**) Cross-sectional view showing the plasma membrane-derived lipid envelope and embedded E2/E1 heterodimer glycoproteins. (**c**) Model of E2/E1 proteins and underlying capsid proteins (CP) based on crystal structures fit into the electron microscopic map. Adapted from (Zhang et al. 2011) with permission

Virion Structure

VEEV, like other alphaviruses, is a small, spherical virus about 70 nm in diameter (Fig. 2). Its icosahedral nucleocapsid, with $T=4$ symmetry, includes 240 copies of the capsid protein surrounding a copy of genomic RNA that is approximately 11 kB in length (Fig. 1) (Zhang et al. 2011). A host cell plasma membrane-derived lipid envelope, into which trimeric spikes composed of E2/E1 heterodimeric envelope glycoproteins, surrounds the nucleocapsid during its budding from infected cells. Within these heterodimers, E2, which is thought to interact with cellular receptors, forms the tips of the spikes with E1 lying mainly underneath. Interactions between the cytoplasmic tails of the E2 proteins and the capsid proteins impose the icosahedral symmetry of the nucleocapsid on the entire virion.

Viral Genome

VEEV packages genomic RNA, which is a single-stranded, positive or messenger sense, single molecule that is capped and polyadenylated (Fig. 3). Packaging of the genomic RNA occurs in the cytoplasm of infected cells and specificity is imparted by a signal in the nonstructural protein 2 (nsP2) (Kim et al. 2013). The genomic RNA includes an open reading frame (ORF) for the nonstructural polyprotein, which comprises ca. 2/3 of the genome starting just downstream of a 5′ untranslated region (5′UTR). For VEEV and many other alphaviruses, there is a stop codon near the 3′ end of the nsP3 gene that undergoes read-through to produce an nsP1–4 polyprotein

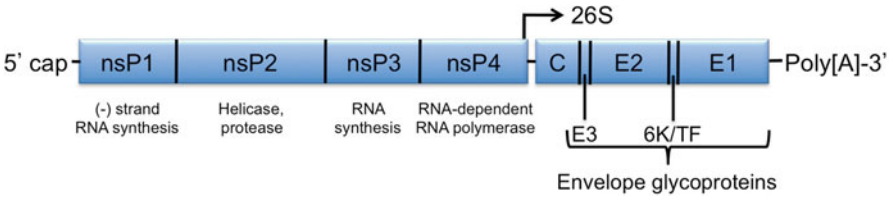

Fig. 3 Organization of the VEEV genome including the two open reading frames and names and functions of the seven major proteins produced

as well as nsP1–3. A second ORF is found in the 3′ ca. 1/3 of the VEEV genome and encodes the structural proteins: capsid, E3, E2, 6K/TF, and E1. However, this ORF is only translated from a subgenomic RNA transcribed from the minus strand copy of the genomic RNA. Like the genomic RNA, the subgenomic RNA is capped and polyadenylated for translation using the host cellular apparatus.

Viral Replication

Like other alphaviruses (Kuhn 2007), VEEV replicates principally in the cytoplasm of susceptible cells. Entry occurs through receptor-mediated endocytosis, although the receptor(s) for VEEV remain poorly understood. Once internalized through clathrin-coated vesicles, the E2/E1 heterodimers undergo a conformational rearrangement within acidic endosomes, which exposes a hydrophobic fusion peptide within the E1 protein to mediate fusion between the virion envelope and the endosomal membrane. Upon release of the nucleocapsid into the cytoplasm, it binds to ribosomes to initiate uncoating and translation of the nonstructural polyprotein. This polyprotein is cleaved in a temporally regulated manner to generate partially followed by completely cleaved nsPs, which combine with host factors within replication complexes to produce minus strand copies of the genome, followed by positive strand genomic and subgenomic RNAs. Minus strand replication is favored by the partially cleaved nonstructural polyprotein early during the replication cycle, while positive strands are favored by completely cleaved nsPs during later stages of replication.

Once the VEEV subgenomic RNA is produced, the structural polyprotein is translated and the capsid self-cleaves in the cytoplasm through its own protease activity. The envelope glycoproteins are translocated into the endoplasmic reticulum where a series of cleavages and glycosylation events occur through the secretory pathway, resulting in the deposition of the E3/E2 and E1 proteins as heterodimers into the membranes. The final cleavage event, of E3 from E2, precedes interactions between the E2 cytoplasmic tails and capsid proteins to initiate the budding process at the plasma membrane.

In vertebrate cells, VEEV is highly cytopathic, whereas in mosquito cells there is generally little or no indication of cytopathic effects (Kuhn 2007). In vertebrate

cells, VEEV like many other viruses has evolved mechanisms to evade the host cell response to its replication. These include a capsid protein with nuclear localization signals for import into the nucleus, where it interferes with the transcription of host cell mRNAs (Garmashova et al. 2007a). This allows the virus to hijack the expression of genes for its own replication and to shut off the production of interferons and other proteins designed to counteract viral replication. Also, the 2′-O methylation of the 5′ cap of VEEV counteracts vertebrate cell antiviral responses by evading the restriction of Ifit1, an IFN-stimulated gene that regulates translation. The secondary structure within the VEEV 5′UTR alters Ifit1 binding and its impact on translation (Hyde et al. 2014). In mosquito cells, VEEV does not interfere with host cell gene expression and persistent infections occur both in vitro and in vivo.

General Description of Illness

VEEV is the etiologic agent of a flu-like syndrome characterized by high fever, myalgia, headache, malaise, rash, and often gastrointestinal disease. The vast majority of infections, which are typically misdiagnosed as dengue, are self-limited. In a very small percentage of cases (0.5 % in adults and about 4 % children) encephalitis occurs (Weaver et al. 2004). Symptoms of central nervous system involvement include photophobia, headache, ataxia, seizures, somnolence, paralysis, and sometimes coma. The mortality rate for VEE complicated by encephalitis in adults is approximately 10 % and in children up to 35 % (Weaver et al. 2004; Johnson and Martin 1974; Briceno Rossi 1967). Although most cases of human encephalitis are detected during equine-amplified epidemics, there is no compelling evidence for a difference in virulence between the epidemic subtype IAB and IC strains compared to many enzootic subtypes and lineages (Johnson et al. 1968; Aguilar et al. 2009, 2011; Quiroz et al. 2009).

VEEV Strain Diversity and Phylogenetics

VEEV is one species within the VEE complex of alphaviruses (Calisher et al. 1980; Powers et al. 2011). Originally categorized as antigenic subtypes and varieties based on hemagglutination inhibition and other serological assays (Young and Johnson 1969a), members of the complex are now also recognized to comprise several distinct species (Fig. 1; Table 1). Most of these species and VEEV subtypes are considered “enzootic” viruses that circulate continuously in tropical forest or swamp habitats, most using rodents as reservoir/amplification hosts and mosquitoes in the subgenus *Culex* (*Melanoconion*) as vectors (Weaver et al. 2004). Like the epizootic/epidemic strains described below, many of these enzootic viruses cause febrile illness that can progress to neurologic disease when humans are infected via direct spillover from the enzootic cycles (Aguilar et al. 2011). However, the enzootic VEE

Table 1 Alphaviruses in the Venezuelan equine encephalitis complex

Species	Subtype	Variety	Disease syndrome	Distribution
VEEV	I	AB	Febrile illness, encephalitis	North, Central, South America
		C	Febrile illness, encephalitis	South America
		D	Febrile illness, encephalitis	South America, Panama
Mosso das Pedras (MEDV)		E	Febrile illness, encephalitis	Central America, Mexico
		F	None recognized	South America
Everglades (EVEV)	II		Febrile illness, encephalitis	Florida (USA)
Mucambo (MUCV)		A	Febrile illness, myalgia	South America, Trinidad
		C (strain 71D1252)	None recognized	Peru
		D	Febrile illness	Peru
Tonate (TONV)	V	(IIIB)	Febrile illness, encephalitis	Brazil, Colorado (USA)
Pixuna (PIXV)	IV		None recognized	Brazil
Cabassou (CABV)	V		None recognized	French Guiana
Rio Negro (RNV)	VI		None recognized	Argentina

complex alphaviruses are generally avirulent for equids, with the exception of VEEV subtype IE strains implicated in recent Mexican epizootics (Gonzalez-Salazar et al. 2003; Sahu et al. 2003). Only two of the VEEV subtypes, IAB and IC, are almost uniformly virulent for equids, and occur in epizootic cycles where equids serve as amplification hosts and a variety of mosquitoes in genera such as *Aedes* and *Psorophora* serve as vectors.

All recent phylogenetic analyses indicate that the VEE complex comprises a monophyletic group descended from a common ancestor (Forrester et al. 2012b). All of the species in the VEE complex also comprise monophyletic groups except for VEEV, which is paraphyletic with Everglades virus grouping with VEEV subtype ID (Fig. 3). Within the well-represented subtypes such as ID and IE, independently evolving lineages are generally defined by geographic regions, suggesting that the limited dispersal of the mosquito vectors and rodent hosts result in limited movement of individual lineages. The subtype IAB and IC strains of VEEV occupy three distinct clades that fall within one particular lineage of subtype ID strains, which occurs in northern South America. This relationship, along with reverse genetics and experimental equine infections, supports the hypothesis that the epizootic strains evolve periodically from enzootic ID progenitors, which undergo antigenic alterations along with adaptation for equine viremia (Anishchenko et al. 2006; Powers et al. 1997).

Epidemiology

Outbreaks of equine encephalitis were first recognized in Venezuela and Colombia during the 1920s (Lord 1974), but the etiologic agent, VEEV, was not isolated and characterized until 1938 in Venezuela (Kubes and Rios 1939; Beck and Wyckoff 1938). Although there was undoubtedly spillover from the equine-mosquito cycles to humans during these early epizootics, VEEV was not recognized as a human pathogen until the 1950s when infections were recognized in enzootic regions of Colombia (Sanmartin-Barberi and Osorno-Mesa 1954). Then, infections of large numbers of people were recognized to occur during equine epizootics during the 1960s (Fig. 4) (Johnson and Martin 1974; Suarez and Bergold 1968). These earlier studies as well as more recent ones indicate that, although humans become viremic and also shed VEEV into the nasopharyngeal fluids and saliva, direct human-to-human transmission cannot be detected (Rivas et al. 1997). These equine

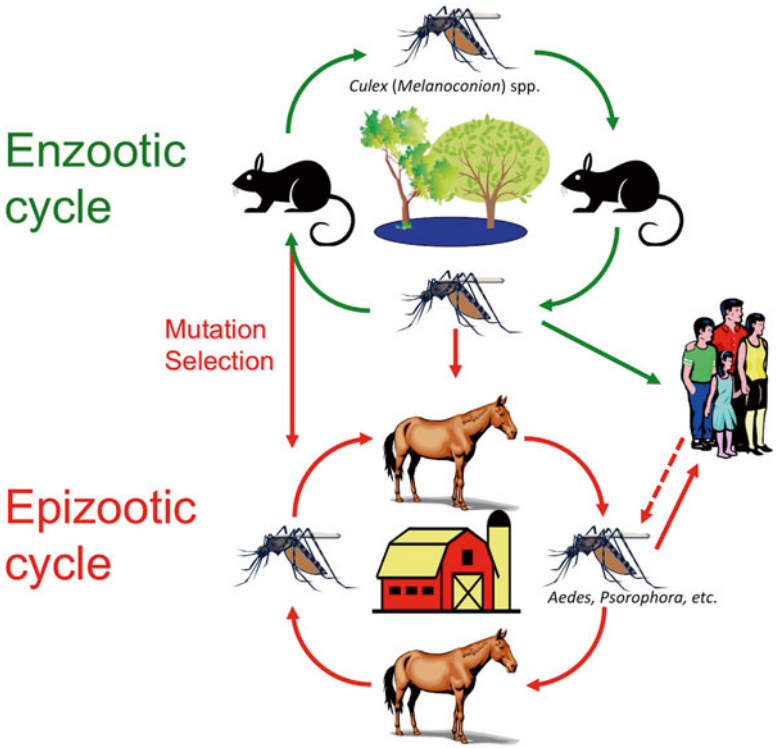

Fig. 4 Cartoon depicting the enzootic and epizootic transmission cycles of VEEV. *Red arrows* indicate host range changes involving adaptive E2 envelope glycoprotein amino acid substitutions that adapt epizootic strains for equine amplification or transmission by epizootic vectors

epizootics/epidemics were also recognized to occur at intervals of approximately 10–20 years, presumably because equine mortality and immunity in survivors following outbreaks left herd immunity too high for efficient amplification until populations turned over. Retrospective sequencing studies later supported the origin of several epizootics between 1943 and 1973 involving subtype IAB strains from incompletely inactivated vaccines generated during that era from these equine-virulent strains (Weaver et al. 1999b). However, more recent outbreaks in 1992–1993 and 1995 involved subtype IC strains that are not known to have been used for vaccine production.

Although human infection in the absence of equine disease was recognized decades ago, the burden of human disease due to direct spillover from the continuous enzootic cycles (transmission from rodents to humans by enzootic mosquito vectors; see Fig. 4) was not appreciated until human serosurveillance and disease surveillance were implemented more recently in several Latin American locations. These seroprevalence studies show relatively high rates of human seroprevalence in enzootic regions despite little or no diagnosis of human infections, and robust surveillance has detected hundreds of infections per year in some locations (reviewed in (Aguilar et al. 2011)). Because human infections are associated with high apparent:inapparent case ratios, these studies indicate that human VEE typically goes undiagnosed, probably usually confused with dengue and other tropical infectious diseases with undifferentiated signs and symptoms. This situation is unlikely to change until affordable, point-of-care diagnostics become available and are implemented in enzootic regions.

Mosquito Vectors

Although a few other arthropods (Linthicum et al. 1991; Linthicum and Logan 1994) are susceptible to infection with VEEV, natural transmission has only been attributed to mosquito vectors. During equine-amplified epizootics, several different species of mosquitoes in diverse taxa have been implicated in transmission and epizootic strains appear to be opportunistic in their vector usage (Fig. 4) (Weaver et al. 2004). Infected equids can generate high levels of viremia that last several days, permitting mosquitoes that are only modestly susceptible but are abundant to propagate the transmission cycle. In contrast, enzootic strains of VEEV and VEE complex viruses are mainly transmitted by very closely related mosquitoes in the Spissipes section of the subgenus *Culex* (*Melanoconion*). These are mostly forest or swamp dwelling mosquitoes that are often associated with small rodents that serve as enzootic amplification hosts. In some locations a single species appears to serve as the principle enzootic vector (Cupp et al. 1979) while in others up to three species transmit at similar levels (Ferro et al. 2003). Horizontal transmission among rodents or equids by VEEV vectors appears to be the only mode of circulation, with no evidence of vertical transmission among mosquitoes.

VEEV Infection and Replication in Mosquitoes

Infection of the mosquito vector begins with the ingestion of viremic blood from an infected rodent or equid, with initial infection of midgut epithelial cells during blood digestion (Weaver 1986; Smith et al. 2008). Following replication in these cells and spread to others in the midgut, VEEV must traverse a surrounding basal lamina to gain access to the hemocoel or open body cavity, where it can disperse freely to other organs and tissues. The fat body appears to be an important site of secondary replication. Ultimately, typically within 3–5 days of oral infection, VEEV infects the salivary glands and is shed into the apical cavities of the acinar cells. Then, upon a subsequent blood meal, saliva with virus is injected into a vertebrate host during probing and feeding to complete transmission. Although there is evidence of pathogenic effects in some vectors of other alphaviruses (Weaver et al. 1988, 1992), no evidence has been generated to indicate that VEE adversely affects its mosquito hosts.

Adaptation for Vector-Borne Transmission

The highly susceptible nature of some enzootic vectors to low doses of VEEV in viremic blood meals (Scherer et al. 1981; Turell et al. 2000; Galindo and Grayson 1971) suggests that enzootic strains have adapted evolutionarily for efficient infection. This characteristic, combined with the lack of overt disease or mortality in experimentally infected rodent hosts, also suggests evolution of both natural rodent (Carrara et al. 2005; Young and Johnson 1969b; Deardorff et al. 2009) and mosquito hosts for resistance to disease caused by infection. However, arboviruses like VEEV face additional challenges for continuous horizontal transmission. One of these is the many physical barriers that can result in viral population bottlenecks during the cycle. These bottlenecks, which can result in fitness declines in RNA viruses including alphaviruses due to Muller's ratchet (Weaver et al. 1999a), have been shown to occur repeatedly during VEEV infection of mosquito vectors and transmission via the saliva (Forrester et al. 2012a; Smith et al. 2005, 2008) but do not appear to result in the accumulation of deleterious mutations in nature. This apparent conundrum probably reflects our incomplete understanding of mechanism whereby VEEV and other arboviruses regain genetic diversity in their quasispecies populations during natural infections.

Pathophysiology

As mentioned in previous sections VEEV causes a flu-like illness, which in a minority of cases leads to central nervous system involvement (Weaver et al. 2004). In naturally acquired disease the virus is transmitted by the bite of an infected mosquito. After inoculation the virus is transported to the lymph nodes where the virus infects

lymphoid cells and produces viremia. During this lymphotropic phase of disease the virus is spread to other lymphoid tissues: spleen, gastrointestinal tract, and lymph nodes distant from the site of inoculation, in addition to spreading to the major viscera. It is during this phase that the individual would manifest flu-like symptoms (Schoneboom et al. 1999, 2000; Grieder et al. 1995, 1997; Garcia-Tamayo et al. 1979; Gorelkin 1973; Ryzhikov et al. 1995; de la Monte et al. 1985; Leon 1975). Also during this phase the adaptive immune system is activated and the first neutralizing antibodies begin to appear. In those cases complicated by encephalitis this flu-like first phase is followed by a second phase with neurologic manifestations: photophobia, headache, ataxia, seizures, paralysis, and sometimes coma. During this neurotropic phase viremia has either resolved or is resolving. It appears that instead of directly crossing the blood–brain barrier VEEV gains access to the brain via the olfactory mucosa. This apparently occurs through infection of the olfactory nerves of the cribriform plate via the blood circulation (Charles et al. 1995). After the virus gains entry into the brain it disseminates and infects astrocytes and neurons. Once the brain is infected, the signs and symptoms of encephalitis appear. In the majority of cases (90 % in adults and about 70 % in children) the encephalitis resolves, though a minority of patients suffer long-term sequelae such as paralysis, paresthesia, ataxia, and other neurologic deficits (Weaver et al. 2004; Johnson and Martin 1974; Briceno Rossi 1967).

In addition to the naturally acquired mosquito-borne infection, there have been instances of laboratory acquired infection. The virus can be contracted via needle stick and via aerosol. VEEV is not only very easily aerosolized it is easily transmitted via aerosol. Even with this ease of transmission via aerosol, the illness is usually not fatal (Hanson et al. 1967; Slepushkin 1959). The easy aerosol transmissibility led both the USA and the Soviet Union to research and weaponized VEEV in the 1950s and 1960s (Sidwell and Smee 2003). These programs have since been ended.

Diagnosis of VEE can be difficult due to the nonspecific nature of its symptoms. Diagnosis is also hampered by other diseases which share many of these same symptoms, although during major, equine-amplified outbreaks most physicians are aware of the likelihood of human infections. Many cases of VEE, though, occur from spillover infections in people living near enzootic foci, with no equine involvement, and these infections are typically misdiagnosed as dengue or other tropical diseases which are symptomatologically similar to VEE (Aguilar et al. 2011). If VEE is suspected, virus may be isolated from the serum of the patient if caught early enough in the course of disease. Other methods of detection would include RT-PCR of bodily fluids and hemagglutination assays. In addition, the plaque reduction neutralization test can be used to quantify the titer of neutralizing antibodies. Using ELISA one can identify anti-VEEV antibodies such as IgM or IgG. During early infection, VEEV specific IgM levels rise with a concomitant decrease in viral titer. At about 7–10 days IgG begins to appear and can be positive for months to years (Juarez et al. 2013; Shipley et al. 2012; Kirsch et al. 2008; Caceda et al. 2007; Coffey et al. 2006).

Histologic changes were seen and analyzed in a series of 21 autopsies from fatal VEE infections (de la Monte et al. 1985). Pathologic changes occurred predominantly in the brain, spleen, lymph nodes, liver, gastrointestinal tract, and lungs.

Because the brain can respond to infection in only a limited number of ways, the pathologic changes, like the illness, are rather nonspecific. Changes in the brain consisted of meningoencephalitis, perivascular cuffing, hemorrhage, vasculitis, and in some cases loss of neurons. Pathologic changes in the lymph nodes, spleen, and gastrointestinal tract consisted mostly of depletion of lymphocytes and necrosis of lymphoid follicles. Livers of most of the patients demonstrated individual hepatocyte degeneration and focal areas of hepatocellular necrosis. The inflammatory infiltrate was relatively mild in most of the cases and was predominantly composed of lymphocytes and neutrophils. Lesions in the lungs most often consisted of an interstitial pneumonia with pulmonary congestion and edema. Several of the lungs demonstrated intra-alveolar hemorrhage and a superimposed acute bronchopneumonia (de la Monte et al. 1985; Johnson et al. 1968).

Animal Models

Mice

Because of the lack of clinical and autopsy samples, the human pathogenesis of VEE is still not well understood, though animal models have given us clues to its pathogenesis. By far the most common animal model used to study VEEV is the mouse (Davis et al. 1994; Charles et al. 1995; Steele and Twenhafel 2010). Though there are a few similarities with human disease, there are major differences. VEE is uniformly fatal in mice. Generally neither the subtype of virus nor the age of the mouse makes any difference in mortality (Ludwig et al. 2001; Steele et al. 1998). Mice begin to show the first signs of illness by approximately day 3 postinfection: weight loss, lethargy, and ruffled fur. The severity of these signs of illness worsen over the next three or so days at which time some mice begin to exhibit signs of central nervous system involvement including tremors, paralysis, and hypersensitivity to touch. Viremia in mice lasts for approximately the first 4 days postinoculation usually peaking about day 2. Histologic changes seen in the brain become evident early in the illness (day 3) and include neuronal death, perivascular cuffing, hemorrhage, edema, meningitis, and infiltration of the parenchyma by inflammatory cells (neutrophils, lymphocytes, and macrophages) (Fig. 5). Vasculitis is not usually present. Lesions outside of the brain include: lymphoid depletion and necrosis in bone marrow, lymph nodes, gastrointestinal tract, and spleen. The depletion of lymphocytes is another commonality between disease in the human and mouse. After the initial depletion of lymphocytes, these organs recover and develop lymphoid hyperplasia.

Although the VEE in mice is in some respects very different from that of humans, studies in mice are still very valuable. They can give insights into which portions of the immune system are important in viral control and can give indications as to which proteins act as virulence factors. In addition, since the disease is so virulent in mice, this makes them a very valuable tool in testing vaccine efficacy and safety. This would also make mice valuable in testing the efficacy of other specific treatments (Steele and Twenhafel 2010).

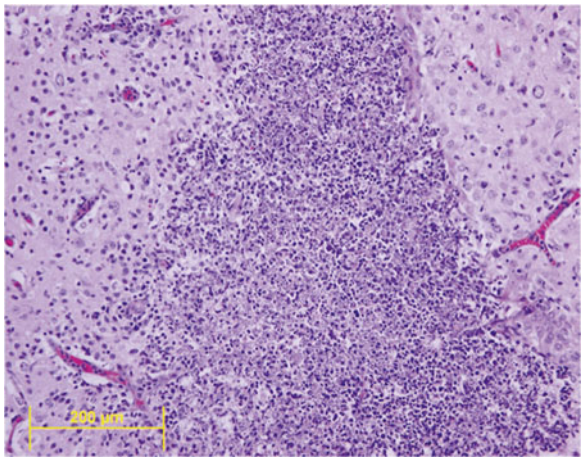

Fig. 5 Hematoxylin and Eosin stain of an infant mouse brain infected with VEEV strain TC-83. The micrograph demonstrates an inflammatory infiltrate composed predominantly of neutrophils and mononuclear cells. There is perivascular cuffing and necrosis

Nonhuman Primates

The other animal model used in the study of vaccine and treatment efficacy in addition to virulence is the nonhuman primate (Steele and Twenhafel 2010; Gleiser et al. 1962; Danes et al. 1973; Dupuy and Reed 2012). The course of disease in *Cynomolgus macaques* is almost identical to that in humans, with most cases being self-limited and the vast majority surviving infection. Typically they too show a biphasic illness and initially develop fever, viremia, and lymphopenia. Like humans viremia is highest during this phase of illness. IgM begins to rise and then viremia declines. In the neurotropic phase a minority of animals develop signs of encephalitis a few days later. During serial sacrifice studies brain lesions were most prominent in the midbrain and olfactory cortex followed by dissemination to the remainder of the brain. Most of these lesions demonstrated perivascular cuffing by lymphocytes, evidence of gliosis, and mild neuronal damage. Most of the lesions ultimately resolved. In this study there was no evidence of hemorrhage, vasculitis, meningitis, demyelination, and parenchymal infiltrates of neutrophils, whereas humans have evidence of hemorrhage, vasculitis, and meningitis (Steele and Twenhafel 2010; Gleiser et al. 1962; Danes et al. 1973; Monath et al. 1992).

Immune Responses to Infection

Antibodies

As for most of the alphaviruses, neutralizing antibodies play a critical role in control and clearance of VEEV (Smith et al. 2009). During the early phase of illness, neutralizing antibodies begin to appear in the serum. These antibodies are predominantly of the IgM class and are usually detectable by days 4–6 postinfection. Once

this occurs, viremia declines sharply. Over the next few weeks IgM slowly declines. At about days 8–11 postinfection neutralizing antibodies of the IgG class begin to appear. At this point IgG becomes the predominant antigen-specific antibody against VEEV. The affinity for IgG for the viral particle is generally much higher than that of the initial IgM produced. By its nature the neutralizing antibody regardless of class inhibits the virus from attaching and entering cells. Not all antibodies produced are neutralizing and can help the response to the virus in numerous different ways. Antibodies help the phagocytic cells, predominantly monocytes/macrophages, through opsonization. In the process of opsonization antibodies attach to the virion and phagocytic cells recognize the Fc portion of the antibody through various Fc receptors (CD16, CD32, and CD64). This interaction makes it easier for the phagocytic cell to ingest the virus removing it from the circulation or tissues. Antibodies also promote opsonization through the fixation of complement. During the activation of the complement cascade numerous split products are produced which attach to the surface of the virion. Phagocytic cells have receptors for many of these split products thus helping them ingest and destroy the virus. Another function of the antibody is fixation of complement. During this process, which is classically thought of with bacteria, the complement cascade goes to completion with the production of the membrane attack complex which opens pores in the lipid bilayer destroying the virion (Schoneboom et al. 1999, 2000; Grieder et al. 1995, 1997; Garcia-Tamayo et al. 1979; Gorelkin 1973; Ryzhikov et al. 1995; Juarez et al. 2013; Shipley et al. 2012; Kirsch et al. 2008; Caceda et al. 2007; Coffey et al. 2006; Johnson KM et al. 1968; Charles et al. 1995; Ludwig et al. 2001; Steele et al. 1998, 2006; Steele and Twenhafel 2010; Danes et al. 1973; Victor et al. 1956; Monath et al. 1992; Phillpotts et al. 2002, 2005; Bennett et al. 1998).

T Cells

Although the humoral immune response is critical in protection of a vaccinated host, the T cell response is still important, though less well characterized. Studies with TC83 indicate that the T cell response is predominantly Th1 (Bennett et al. 1998, 2000). However, mice vaccinated parenterally with TC83 lack a detectable CD8+ response in the spleen or draining lymph nodes (Jones et al. 2003). Vaccination studies indicate that the neutralizing antibody response is important in fending off infection in the periphery while T cells are important in the immune response during CNS infection (Paessler et al. 2006, 2007). These findings indicate that CD4+ T cells are critical in viral clearance in the CNS and can do so in the absence of antibodies. Others have also shown a role for CD4+ T cells in the alleviation of CNS disease in vaccinated animals in the absence of antibodies (Yun et al. 2009). It is of interest that vaccinated animals deficient in γ/δ T cells have been shown to be protected from lethal challenge but have viral persistence in the brain (Paessler et al. 2007). The role of CD8+ T cells is less well understood. There is speculation that they are less important in the viral clearance from the brain due to the cytotoxic/lytic mechanisms of their effector functions. This is important, especially

considering that a major target of VEEV infection in the brain is neurons and T cell mediated neuronal loss would be detrimental. In certain inbred mouse strains NK cells contribute to the CNS pathophysiology seen in VEE (Taylor et al. 2012).

Innate Immune Responses and Control of Disease

As with all alphaviruses type I interferons play a critical role in host defense (Smith et al. 2009). During an infection cells produce type I interferons to set up an antiviral state in the infected cells and in neighboring cells. A lack of type I interferon production or the inability of cells to respond to interferons renders the host extremely susceptible to the effects of the virus. In the normal host these interferons help keep the virus under control until the adaptive immunes response takes over (Lukaszewski and Brooks 2000; Anishchenko et al. 2004; White et al. 2001; Grieder and Vogel 1999; Konopka et al. 2009). The virus has evolved ways to combat the effects of interferon on the cell. An important mechanism by which VEE combats the effects of type I interferons is to shut off host protein translation. VEE uses the capsid protein to shut off most host cell translation thus eluding the innate immune system (Garmashova et al. 2007a, b; Schoggins et al. 2011). VEEV has also developed the ability to interfere with interferon signaling via inhibition of the STAT1 protein (Simmons et al. 2009). The complement system also plays a role in the host's response to infection (Mathews et al. 1985; Brooke et al. 2012).

The immune response to VEEV, like with most pathogens, is multifaceted. Due to variations in outbred populations such as horses or humans, the relative contribution of each arm of the immune response likely varies among individuals.

Treatments and Control of Outbreaks

Supportive Treatment

Currently there are no specific licensed treatments for VEE once an individual or animal is exposed to the virus (Smith et al. 2009). All treatment at that point is supportive to keep the patient hydrated, breathing, to prevent circulatory collapse and to manage neurologic symptoms through the administration of medications and monitoring intracranial pressure. All of these treatments are designed to keep the patient alive long enough for them to fight off the virus on their own.

Vaccines

Vaccines for VEEV have been reviewed previously (Weaver and Paessler 2009). Vaccine strategies against VEEV have included live-attenuated and inactivated versions. These strategies have met with varying degrees of success. The Trinidad

donkey strain of VEEV, which belongs to IAB subtype, was serially passaged 83 times on guinea pig heart cells to produce the attenuated TC83 (Berge et al. 1961). During the 1971 Texas VEE outbreak, TC83 was administered to thousands of equids. Although it produces some fever, leukopenia, and viremia in equids, it also induces the production of neutralizing antibodies and protects from VEEV challenge (Walton et al. 1972). During this outbreak, however, it was noticed that TC83 did produce viremia in equids and the vaccine strain was discovered in mosquitoes collected in Louisiana (Pedersen et al. 1972). This led to the conclusion that TC83 could potentially spread from vaccinated horses to mosquitoes and other hosts. In addition, it has been demonstrated that of the 12 mutations identified in TC83 only two are attenuating (Kinney et al. 1993). Considering the mutability of RNA viruses, this suggests the possibility of TC83 reverting to a wild-type, virulent, equine amplification-competent strain that could initiate epizootics/epidemics (Kinney et al. 1993). Live TC83 continues to be produced and used in equids in Latin America; however, only inactivated TC83 is available for horse vaccinations in the USA.

Due to the development of VEEV as a biological weapon by the USA and the former USSR during the cold war and VEEV laboratory infections (Laboratory safety for arboviruses and certain other viruses of vertebrates. The Subcommittee on Arbovirus Laboratory Safety of the American Committee on Arthropod-Borne Viruses 1980), TC83 was used in humans as an investigational new drug (IND). In humans TC83 is very reactogenic causing mild to moderate flu like symptoms (Pittman et al. 1996). Seroconversion is attained in approximately 80 % of those receiving the vaccine. For those people who fail to seroconvert an inactivated TC83 (C84) is administered. These vaccines are administered by the U.S. Army Special Immunization Program to laboratory workers and others expected to come into close contact with VEEV. Though immunogenic, TC83 and C84 only provide partial protection to aerosol challenge in nonhuman primates and afford lesser protection to VEEV subtypes other than IAB (Jahrling and Stephenson 1984; Pratt et al. 1998; Paessler and Weaver 2009). Another strain derived from the parent of TC83, V3526, has been found to be immunogenic and safer in rodents and nonhuman primates. It also appears to have a reduced chance of transmission from a vaccinated host to the environment (Pratt et al. 2003; Hart et al. 2000; Ludwig et al. 2001; Turell et al. 2003; Reed et al. 2005; Fine et al. 2008; Rao et al. 2004). However, Phase I trials demonstrated some reactogenicity (Martin et al. 2010).

Another strategy to reduce the chances of transmission of a live-attenuated vaccine strain from vaccinated animals or mosquitoes and humans is to employ the internal ribosome entry site (IRES) of the encephalomyocarditis virus to control translation of one of the viral ORFs (Volkova et al. 2008). In this strategy the subgenomic promoter is inactivated with 13 synonymous mutations and an IRES element is inserted just downstream of the inactivated subgenomic promoter. This results in the IRES element controlling the transcription of subgenomic RNA and thus the structural proteins. The end result is an attenuated virus completely unable to infect mosquitoes. VEEV/IRES vaccine candidates have been shown to produce neutralizing antibodies in both rodents and nonhuman primates and to protect rodents from VEEV challenge and protect nonhuman primates from aerosol challenge (Rossi et al. 2013, 2015; Guerbois et al. 2013).

Another live attenuated strategy involves a recombinant Sindbis/VEEV construct (SIN/VEEV). The vaccine contains the nonstructural proteins and cis-acting RNA elements necessary for replication and subgenomic RNA transcription from Sindbis virus (SINV), and contains the structural genes of VEEV. This vaccine strategy has shown promise in mouse models of VEE and demonstrates the induction of neutralizing antibodies and protection from challenge with wild-type VEEV (Paessler et al. 2003, 2006).

Alphavirus replicons have been used to immunize mice and nonhuman primates. These replicons use a system whereby the genomic RNA containing the nonstructural protein ORF but lacking one or more structural protein genes is packaged in a virion with capsid and envelope proteins expressed from a helper RNA (Pushko et al. 2000). This packaged genome only contains the nonstructural cassette. This virus-like particle can enter the cell and uncoat. However, since the genome lacks the structural protein ORF, the virus-like particle cannot generate additional virus and spread. This system has shown promise in eliciting protective immunity in both rodents and nonhuman primates. This strategy carries the advantage of the replicon not being able to cause viremia or otherwise amplify in a vaccinated animal or human, thus not allowing the virus to spread to other hosts (Konopka et al. 2009; Pushko et al. 1997; Reed et al. 2014; Maruggi et al. 2013). Adenovirus based strategies are also being investigated (Paessler and Weaver 2009).

Inactivated vaccine candidates have also been generated against VEEV. As mentioned previously inactivated TC83 (C84) is given to horses in North America and to humans who fail to seroconvert to TC83 under an IND protocol (Pittman et al. 1996). Inactivated vaccines do show promise in generating protective immunity in rodents and nonhuman primates. The major advantage of the inactive vaccines is no live virus is used. This limits the chance of transmission and or reversion to wild-type. On the other hand inactivated vaccines usually do not produce the breadth of immune response as live-attenuated vaccines. This may require several inoculations to produce seroconversion, whereas, live-attenuated vaccines usually require one to two vaccinations. The other drawback to inactivated vaccines is the inactivation process. If a virulent strain of virus is used and incompletely inactivated, the possibility exists of causing disease in the vaccinee and/or environmental transmission (Weaver et al. 1999b; Sutton and Brooke 1954).

DNA-based vaccines have been developed for VEEV and have shown efficacy in protecting rodents and nonhuman primates from challenge. One of these DNA strategies uses intramuscular delivery of VEEV envelope protein cDNA (Dupuy et al. 2009, 2010, 2011). Another uses intramuscular delivery of a eukaryotic plasmid which contains a complete TC83 cDNA genome (Tretyakova et al. 2013).

Vector Control

The control of mosquito vectors is one of few approaches currently available to control equine-amplified epizootics and epidemics, as well as endemic spillover disease. During epizootics, aerial applications of insecticides have been used in an

attempt to reduce vector populations, but conclusive evaluation of efficacy is lacking. These outbreaks typically occur during unusually rainy seasons in northern South America, resulting in explosive populations of floodwater mosquitoes that transmit efficiently more due to their large numbers than susceptibility. This suggests that effective control should have a significant impact on circulation and spread. The prevention of endemic spillover infections from the sylvatic enzootic cycles represents a far greater challenge because these cycles are widespread and often occur in remote locations. In addition, although the enzootic vectors are typically considered forest species, they have also adapted to towns and cities where enzootic circulation has been detected in several locations (Ferro et al. 2008; Watts et al. 1997; Morrison et al. 2008; Forshey et al. 2010). In these urban locations, vector control could be more effective in reducing human infections.

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Neurotropic Flaviviruses

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Flaviviruses are small, positive-strand RNA viruses which are transmitted from infected to susceptible vertebrate hosts primarily by arthropods (Burke and Monath 2001; Gubler et al. 2007). 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 (Burke and Monath 2001; Solomon 2004; Monath 1989, 1999).

The genus *Flavivirus* is within the *Flaviviridae* family, which also includes the *Pestivirus* and *Hepacivirus* genera. The *Flavivirus* genus consists of nearly 80 viruses, approximately half of which are associated with human disease (Burke and Monath 2001; Calisher and Karabatsos 1988; Roehrig 2003; Lindenbach et al. 2007). The majority of flaviviruses are *ar*thropod-*bo*rne viruses, or arboviruses, with over half transmitted by mosquitoes, and approximately one third transmitted by ticks (Fig. 1). Five flaviviruses have no known vector (Burke and Monath 2001). 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 (Burke and Monath 2001; Mackenzie et al. 1996; Mackenzie and Field 2004).

Flaviviruses were originally characterized serologically and divided into 8 antigenic complexes and 12 subcomplexes based on cross-neutralization assays with hyperimmune antisera (Burke and Monath 2001; Roehrig 2003; McMinn 1997; Calisher et al. 1989; King et al. 2012). Phylogenetic analyses of genomic sequences

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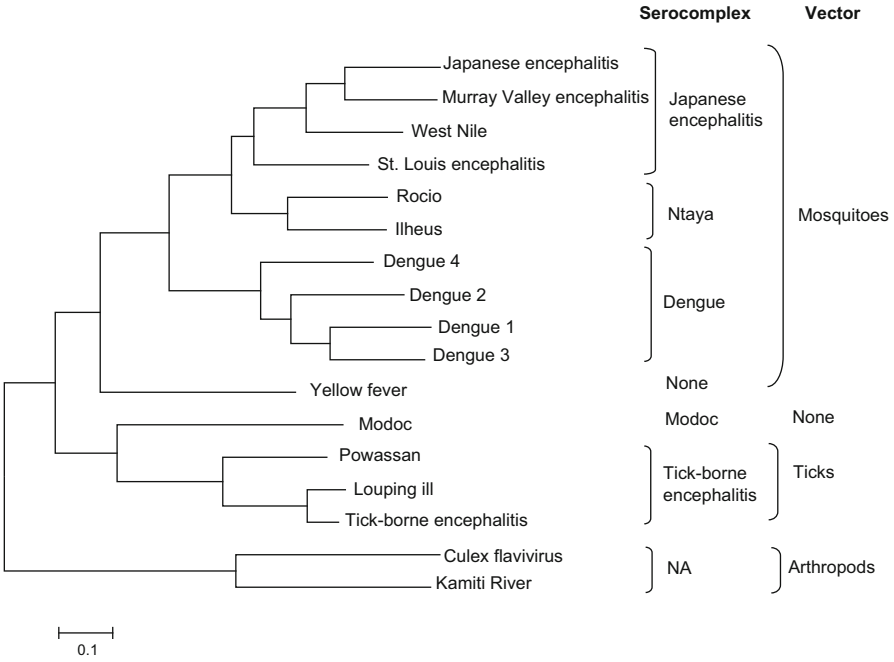

Fig. 1 General organization of a selection of flaviviruses based on phylogenetic analysis of complete genome sequences (King et al. 2012; Kuno and Chang 2005). The serological complexes and arthropod vectors are shown on the *right* (Burke and Monath 2001; Kuno et al. 1998). Nucleotide sequences were aligned using CLUSTALW in MegAlign (Lasergene 12, DNASTAR, Madison, WI); phylogeny generated using maximum likelihood (MEGA5) (Tamura et al. 2011)

from archival and recent flavivirus isolates have, in most cases, confirmed the serologically derived antigenic relationships (Fig. 1) (Burke and Monath 2001; Lindenbach et al. 2007; King et al. 2012; Kuno and Chang 2005). Although there are regions of the flavivirus genome that are highly conserved, particularly the genes that code for the antigenic epitopes in the envelope protein, there is also considerable genetic diversity within the genus, with the most distantly related flaviviruses having only about 40 % sequence homology (Burke and Monath 2001; Calisher et al. 1989).

In regions where multiple flaviviruses co-circulate a person may be at risk of serial flavivirus infections, as a prior flavivirus infection does not prevent infection by a different flavivirus. However, antibodies raised in the primary flavivirus infection may modulate the second infection, resulting in milder or more severe illness (Gubler 1998a, b; Porterfield 1986). Experimental evidence suggests that there may be some degree of cross-protection between flaviviruses, as antibodies elicited in the first flavivirus infection produce an anamnestic response and partially neutralize the second flavivirus, leading to reduced clinical symptoms (Tesh et al. 2002). This is hypothesized to be the mechanism for the lack of West Nile encephalitis cases in Central and South America compared to North America. In Central and South America many flaviviruses are known to co-circulate and serosurveys have shown that a high proportion of the population has been previously exposed to a flavivirus. In comparison, in the United States and Canada,

West Nile and St. Louis viruses have limited geographic ranges, and flavivirus seroprevalence is low. In contrast, in secondary dengue infections, the anamnestic response may enhance the entry of the second dengue serotype virus into cells, in a process call antibody-dependent enhancement of infection, which may result in more serious disease syndrome. It has been shown that in areas where transmission of multiple dengue serotypes is occurring, most dengue infections are secondary infections, and there is a higher incidence of the more serious dengue hemorrhagic fever/dengue shock syndrome in comparison to areas where a single dengue virus serotype has recently emerged and infected a naïve population (Gubler 1998a, b; Porterfield 1986).

Medically important flaviviruses are associated with three clinical syndromes: encephalitis and meningitis; hemorrhagic fever; or fever, arthralgia, and rash (Burke and Monath 2001; Gould and Solomon 2008). 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 (Burke and Monath 2001; McMinn 1997; Monath 1986; Gritsun et al. 1995). The neurotropic flaviviruses that cause neuroinvasive disease belong primarily to two groups: mosquito-borne viruses in the Japanese encephalitis serocomplex and tick-borne viruses in the tick-borne encephalitis serogroup (Fig. 1) (Burke and Monath 2001). 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, and Murray Valley encephalitis virus in the Japanese encephalitis serocomplex; in the tick-borne encephalitis serocomplex, Powassan virus and tick-borne encephalitis virus subtypes Far Eastern, Siberian, and European (Burke and Monath 2001; McMinn 1997). 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, with the exception of Japanese encephalitis virus, which will be presented in the chapter entitled "Japanese Encephalitis Virus." Dengue viruses, which usually cause febrile illness or hemorrhagic manifestations, occasionally present as meningoencephalitis, and are discussed in the chapter entitled "Japanese Encephalitis Virus. "Note added in proof: Zika virus, previously considered to cause a mild febrile illness, has recently been associated with neurological disease in French Polynesia and the Americas. Much research will be needed to determine the mechanisms of pathogenesis and the resultant clinical disease of this emerging virus."

Ecology and Epidemiology

Japanese encephalitis serocomplex viruses (Fig. 1) 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 viremia is brief and low, and rarely of sufficient 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 (Calisher et al. 1989; Kuno et al. 1998; Grard et al. 2006). 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 the chapter entitled "Influences of Arthropod Vectors on Encephalitic Arboviruses." prospective multi-center study; no evidence was. Although in general these viruses are not transmitted in nature directly from host to host, cases of human-to-human transmission have been reported to occur through blood transfusions and organ donations during epidemics of intense transmission activity (Montgomery et al. 2006; Iwamoto et al. 2003; Centers for Disease Control and Prevention 2003, 2004; Cushing et al. 2004). Possible cases of sexual and congenital transmission have also been reported (Musso et al. 2015; Foy et al. 2011; O'Leary et al. 2006)

Flaviviruses from the two neurotropic flavivirus groups are distributed widely throughout temperate and tropical regions of the world (Fig. 2). In Asia alone over three 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 is the leading cause of pediatric encephalitis throughout Asia (Campbell et al. 2011; Hills et al. 2014). In addition, flaviviruses such as West Nile virus have emerged for the first time in areas

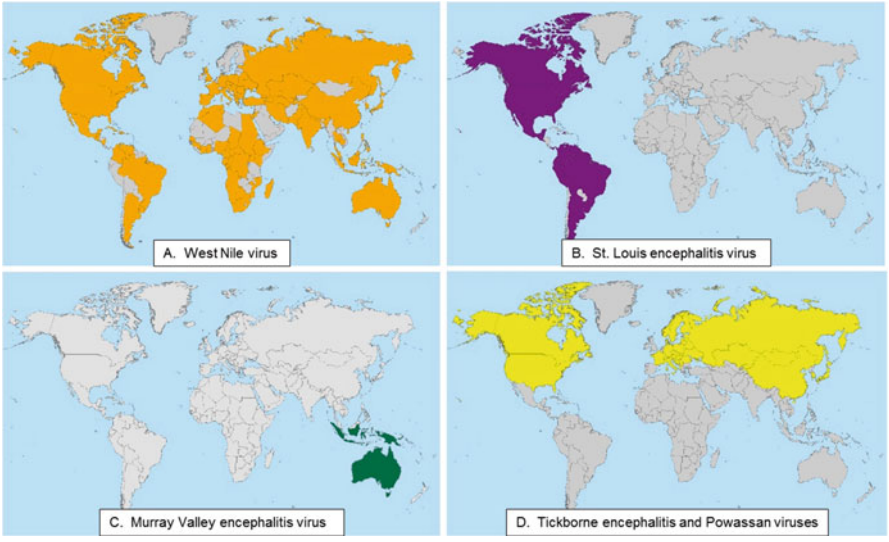

Fig. 2 Countries with historic reports of disease cases or other virus activity as of August 2015. Includes human disease cases, virus-specific antibodies in humans or other animals, or virus or viral RNA detected in mosquitoes or vertebrate animals. Geographical distribution by state or province of (**a**) West Nile virus, (**b**) St. Louis encephalitis virus, (**c**) Murray Valley encephalitis virus, and (**d**) tick-borne encephalitis flaviviruses. The distribution of St. Louis virus in Canada in Ontario, Manitoba, and Quebec was based on a single North American outbreak in 1975–1976

where competent arthropod vectors and susceptible vertebrate hosts have provided the conditions necessary for establishment of virus transmission in new ecological niches. The introduction of West Nile virus into New York in 1999 and the spread of the virus throughout North America since then has resulted in the largest outbreaks of meningitis and encephalitis in the Western Hemisphere (Solomon and Winter 2004). In contrast, although there is evidence of West Nile virus in Central and South America, there have been only sporadic reports of West Nile neuroinvasive disease cases. Powassan virus is being detected with greater frequency in wider geographical regions, probably due to increased laboratory-based surveillance for arboviruses in general since the introduction of West Nile virus.

Flavivirus Structure and Replication

Flaviviruses are small, spherical viruses with icosahedral symmetry, approximately 50 nm in diameter (Lindenbach and Rice 2001; Chambers et al. 1990) (Fig. 3). The virion is smooth, with no spikes or surface projections and comprises viral envelope and membrane proteins arranged in head-to-tail heterodimers, embedded in a host cell-derived lipid bilayer, surrounding a nucleocapsid core. The nucleocapsid consists of multiple copies of the capsid protein, arranged in an icosahedral, anchoring the RNA genome (Lindenbach et al. 2007; Kuhn et al. 2002; Mukhopadhyay et al. 2003). The single-stranded, positive-sense RNA genome, approximately 11-kb in length, functions as an mRNA, with a single open reading frame. The genome is flanked at both the 5′ and 3′ ends by untranslated regions, and capped at the 5′ end (Fig. 4). Viral proteins are translated in a polyprotein that is co- and posttranslationally cleaved by cellular and viral proteases and glycosylated by cellular glycosyltransferases into three structural proteins: capsid, premembrane, and envelope; and seven nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Lindenbach and Rice 2001; Zhang et al. 2003). The structural proteins make up the virus particle; the

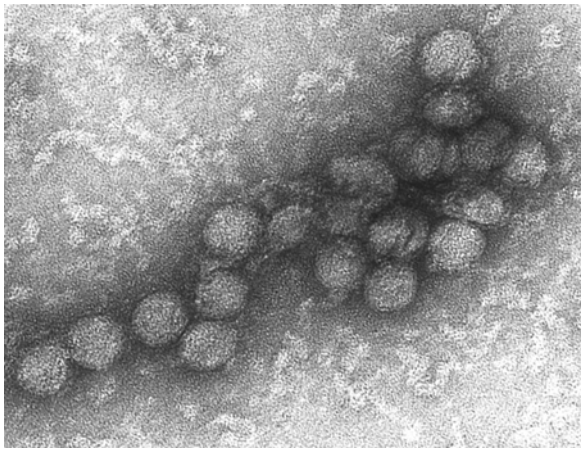

Fig. 3 Negative staining electron micrograph of West Nile virions, approximately 45 nm in diameter (×216,000). The virus was isolated in Vero E6 cells from an organ transplant recipient who died in 2002 (Cushing et al. 2004) (From C. Goldsmith, CDC Public Health Image Library)

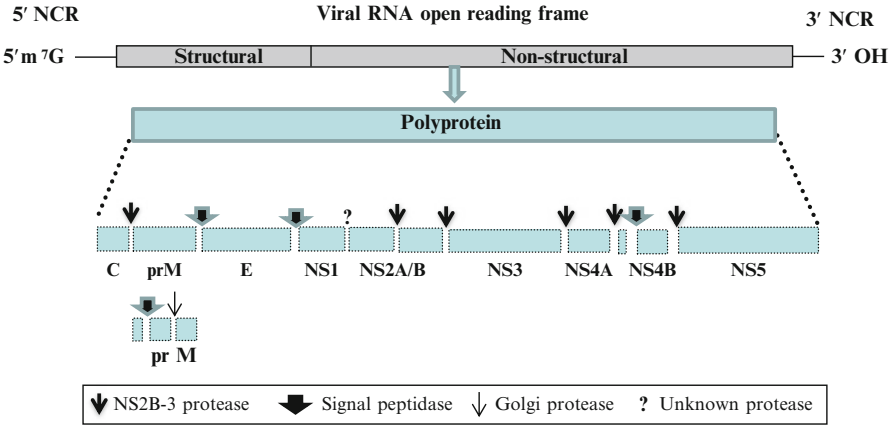

Fig. 4 Flavivirus genome organization. The single-stranded, positive sense RNA genome functions as a messenger RNA consisting of a single open reading frame that codes for a single polyprotein, flanked by noncoding regions at the 5′- and 3′-terminal ends. The polyprotein is co- and posttranslationally cleaved by cellular and viral proteases into three structural and seven nonstructural proteins. The prM is cleaved upon virus particle release through the cell plasma membrane. *C* capsid, *E* envelope, *NCR* noncoding region, *NS* nonstructural, *prM* premembrane

nonstructural proteins function primarily in viral replication and virion assembly, together with host cell factors (Lindenbach and Rice 2001; Chambers et al. 1990). The envelope glycoprotein 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 and is involved with membrane fusion and entry; thus it is an important determinant of tissue tropism and virulence (Roehrig 2003; Heinz and Roehrig 1990). The envelope protein is also the major viral antigen against which host protective antibodies are elicited (Roehrig 2003; Heinz and Roehrig 1990; Guirakhoo et al. 1992). Comparison of flavivirus envelope protein sequences has shown both highly conserved and highly variable subregions (Burke and Monath 2001; Kuno and Chang 2005). 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 (Fig. 5). The virus binds to the surface of host cells through an interaction between the envelope protein and specific cellular receptors (Lindenbach and Rice 2001; Chambers et al. 1990; Seligman and Bucher 2003; Koschinski et al. 2003). 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 (Lindenbach and Rice 2001; Heinz and Roehrig 1990). 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

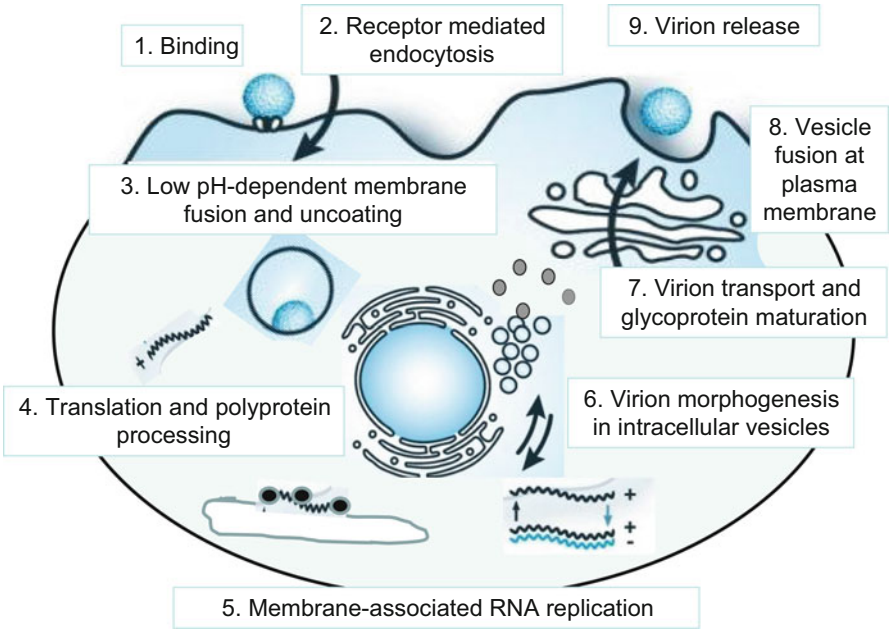

Fig. 5 Flavivirus life cycle [Modified from Lindenbach et al. (Lindenbach et al. 2007)]

(Lindenbach and Rice 2001). Virion morphogenesis is hypothesized to occur in association with intracellular membranes, probably the endoplasmic reticulum (Lindenbach and Rice 2001; Chambers et al. 1990). Immature virions assemble in membrane-bound vesicles in a premembrane and envelope protein heterodimer conformation which prevents 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 that used for synthesis of host plasma membrane glycoproteins (Lindenbach and Rice 2001). 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 (Fig. 4) (Guirakhoo et al. 1992).

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 "Influences of Arthropod Vectors on Encephalitic Arboviruses"). 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 (Burke and Monath 2001; Lindenbach and Rice 2001).

In natural infections, an infected arthropod inoculates the flavivirus into vertebrate skin along with the saliva during a bite. Initial virus replication occurs at the site of inoculation in keratinocytes, newly recruited neutrophils, and skin dendritic cells, specifically Langerhans cells (Johnston et al. 2000). Virus is then transported in migrating dendritic Langerhans cells and neutrophils to lymph nodes and from the lymphatic system to the thoracic duct and into the bloodstream (Burke and Monath 2001; Monath 1986; Johnston et al. 2000). This primary viremia is the source of infection of peripheral tissue such as spleen, liver, and kidney. Viremia continues for several days due to release of virus from these tissues back into the bloodstream (Burke and Monath 2001).

The vast majority of infections by neurotropic flaviviruses are self-limiting and the person is either asymptomatic or 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 (Fig. 6) (Burke and Monath 2001). In addition, the T-cell helper and cytotoxic immune response is elicited against infected lymphoblastoid cells (Roehrig 2003; Seligman and Bucher 2003). Virus is usually not detectable in serum collected at the time of onset of symptoms.

Neuroinvasive disease occurs in approximately 1 in 100 to 1 in 1000 neurotropic flavivirus infections and is dependent on viral factors, vector competence, and host factors. The viral factors that are hypothesized to contribute to neuroinvasive disease include the level of viremia and the genetic differences in virus strain neurovirulence (Burke and Monath 2001; McMinn 1997; Monath 1986; Gritsun et al. 1995). Single mutations in the envelope gene have been shown to alter neurovirulence phenotype (McMinn 1997). Arthropod vector competence is another factor that contributes to neurovirulence, and is described in detail in "Influences of Arthropod Vectors on Encephalitic Arboviruses." In the 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 disease severity (Burke and Monath 2001; Libraty et al. 2002). 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, the risk of St. Louis encephalitis and West Nile virus infections resulting in neuroinvasive disease is higher in those over 55 years of age (Burke and Monath 2001; Sejvar and Marfin 2006). 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 (Solomon 2004).

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, West Nile cases from North America, and experimental infections in animal

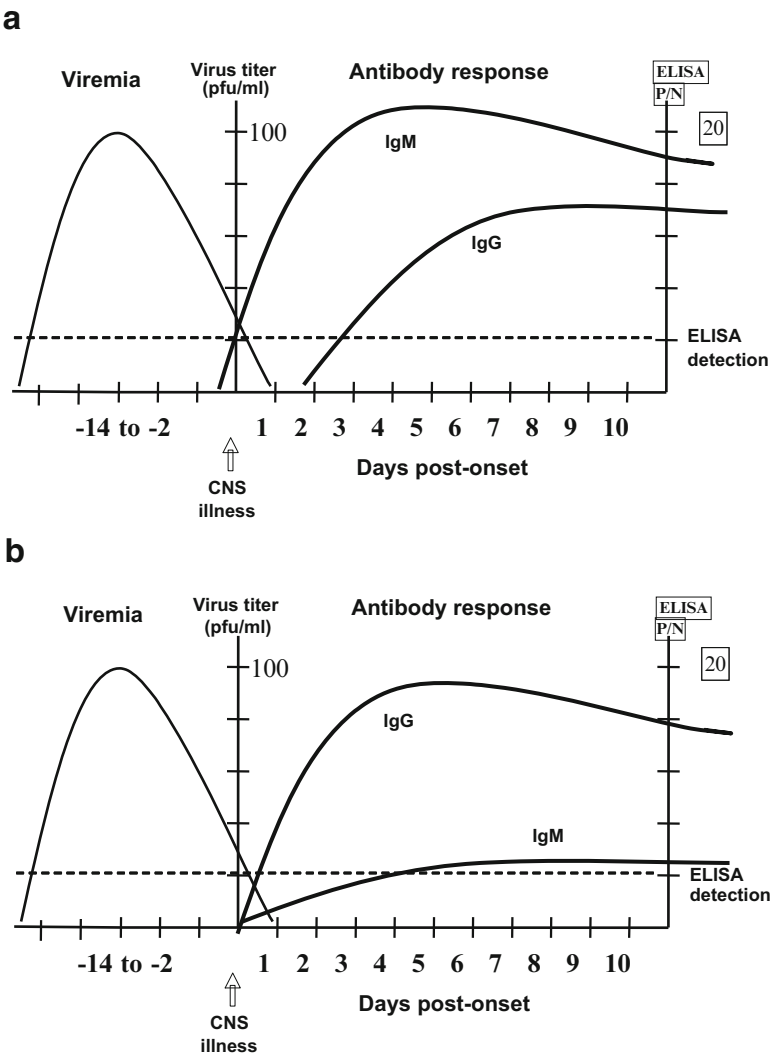

Fig. 6 Graphical representation of the course of viremia and IgM and IgG antibody immune response in a (**a**) primary and (**b**) secondary neurotropic flavivirus infection

models. The exact mechanisms by which flaviviruses enter the central nervous system have not been definitively identified, but hypothesized pathways 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 (Burke and Monath 2001; Gubler et al. 2007;

McMinn 1997; Sejvar and Marfin 2006; Kramer 2007; Hayes et al. 2005; Campbell et al. 2002). Whether the virus enters at a single site or at multiple locations is also unknown.

Once in the central nervous system, the virus replicates and spreads rapidly. 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 (Burke and Monath 2001; Kuno and Chang 2005; Campbell et al. 2002; Solomon 2003). Virus tropism for specific brain areas may vary and could explain the different clinical presentations. 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 (Fig. 7) (Cushing et al. 2004). The inflammatory immune response of natural killer cells, macrophages, and T-lymphocytes 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 (Gubler et al. 2007; Hayes et al. 2005). Damage to these neuronal cells is characterized by central chromatolysis, cytoplasmic eosinophilia, cell shrinkage, and neuronophagia, and by the formation of cellular nodule formation composed of activated microglia and mononuclear cells (Burke and Monath 2001; Sejvar and Marfin 2006; Kramer 2007; Hayes et al. 2005; Campbell et al. 2002). Apoptosis of motor neurons in the anterior horn of the spinal cord results in flaccid paralysis.

Persistent and long-term pathological changes are often seen following neuroinvasive flavivirus infection, such as residual neurological deficits, electroencephalographic changes, and psychiatric disturbances (Burke and Monath 2001). Long-term follow-up studies in Japanese encephalitis cases in children have shown neuronal loss and dense microglial scarring resulting in recurrent neurological disease (Solomon 2003, 2004; Monath 1986). Chronic progressive encephalitis has been observed years after infection with tick-borne encephalitis virus (Burke and Monath 2001; Monath 1986). 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 (Sejvar 2014; Sejvar et al. 2003a, 2010; Staples et al. 2014).

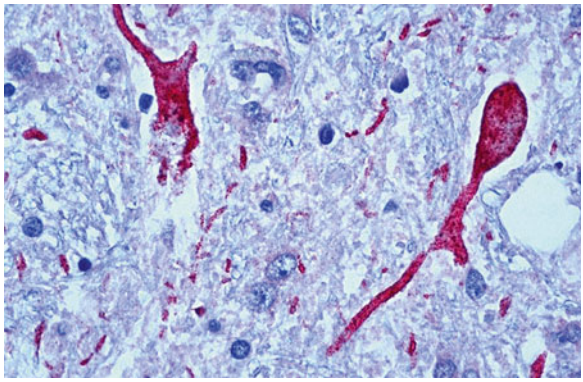

Fig. 7 Photomicrograph of immunohistochemical staining of brain tissue from a fatal West Nile encephalitis case, showing West Nile antigen-positive neurons and neuronal processes in the brain stem and anterior horn cells (in *red*) (From W.-J. Shieh and S. Zaki, CDC Public Health Image Library)

Clinical Presentation

The majority of flavivirus infections are asymptomatic or subclinical (Solomon 2004; Kramer 2007; Sejvar 2014). Clinical disease ranges from a mild febrile illness to a severe neurological syndrome following an incubation period of 2–14 days (Mackenzie et al. 1996). Febrile illness is characterized by fever, chills, headache, back pain, myalgia, and anorexia, as well as eye pain, pharyngitis, nausea, vomiting, and diarrhea (Mackenzie et al. 1996; Campbell et al. 2002). A transient maculopapular rash over the trunk and limbs is also common. 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 (Solomon 2004; Sejvar and Marfin 2006; Campbell et al. 2002).

Fever symptoms may be followed in 1–4 days by acute or subacute appearance of meningeal and neurological signs (Table 1). The neurological syndrome depends on the part of the nervous system that is infected: the parenchyma of the brain, which causes encephalitis; the meninges, which causes meningitis; or the anterior horn cells of the spinal cord, which causes myelitis (Burke and Monath 2001; Solomon 2004; Sejvar and Marfin 2006; Hayes et al. 2005; Sejvar 2014; Hayes and Gubler 2005; Petersen and Marfin 2002; Petersen et al. 2002; Sejvar et al. 2003b) The primary clinical presentations may overlap, and include a reduced level of consciousness, often associated with seizures, flaccid paralysis, and parkinsonian

Table 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 (parkinsonian syndrome including mask-like faces, 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/mm^3; mostly lymphocytes), elevated protein concentration (80–105 mg/dL), normal glucose concentration

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

Modified from Burke et al. 2001 (Burke and Monath 2001; Solomon 2004; Monath 1986; Sejvar and Marfin 2006; Kramer 2007)

movement disorders (Solomon 2004; Halstead and Jacobson 2003). Encephalitis is more common than meningitis, with 50–85 % of patients presenting with encephalitis, compared to 5–50 % with meningitis (Solomon 2004; Sejvar 2014). 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 (Solomon 2004; Mackenzie et al. 1996; Solomon et al. 2000). Motor weakness occurs in 10–50 % of flavivirus neuroinvasive cases, with acute asymmetric flaccid paralysis similar to that seen in poliomyelitis (Solomon 2004; Hayes et al. 2005). 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 (Campbell et al. 2002; Sejvar et al. 2010). 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 oculocephalic reflex abnormalities (Solomon 2004; Solomon et al. 2000). 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 (Hayes et al. 2005; Solomon et al. 2000; Douglas et al. 2006). 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 (Murgod et al. 2001). Many patients with poliomyelitis do not recover, although limb strength may improve over time (Hayes et al. 2005; Staples et al. 2014). Since the introduction of West Nile virus in 1999, in follow-up studies on short- and long-term outcomes of West Nile neuroinvasive infections, <50 % of patients had full cognitive and functional recovery after 1 year. Frequent complaints included fatigue, muscle aches, and difficulties with memory and concentration, suggesting a subcortical type of cognitive dysfunction based on prominent thalamic and basal ganglia involvement (Sejvar 2014). Case fatality rate of hospitalized patients with West Nile disease is 4–16 %, with a further two- to threefold increase in mortality observed up to 3 years after acute illness, and possibly longer (Staples et al. 2014).

The clinical course of tick-borne encephalitis virus infections may be distinct from the mosquito-borne flaviviruses, with many infections taking a biphasic course. Onset of illness may be more gradual, with patients experiencing an influenza-like illness for approximately 1 week, with fever, headache, malaise, and myalgia, followed by an asymptomatic period of up to a week (Gresikova and Calisher 1989; Leonova et al. 2014; Lindquist and Vapalahti 2008). A second phase involving the central nervous system includes clinical symptoms ranging from mild meningitis to severe encephalitis, with or without myelitis and spinal paralysis (Burke and Monath 2001; Gresikova and Calisher 1989). Generally, symptoms are more severe in adults than in children (Lindquist 2008; Logar et al. 2000). Long-term neurological problems are similar to those resulting from other neuroinvasive flavivirus infections (Lindquist and Vapalahti 2008).

Laboratory Diagnosis

Flavivirus infections may present with clinical symptoms similar to those of other virological or treatable bacterial infections, such as a flu-like illness, encephalitis, or polio-like myelitis (Burke and Monath 2001). In addition, vaccine preventable diseases such as Japanese encephalitis and tick-borne encephalitis may be clinically indistinguishable and cause similar disease syndromes to neurotropic flavivirus infections for which there is no effective vaccine. Therefore, laboratory diagnosis is necessary to identify etiology and differentiate between other bacterial or viral pathogens, guide effective clinical management and/or treatment, as well consider public health responses such as vaccination. Virus isolation or viral RNA detection in serum, cerebral spinal fluid, or tissue is the gold standard for diagnosis 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 (Fig. 6).

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 (Martin et al. 2000; Nasci et al. 2002). 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 (Fig. 6) (Iwamoto et al. 2003; Martin et al. 2000; Johnson et al. 2000, 2005a; Wong et al. 2003, 2004).

CSF is the preferable specimen for diagnosis of flavivirus neuroinvasive infections, as antiflavivirus IgM antibody may be present in serum, but may not be the cause of encephalitis, such as in inapparent or mild infections or following a recent flavivirus vaccination (Burke et al. 1982, 1985). This is especially prevalent in areas where there is high background immunity in the population, such as in Asia where a large proportion of the population have 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 (Roehrig et al. 2003). However, IgM antibodies elicited in non-neuroinvasive flavivirus infections or following flavivirus vaccination do not enter the CSF; therefore by testing the CSF, the effect of background IgM antibodies in the serum is eliminated (Mackenzie et al. 1996; Martin et al. 2000, 2002; Johnson et al. 2000; Burke et al. 1982; Chanama et al. 2005). 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 (Fig. 6) (Johnson et al. 2000; Burke et al. 1982). 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 (Martin et al. 2000; Lindsey et al. 2012).

Cross-reactivity in serological assays, including the IgM ELISA, is a problem in flavivirus diagnostics. Antibodies within a flavivirus serocomplex are highly cross-reactive; those between serocomplexes are less cross-reactive but still may confound

accurate diagnosis (Martin et al. 2002). 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 serocomplex or flavivirus 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. For example, dengue and West Nile viruses, which are not in the same serocomplex, co-circulate in Africa and Asia. Dengue virus does not cause encephalitis but may have a clinical presentation of encephalopathy, and a specimen from a dengue patient submitted for West Nile testing may have a positive result in a West Nile virus IgM ELISA (Hogrefe et al. 2004; Niedrig et al. 2007). 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.

In secondary flavivirus infections, an anamnestic reaction may occur, in which antibodies from the first flavivirus infection are elicited and cross-react in the IgM ELISA with antigens to the most recent infection, producing false-positive results (Porterfield 1986; Johnson et al. 2005b). In addition, the plaque neutralization test has less specificity in secondary flavivirus infections, as neutralizing antibody from the primary flavivirus infection rises quickly and the titer may be equal to or higher than that of the neutralizing antibody titer elicited in the acute flavivirus infection (Johnson et al. 2005b). In addition, IgM antibody to the second infecting virus may not rise to levels detectable by the IgM ELISA, producing a false-negative result (Fig. 6b).

Diagnostic assays such as the microsphere immunoassays have been shown to have improved specificity and sensitivity, and can be used for differential diagnosis (Johnson et al. 2005a; Wong et al. 2003, 2004). These assays, based on Luminex™ 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.

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 (Cushing et al. 2004). In addition, viral nucleic acid detection in CSF has also proved useful in up to 50 % of very acute infections, which when used together with IgM ELISA enhances sensitivity (Tilley et al. 2006; Lanciotti et al. 2000; Lanciotti and Kerst 2001).

Use of the hemagglutination inhibition and the complement fixation assays has decreased in recent years as they require paired specimens and lack specificity. The CT scan has not been shown to be an effective diagnostic method for identifying flavivirus encephalitis cases, and the MRI yields characteristic abnormal results in only 25–35 % of cases, and these may be nonspecific (Sejvar and Marfin 2006; Hayes et al. 2005; Campbell et al. 2002; Sejvar et al. 2003a; Solomon et al. 2000).

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 (Solomon 2006). 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. High-dose corticosteroid treatment may be contraindicated because of the risk of depressing the immune system before the virus is cleared (Hayes et al. 2005).

Prevention

Vector control programs up to the early 1970s successfully eradicated mosquitoes such as *Aedes aegypti*, the primary mosquito vector of dengue and yellow fever viruses, from most of Central and South America (see "Influences of Arthropod Vectors on Encephalitic Arboviruses"). However, these programs were not sustainable, and as a result *Aedes aegypti* has re-infested 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 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 prevent Japanese encephalitis and tick-borne encephalitis and will be described in more detail in the chapter entitled "Japanese Encephalitis Virus" and below, respectively (Solomon 2006; Marfin et al. 2005; Monath 2001, 2002).

Brief Descriptions of Specific Neurotropic Flaviviruses

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. The virus is separated into multiple lineages based on phylogenetic analysis of the complete West Nile virus genomes; lineage 1 and 2 strains have been most often associated with outbreaks in humans and equines (Lanciotti et al. 1999, 2002; Donadieu et al. 2013). Lineage 1 consists of strains from Western Africa, Eastern Europe, the Middle East, and recently, North America, and includes Kunjin virus from Australia (Fig. 2a) (Lanciotti et al. 2002). West Nile lineage 1 virus strains 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

(Solomon 2004; Sejvar and Marfin 2006). In West Nile virus endemic areas of Africa, 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, a similar pattern to that of Japanese encephalitis virus in Asia (Burke and Monath 2001). However, a higher proportion of adults may become infected when West Nile virus moves to new areas such as North America, or when susceptible adults, particularly travelers, enter an area of transmission activity. Lineage 2 strains historically have been considered less pathogenic than lineage 1 strains, causing febrile illness, but until recently have not been associated with neuroinvasive disease. The geographical range of West Nile lineage 2 strains was thought to be restricted to sub-Saharan Africa and Madagascar. Recently however, lineage 2 strains have been shown to be responsible for outbreaks in humans and equines in South Africa, as well as in eastern and southern Europe (Donadieu et al. 2013).

West Nile virus is transmitted in an enzootic cycle between mosquitoes, primarily *Culex* spp., and birds, particularly water birds and birds in the corvid family (see "Influences of Arthropod Vectors on Encephalitic Arboviruses") Volume 2. Humans and horses become infected through the bite of an infected mosquito, and viremia is generally 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 (Campbell et al. 2002; Nasci et al. 2002).

West Nile virus was identified as the cause of a meningoencephalitis epidemic of 59 cases and 7 deaths in New York that began in August in 1999 (Campbell et al. 2002). The North American West Nile virus is most closely related to and probably originated from a lineage 1 strain from Israel (Lanciotti et al. 1999). Since its introduction, the range of West Nile virus has extended across the United States, north into Canada, and into Central and South America (Gonzalez-Reiche et al. 2010; Morales et al. 2006; Morales-Betoulle et al. 2013). Through 2013, nearly 40,000 West Nile infections have been reported throughout the continental United States. Approximately 17,000 of those are neuroinvasive disease cases, and there have been over 1500 deaths (http://www.cdc.gov/westnile/statsMaps/index.html) (Hayes et al. 2005). In Canada there have been more than 5000 West Nile infections reported since the first cases were detected in Ontario in 2002, approximately 240 of which caused neuroinvasive disease (http://www.phac-aspc.gc.ca/wnv-vwn/mon-hmnsurv-archive-eng.php#a2002_07). Since 2006, probable human West Nile cases based on serology have been identified in Central and South America, and West Nile virus has been isolated from mosquitoes in Guatemala and equines in Argentina, but large West Nile encephalitis epidemics have not been reported from these areas (Gonzalez-Reiche et al. 2010; Morales et al. 2006; Komar and Clark 2006; Komar et al. 2005; Mattar et al. 2005). In Europe, epizootics resulting from autochthonous transmission of West Nile virus have occurred for over 20 years, with human cases reported sporadically (Sambri et al. 2013; Rossini et al. 2008).

Since its introduction in 1999, the ecology, epidemiology, and pathology of North American West Nile virus have been intensely studied. The many competent mosquito vectors and susceptible vertebrate hosts, 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 "Influences of Arthropod Vectors on Encephalitic Arboviruses") (Brault et al. 2004). In addition, the combination of flood irrigation

practices in the Great Plains with the preponderance of the highly competent vector *Culex tarsalis* in this region has resulted in the highest annual and cumulative incidence of neuroinvasive disease in the northern Great Plains states, along with the Louisiana–Mississippi Gulf region. The multiple determinants of vector-borne disease make it difficult to predict over the long term 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 the total case count varies considerably from year to year. Seroprevalence in North America and Europe remains low, between 2 and 3 % a year in areas where outbreaks have occurred, which is similar to that of St. Louis virus in North America (Campbell et al. 2002).

Similar to other flaviviruses in the Japanese encephalitis serocomplex, the majority of West Nile virus infections are asymptomatic, with 1 in 5 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. Symptoms usually resolve within 60 days; however, long-term effects have been reported (Hayes et al. 2005; Campbell et al. 2002; Petersen and Marfin 2002). 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 (Sejvar 2014; Staples et al. 2014).

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 (Campbell et al. 2002; Sejvar 2014). Acute asymmetric flaccid paralysis has been reported in approximately 13 % of patients with West Nile neuroinvasive disease (Hayes et al. 2005). 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 (Sejvar and Marfin 2006; Sejvar et al. 2003b). The case fatality rate is approximately 4–18 % of hospitalized patients, with mortality higher in the elderly and in immunocompromised persons (Sejvar and Marfin 2006; Petersen et al. 2003). Long-term neurological problems in survivors and muscle weakness patients with paralysis have been reported (Sejvar and Marfin 2006; Hayes et al. 2005; Sejvar et al. 2003a, 2010).

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 (Montgomery et al. 2006; Centers for Disease Control and Prevention 2009). A probable case of transplacental infection has also been reported (Sejvar and Marfin 2006). As a result, routine blood and organ screening by highly sensitive and specific nucleic acid amplification tests has been implemented (Centers for Disease Control and Prevention 2003, 2004).

Vaccines to protect against West Nile virus infection have been developed for the veterinary market and are commercially available for horses and birds (Davis et al. 2001). Consideration of the risk–benefit ratio of human West Nile virus vaccination is ongoing but the development of a commercial vaccine is unlikely, because the

percentage of the population at risk is very low, and the elderly, which are the most likely to have severe clinical manifestations and thus the group which would most benefit from a vaccine, are also the most likely to have higher risk of adverse effects from the vaccine. 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 Missouri in 1932 and 1933, and was first recognized in South America in the 1960s (Gubler et al. 2007; Tsai and Mitchell 1989; Brinker et al. 1979). St. Louis encephalitis virus is widely dispersed throughout the Americas (Fig. 2b). The largest epidemic in the United States, with nearly 2000 cases, occurred throughout the Midwest in 1975. In the most recent outbreak in 1990 there were nearly 250 cases, primarily in Florida and Texas. An outbreak of 46 human cases of St. Louis encephalitis was reported in Argentina in 2005 and in the same year, St. Louis encephalitis virus was isolated from the serum of a suspected dengue case in Brazil (Spinsanti et al. 2008; Rocco et al. 2005). St. Louis encephalitis virus activity has been characterized by periodic outbreaks interspersed with long periods of sporadic cases (Brinker et al. 1979). 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. There have been approximately 90 cases reported since 2004, the majority of which were neuroinvasive disease (http://www.cdc.gov/sle). This 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 primary role in the transmission cycle, and there is no morbidity or mortality associated with St. Louis encephalitis virus infection in horses (Tsai and Mitchell 1989). Despite an expansive geographical range throughout the temperate and tropical regions in the Americas, rates of St. Louis encephalitis virus 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 urban outbreaks; in the 1975 outbreak the largest number of cases occurred in the Chicago metropolitan area. However, even in these cases vegetated parkland areas were shown to be the sites of transmission (Burke and Monath 2001). Transmission is seasonal, corresponding to mosquito activity during the late summer months (see 26”Influences of Arthropod Vectors on Encephalitic Arboviruses”).

St. Louis encephalitis is in the Japanese encephalitis serocomplex, and genetically closely related to West Nile virus (Fig. 1) (Tsai and Mitchell 1989). In Central and South America, where St. Louis encephalitis and West Nile viruses co-circulate, it may not be possible to differentiate between the two viruses in serological assays

due to the cross-reactivity, although this is the primary method of diagnosis, as virus isolation from either West Nile or St. Louis virus infected human patients is rare.

The majority of human St. Louis encephalitis virus infections are subclinical, with the ratio of encephalitis cases to asymptomatic infections approximately 1:85 to 1:800 in adults and children, respectively. Adults are more likely to become infected in North America due to low seroprevalence rates. 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. From 30 to 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 (Tsai and Mitchell 1989). 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 were reported in the Murray Valley of Australia in the early 1900s, although the virus was not identified or characterized as being distinct from Japanese encephalitis virus until 1951 (Burke and Monath 2001; Solomon 2004; Marshall 1988). Like Japanese encephalitis and West Nile viruses, Murray Valley encephalitis virus infections can cause 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 (see "Influences of Arthropod Vectors on Encephalitic Arboviruses") (Marshall 1988). Similar to Japanese encephalitis serogroup viruses, large water birds such as herons, egrets, and pelicans are the primary vertebrate hosts (Mackenzie et al. 1996; Marshall 1988). Mammals such as kangaroos and rabbits also may be significant viremic hosts in the transmission cycle (Burke and Monath 2001). 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 (Fig. 2c). Similar to the other neurotropic flaviviruses, humans are infected incidentally and probably do not contribute to the transmission cycle (Douglas et al. 2006). Outbreaks of Murray Valley encephalitis in 1951 (45 cases), 1974 (58 cases), and 2011 (17 cases) have been interspersed with sporadic cases; approximately 40 cases have been reported in the last 25 years (Selvey et al. 2014).

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 (Douglas et al. 2006). The clinical patterns of the disease include rapid onset of fatal encephalitis, flaccid paralysis, tremor, or encephalitis with complete recovery, simi-

lar to those of the other Japanese encephalitis complex viruses. Clinical illness is generally seen in young children and nonimmune adults (Solomon 2004; Douglas et al. 2006).

(Solomon 2004; Selvey et al. 2014). The case fatality rate is 31 %; a third of survivors experience long-term neurological deficits (Douglas et al. 2006). 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

Scandinavian church records from the eighteenth century describe a tick-borne encephalitis-like illness occurring annually in spring time. Russian spring-summer encephalitis was first described as a clinical illness in the far-eastern region of the Soviet Union in 1935. Tick-borne encephalitis virus was isolated from a human brain and ticks were shown to be the arthropod vectors in 1937 (Burke and Monath 2001; Gresikova and Calisher 1989; Lindquist and Vapalahti 2008; Gritsun et al. 2003). Since then three subtypes of tick-borne encephalitis virus have been identified: Far Eastern (Russian spring-summer), Siberian, and European (Lindquist and Vapalahti 2008). The geographical ranges of the tick-borne encephalitis viruses extend from western Europe to the east coast of Japan, corresponding to those of the tick hosts (Fig. 2d) (Burke and Monath 2001; Calisher et al. 1989; King et al. 2012; Grard et al. 2006; Lindenbach and Rice 2001; Gresikova and Calisher 1989; Tsai and Mitchell 1989; Reid 1988).

Tick-borne encephalitis virus is transmitted to humans through the bite of an infected tick of the *Ixodes* spp., primarily *I. ricinus* for the European subtype and *I. persulcatus* for the Siberian and Far Eastern subtypes. In addition to being vectors of the virus, and because of their longer life span compared to mosquitoes, ticks are also the main virus reservoir. Small rodents are the primary vertebrate amplifying, reservoir, and overwintering hosts (see "Influences of Arthropod Vectors on Encephalitic Arboviruses") (Lindquist and Vapalahti 2008). 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 (Gresikova and Calisher 1989). Transmission has been infrequently reported during slaughtering of viremic animals, and directly person-to-person, through blood transfusion and breastfeeding.

The risk of acquiring tick-borne encephalitis in forested areas through activities such as camping, hiking, or military training peaks in early and late summer when ticks are active; however, cases have been reported during the hot summer months despite lower tick activity, as people increasingly come into contact with ticks during outdoor activities. The risk is negligible for people who remain in urban or unforested areas (Lindquist and Vapalahti 2008).

Tick-borne encephalitis viruses are the most medically important arbovirus in Europe, with more than 10,000 cases of tick-borne encephalitis occurring annually, 3000 of which require hospital treatment (Lindquist and Vapalahti 2008). Western Siberia has the largest annual number of reported cases, but the endemic area extends throughout Eurasia (Fig. 2d) (Palo 2014; Yun et al. 2011). During the past two decades new endemic foci and increased cases have been reported in Europe, and because of its emergence, tick-borne encephalitis needs to be considered outside the traditional endemic areas (Lindquist and Vapalahti 2008).

Clinical disease is biphasic and disease severity varies between the three tick-borne encephalitis virus subtypes (Burke and Monath 2001; Calisher et al. 1989; King et al. 2012; Grard et al. 2006; Lindenbach and Rice 2001; Gresikova and Calisher 1989; Tsai and Mitchell 1989; Reid 1988; Belikov et al. 2014). The European subtype is associated with milder disease. 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 (Gresikova and Calisher 1989; Leonova et al. 2014; Lindquist and Vapalahti 2008). Following an asymptomatic period of up to a week, 20–30 % experience a second phase involving the central nervous system, with the clinical symptoms ranging from mild meningitis to severe encephalitis, with or without myelitis and spinal paralysis (Burke and Monath 2001; Gresikova and Calisher 1989). The reported case fatality rate ranges from 1–5 %, and 10–20 % of survivors, generally those with the more severe clinical symptoms, have long-term neurological problems (Lindquist and Vapalahti 2008).

The more severe course of disease results from infection by the Far-Eastern and Siberian subtypes (Gresikova and Calisher 1989; Belikov et al. 2014). The prodromal phase may consist of symptoms similar to those of the Japanese encephalitis complex virus infections: fever, headache, anorexia, nausea, vomiting, and photophobia. In the second phase, infection of the brain stem and upper cervical cord produces stiff neck, ataxia, sensorial changes, convulsions, and in about 20 % of cases, flaccid paralysis (Lindquist and Vapalahti 2008). The case fatality rate is approximately 20 %; 30–60 % of survivors experience residual neuronal damage (Gresikova and Calisher 1989; Kaiser 2002).

Tick-borne encephalitis vaccines are commercially available in Europe, and routine vaccination is recommended for children in many European countries (Gresikova and Calisher 1989; Smit and Postma 2014). Vaccinations may be required for travelers to endemic areas in eastern Russia, where the seroprevalence rate may be as high as 51 % (Wittermann et al. 2015). No tick-borne encephalitis vaccines are licensed or available in the United States.

Powassan Virus

Powassan virus was first isolated in Ontario, Canada from a pediatric case of encephalitis in 1958, retrospectively from ticks collected in Colorado in 1952, and in Russia from ticks in 1996. Powassan virus, the only member of the tick-borne

encephalitis antigenic complex found in North America, is widely distributed in temperate regions in the northern hemisphere (Fig. 2d) (Burke and Monath 2001; Gubler et al. 2007; Artsob 1989). North American Powassan cases are concentrated in New York, Ontario, and Quebec (Centers for Disease Control and Prevention 2001). However, Powassan virus infection has been diagnosed with increasing frequency both within the known range and in areas where Powassan cases had not been previously reported (Ebel 2010). Whether this is due to heightened awareness and increased surveillance for arboviruses following the introduction of West Nile virus into North America, or because Powassan is an emerging virus is unknown.

Powassan virus comprises two closely related lineages: the Powassan virus prototype lineage and the deer tick virus lineage (Ebel 2010). The prototype Powassan virus is principally maintained between *Ixodes cookei* ticks and the groundhog (*Marmota monax*) or striped skunk (*Mephitis mephitis*); the deer tick virus is believed to be maintained between *Ixodes scapularis* ticks and the white-footed mouse (*Peromyscus leucopus*) (Ei Khoury et al. 2013). Both lineages have been linked to human disease, although bites to humans by *I. cookei* ticks are rare, whereas *I. scapularis* tick bites are common (Ebel 2010).

In the United States, disease caused by Powassan virus has occurred sporadically, primarily in the late spring, early summer, and mid-fall when ticks are most active. Approximately 80 Powassan virus infections have been reported since the 1950s, with over 80 % causing neuroinvasive disease. The case fatality rate is approximately 10 % (Ebel 2010). As with other flaviviruses, the majority of Powassan infections are asymptomatic (Gubler et al. 2007). Clinical symptoms can include fever, headache, vomiting, weakness, confusion, loss of coordination, speech difficulties, seizures, and memory loss. Powassan virus can infect the central nervous system and cause encephalitis and meningitis. At least half of survivors have long-lasting sequelae, such as recurrent headaches, muscle wasting, and memory problems.

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 by serological assays 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 or reemerge as significant human pathogens as deforestation and changing agricultural practices bring humans into areas of enzootic transmission cycles.

Rocio virus: Rocio virus was first isolated from a fatal human case during an epidemic of encephalitis in southeastern Brazil in 1975 (Iversson 1989). Between 1975 and 1976, there were over 1000 cases reported, with a 10 % fatality rate, and neurological sequelae were observed in 20 % of survivors. Male adults working in or near the forests were shown to have the highest risk of infection (Iversson 1989).

The transmission cycle of Rocio virus has not been clearly established, although wild birds are believed to be the primary vertebrate host, similar to the other mosquito-borne neurotropic flaviviruses. The virus replicates in *Culex* mosquitoes in the laboratory; however in nature the Rocio virus has been most often isolated from *Psorophora* mosquitoes (Iversson 1989).

Since 1976, despite the continuation of sporadic cases of encephalitis in south-eastern Brazil and serological evidence of Rocio virus infections in horses and humans, only one case of human Rocio virus infection has been identified (Gubler et al. 2007; Silva et al. 2014). This could be due to the difficulty of differentiating by clinical symptoms or serology alone those encephalitis cases caused by Rocio virus from those caused by the closely related St. Louis encephalitis virus, which co-circulates in the same geographical area (Fig. 1) (Burke and Monath 2001; Figueiredo 2000; Medeiros et al. 2007). Likely Rocio virus infections are an under-recognized cause of neuroinvasive illness in the Americas.

Louping ill virus: In Scotland, louping ill neurological disease has been recognized in sheep since the 1700s, and the virus was isolated there in 1929 (Jeffries et al. 2014). The first probable human case was reported in 1934 (Gubler et al. 2007).

Louping ill virus is genetically most closely related to the European subtype of tick-borne encephalitis virus, and similarly is transmitted by *Ixodes* spp. (Fig. 1) (King et al. 2012; Moureau et al. 2015). The geographical range of louping ill virus is in upland grazing areas throughout the United Kingdom, Ireland, Norway, Spain, Greece, and Turkey (Fig. 2d). However, recently the virus has been detected in Scandinavia, and Negishi virus, classified as a genotype of Louping ill virus, was isolated from a human in Japan (Gubler et al. 2007). The natural vertebrate hosts of louping ill virus are speculated to be rodents, deer, and hares, but sheep and cattle are the most important enzootic hosts from an agricultural perspective (Jeffries et al. 2014). Louping ill virus causes neurological disease in sheep, and to a lesser degree, cattle, yet interestingly, the other tick-borne encephalitis viruses do not (Jeffries et al. 2014). Most human infections have occurred through occupational exposure to infected ticks on livestock, such as stockmen, abattoir workers, butchers, and veterinarians (Lindquist and Vapalahti 2008).

There have been 44 reports of human disease caused by louping ill virus, with one fatal case. No human encephalitis cases of been identified since 1991, possibly due to lack of awareness among clinicians for this "forgotten" disease and subsequent lack of specific testing (Jeffries et al. 2014). Serosurveys suggest that at-risk groups are exposed to louping ill virus, but that most infections are asymptomatic or result in an influenza-like illness. Clinical disease is characterized by fever, headache, and some muscle stiffness, which may be followed by more severe neurological signs. Four cases have presented as poliomyelitis-like disease (Jeffries et al. 2014). Vaccination successfully protects livestock but does not eliminate persistence in ticks or virus transmission in wildlife hosts.

Modoc virus: Modoc virus was first isolated from a deer mouse in Modoc County, California in 1958, and subsequently in other regions in western United States and Canada (Burke and Monath 2001). Modoc virus was reported as the etiological

agent in one human aseptic meningitis case (Gubler et al. 2007). Rodents are the primary host and the virus is hypothesized to be maintained in nature by virus shedding in persistently infected rodents and through horizontal transmission (Adams et al. 2013). No arthropod vector has been identified (Fig. 1).

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Neurotropic Dengue Virus Infections

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Introduction

Dengue fever, which is caused by *Dengue virus* (DENV), is currently the most significant vector-borne disease worldwide. Approximately 390 million new cases of DENV infection are diagnosed annually, and roughly 500,000 of these cases will evolve into dengue hemorrhagic fever (DHF), which causes 20,000 deaths per year (Bhatt et al. 2013). The fatality rates of severe dengue cases range from less than 0.2 to 5 %. Population growth, urbanization, and an increase in air travel and trade have all contributed to the emergence and geographical spread of this disease over the past several decades (WHO 2009).

The most common symptoms of dengue, which is an acute febrile viral disease, include headaches, bone, joint and muscular pains, and rash. The clinical manifestations of the disease were recently re-evaluated by WHO (2009), which indicated that severe cases of dengue affect the central nervous system (CNS). Despite this, the neurological complications associated with DENV infection have not been well-described in the literature, although reports of CNS involvement are becoming more frequent (reviewed by Carod-Artal et al. 2013).

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Mosquitoes of the genus Aedes transmit DENV, and the virus is most commonly spread by *Aedes aegypti*, a species that can be found between N°35 and S°35 latitudes worldwide. Mosquitoes have become efficient at spreading dengue because of their high susceptibility to DENV, which is caused by a number of factors. These factors include mosquitoes' preference for feeding on human blood, their laying of eggs in small pools of water around houses, their diurnal habits, and their imperceptible bite, which enables one or several humans to be bitten several times over a short period of time without responding (Gould and Solomon 2008; Black et al. 2002). Thus, dengue transmission results from the interactions that occur between people, mosquitoes, viruses, and environmental factors. For example, as infected people travel throughout their local environment, they become spatiotemporal drivers of viral transmission, which results in the amplification and spread of DENV (Guzman and Harris 2015).

The *Flaviviridae* family currently consists of four genera, *Flavivirus*, *Pestivirus*, *Hepacivirus*, and *Pegivirus*, and includes several pathogens that are responsible for causing disease and mortality in both humans and animals (ICTV 2014). DENV belongs to the *Flavivirus* genus, which is comprised of enveloped viruses that bear single-stranded RNA genomes that are positive in polarity. The RNA genome includes three structural protein genes, which encode the capsid, membrane, and envelope proteins, as well as seven non-structural (NS) protein genes (Mukhopadhyay et al. 2005; Lindenbach et al. 2013).

As a species, DENV is comprised of four antigenically distinct serotypes, which are appointed DENV-1 through DENV-4 based on the order of their discovery (Holmes and Twiddy 2003). The DENV serotypes share a limited identity (approximately 60–75 %) at the aminoacid level. The DENV genotypes differ by approximately 3 % at the aminoacid level and 6 % at the nucleotide level (Rico-Hesse 1990). In a study by Chen and Vasilakis (2011), a phylogenetic history encompassing all of the available E gene sequences for each of the four DENV serotypes was created. DENV-1 is comprised of the Asia, America/Africa, Thailand and South Pacific and Malaysia genotypes (Gonçalvez et al. 2002; Chen and Vasilakis 2011). DENV-2 includes the Asia I, Asia II, Cosmopolitan, America/Asia, and America genotypes (Barcelos Figueiredo et al. 2014), and DENV-3 includes I, II, III, IV, and V genotypes (Araújo et al. 2009). Finally, DENV-4 is comprised of I, II, III, and wild-type genotypes (Weaver and Vasilakis 2009). Based on the above information, we have constructed a phylogenetic tree (Fig. 1), of the genotypes DENV-1 through DENV-4, in addition to the DENV-3 genotype I that was isolated by Barcelos Figueiredo et al. (2008) and deposited in GenBank (EF625835).

The genetic variations between the serotypes and genotypes of DENV are important determinants of its viral fitness, virulence, and epidemic potential (Guzman and Harris 2015). In our studies the DENV-3 genotype I (Fig. 1) has been detected in humans and *Aedes aegypty* alike since 2002, confirming its circulation in Brazil (Ferreira et al. 2010; Barcelos Figueiredo et al. 2008; Vilela et al. 2010). This genotype was described as being neurovirulent in a murine model (Ferreira et al. 2010).

Studies conducted on wild DENV have indicated that each of the DENV serotypes evolved from a common ancestor in a population of non-human primates and that all of the viruses emerged separately in an urban human transmission cycle approximately 500 years ago (Halstead 2007).

Fig. 1 Phylogenetic tree of the envelope gene (E) nucleotides of dengue virus (DENV) genotypes. Tree showing the genotypes of the four DENV serotypes. DENV-1: Asia, America/Africa, Thailand, and South Pacific, Malaysia. DENV-2: Asia I, Asia II, Cosmopolitan, America/Asia and America. DENV-3: I, II, III, IV and V. DENV-4: I, II, III and wild-type. Sequences of DENVs genotypes that were deposited in GenBank were analyzed using neighbor-joining method 4 with the Kimura 2-parameter algorithm in MEGA4 (www.megasoftware.net). The rate of variation among sites was modeled using a gamma distribution (shape parameter = 1). Bootstrap confidence limits (from 1000 replicates) are indicated at each node. DENV-3 genotype I isolate is neurovirulent in mouse models

Clinical Manifestations

DENV infection produces clinical manifestations that range from asymptomatic to severe, including death. A large percentage of these infections are asymptomatic. In symptomatic cases, the severity of clinical manifestation depends on factors such as the DENV serotype and genotype, the virulence of the strain, the nutritional status of the patient, the genetic makeup of the host and the presence of heterotypic DENV infections. After a mosquito bites a susceptible individual, there is an incubation period of 2–7 days during which the virus localizes to and replicates in the lymph nodes, spleen, and liver, ultimately inducing viremia (WHO 2012).

DENV-related illness begins abruptly, and in patients with moderate to severe disease, proceeds through three phases: the febrile phase, critical phase, and recovery phase. Patients will typically develop a sudden high-grade fever. The acute febrile phase usually lasts for 2–7 days and is often accompanied by facial flushing, skin erythema, generalized body ache, myalgia, arthralgia, retro-orbital eye pain, photophobia, rubeliform exanthema and headache (Rigau-Pérez et al. 1998). Some patients may also experience a sore throat, pharyngitis, and conjunctivitis. Anorexia, nausea, and vomiting are common. It can be difficult to clinically distinguish dengue from other non-dengue febrile diseases, especially during the early febrile phase (WHO 2012).

The severity of the disease tends to only become apparent during defervescence (i.e., during the transition from the febrile to the afebrile phase), which often coincides with the onset of the critical phase. During this transition, patients without an increase in capillary permeability will improve without proceeding through the critical phase and their fevers will subside; however, patients with increased capillary permeability may manifest the warning signs that mark the beginning of the critical phase, mostly as a result of plasma leakage (WHO 2012).

Persistent vomiting and severe abdominal pain are early indications of plasma leakage and they become increasingly worse as the patient progresses to the shock state. The patient becomes increasingly lethargic but usually remains mentally alert. These symptoms may persist into the shock state and weakness, dizziness, and postural hypotension may occur during this time (Kalayanarooj et al. 1997). Severe dengue may also cause hepatitis, neurological disorders, and myocarditis (Guzman and Harris 2015).

The recovery phase begins 3 days after defervescence. The general health of the patient improves at this time and pain and fatigue disappear. In some cases, exanthema will develop as white spots, which is characteristic of this phase of the disease. During this phase, platelet and leukocyte value also become normalized (Kalayanarooj et al. 1997).

Changes to the epidemiology of dengue have lead to problems with the existing WHO classification system. Symptomatic DENV infections have traditionally been grouped into three categories: undifferentiated fever, dengue fever (DF), and dengue hemorrhagic fever (DHF). DHF was further classified to have four grades of severity, with grades III and IV being defined as dengue shock syndrome (DSS; WHO 1997).

According to the new proposal, the clinical presentation of dengue should be classified as dengue without warning signs, dengue with warning signs, and severe dengue. Cases of dengue without warning signs generally affect patients who live or travel to dengue endemic areas. In these cases, patients develop a fever in addition to two of the following criteria: nausea (vomiting), rash, aches and pains, leucopenia, a positive tourniquet test, or a laboratory confirmation of dengue. In dengue with warning signs, the warning signs include abdominal pain, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy, liver enlargement (>2 cm), elevated levels of hematocrit, and a rapid decrease in platelet counts. Patients with severe dengue present with severe plasma leakage that leads to shock (DSS) and fluid accumulation with respiratory distress. Additional symptoms

include severe hemorrhage and/or severe organ involvement, such as high levels of alanine aminotransferase (AST) or aspartate aminotransferase (ALT) in the liver, impairment of the heart and CNS involvement including impaired consciousness (WHO 2009).

Neurological Manifestations

Upon admission to the hospital, some dengue patients with neurological symptoms do not show any characteristic features of dengue (Solomon et al. 2000). Previous studies examining the impact of DENV infection on the CNS have primarily focused on the following two objectives: (a) analyzing the neurological manifestations that arise in dengue patients, or (b) searching for the presence of DENV in patients with neurological syndromes, such as encephalitis, meningitis, and myelitis (Puccioni-Sohler et al. 2013; Carod-Artal et al. 2013).

Several neurological symptoms have been associated with dengue in the literature, including depressed sensorium, convulsions, behavioral disorders, nuchal rigidity, positive Kerning's sign, and Brudzinski reflexes, focal neurological deficits, flaccid paraparesis, transverse myelitis, peripheral facial paralysis, hemifacial spasm, a Guillain–Barré syndrome-like illness, tremors, maniac psychosis, depression, dementia, pyramidal tract signs, amnesia, short-term memory loss, decerebration, and coma (Channa and Wasay 2006).

DENV infection affects the CNS by causing dengue encephalopathy, encephalitis, immune-mediated syndromes, dengue-associated muscle dysfunction, neuro-ophthalmic disorders, and rarely, meningitis (Carod-Artal et al. 2013).

DENV infection was first reported to produce neurological manifestation by Sanguansermsri et al. (1976) in a patient presenting with encephalopathy, which is the most commonly reported neurological disorder associated with DENV infection. Encephalopathy leads to a diminished level of consciousness that can be caused by prolonged shock, anoxia, cerebral edema, metabolic disturbances, hemorrhage, and acute liver or renal failure. The cerebrospinal fluid (CSF) of these patients, including measurements of protein, glucose, and cell count, is usually normal (Carod-Artal et al. 2013).

Patients with dengue encephalitis present with diminished consciousness, headache, dizziness, disorientation, seizures, and behavioral symptoms. In severe cases, tetraparesis may be observed. Because the manifestations of dengue encephalitis and dengue encephalopathy are clinically indistinguishable, acute liver failure, hypovolemic shock with metabolic deteriorations, and intracranial hemorrhage must all be ruled out, and the CSF should be examined (Carod-Artal et al. 2013).

Carod-Artal et al. (2013) proposed that at least one of the following signs should be used to identify CNS involvement in dengue: impaired consciousness, neck stiffness, focal neurological signs, or seizure. For a diagnosis of dengue encephalopathy, one of the following dengue-associated complications must also be present: hepatic failure, metabolic acidosis, severe hyponatremia, prolonged shock, disseminated

intravascular coagulation, or brain hemorrhage. Diagnosing dengue encephalitis requires the presence of dengue virus RNA, IgM, or NS1 antigens in the CSF, and pleocytosis in the absence of other neuroinvasive pathogens.

CNS involvement following dengue infection is becoming more frequently reported in the literature. Studies conducted by Domingues et al. (2008), Puccioni-Sohler et al. (2009), and Jackson et al. (2008) found that 21 %, 5 %, and 13 % of total dengue patients had some level of CNS involvement, respectively. Furthermore, it has been found that patients with CNS infection (4 % of a cohort of 378 patients; Solomon et al. 2000), viral encephalitis (9.5 % of a cohort of children; Tan et al. 2010), and death (48 % of 150 fatal cases) caused by CNS malfunction (Araújo et al. 2012a, b) all test positive for dengue.

We recently analyzed CNS samples taken from 71 children (under 12 years old) suspected to have viral meningoencephalitis and detected DENV in six of these cases (11.8 %). From these, four were DENV-2, one was a co-infection of DENV-1 and DENV-3 and one was DENV-3 genotype I (unpublished data).

Although there have been studies into the neurological manifestations of dengue, such as those highlighted above, the true prevalence of the disease remain unknown because of the underrecognition of CNS presentation. Furthermore, the mechanisms driving neurovirulence are only poorly understood (Solomon et al. 2000; Araújo et al. 2012a, b; WHO 2009; Amorim et al. 2012). Therefore, it should be noted that DENV infection may cause encephalitis and other neurological manifestations in regions where dengue is endemic.

Diagnosis

The diagnostic methods used to assess DENV infection in CNS included serological techniques (immunoassays), nucleic acid amplification (NAT), and virus isolation. The CSF is also analyzed for the presence of DENV or antibodies against DENV (Puccioni-Sohler et al. 2013; Carod-Artal et al. 2013; Araújo et al. 2011; Santiago et al. 2013).

An indirect method of measuring specific anti-DENV IgM antibodies in the CSF in early stages of DENV infection (5–7 days) is by IgM-capture using the enzyme-linked immunosorbent assay (ELISA); however, the absence of IgM in the CFS does not exclude DENV infection, as this is normally only produced in low titer. Isolating virus from and detecting RNA in the CSF, without the concurrent detection of IgM is a possibility that has previously been described elsewhere (Puccioni-Sohler et al. 2013; Carod-Artal et al. 2013; Araújo et al. 2011). The presence of DENV non-structural protein 1 (NS1) in the CSF has also been used as a measure of DENV neuroinvasion. NS1 is primarily detected by antigen capture ELISA; however, it should be noted that the inability to detect NS1 does not exclude DENV infection in the CSF (Puccioni-Sohler et al. 2013; Carod-Artal et al. 2013; Santiago et al. 2013). Most of the ELISA tests that have been used toward this end were originally developed to detect dengue-associated-markers in the serum or the

plasma, thus they are not standardized for measuring the CSF (Carod-Artal et al. 2013; Puccioni-Sohler et al. 2009, 2013).

Due to their sensitivity and specificity, the most frequently used assays to detect DENV in the CSF are NATs based on reverse transcription (RT) followed by the polymerase chain reaction (RT-PCR) (Puccioni-Sohler et al. 2013; Carod-Artal et al. 2013). The most frequently used technique is the RT-Real Time PCR "one-step," which is based on technology developed by the "Center for Disease Control and Prevention" (CDC). Although the "Food and Drug Administration" (FDA) has approved this test, it is only standardized for use on human sera and plasma; therefore, when using it to assay the CSF, rigorous restandardization is required (Santiago et al. 2013). Although DENV RNA can be detected in the CSF of patients presenting with acute-phase dengue, it is notable that negative results on the above tests does not exclude the presence of virus in the CNS. Furthermore, some clinical symptoms do not require neuroinvasion.

Another method used to diagnose neurological DENV infection is virus isolation, which is performed using *Aedes albopictus* C6/36 cell cultures. This method is considered a "gold standard" for characterizing viral infections, although it is both less sensitive and more laborious than PCR (Puccioni-Sohler et al. 2013; Carod-Artal et al. 2013; Solomon et al. 2000; Araújo et al. 2012a).

Solomon et al. (2000) analyzed CSF samples taken from 21 patients from Vietnam who had neurological manifestations of DENV infection. Virus isolation verified the presence of DENV in two of these patients (DENV-2), PCR verified it in three (two cases of DENV-3 and one of DENV-2) and seven of them were dengue-specific IgM positive. Only one CSF-sample contained virus, could be detected by both PCR and viral isolation. Araújo el al. (2012a) analyzed 41 CSF samples that were found to contain DENV-3 by viral isolation. Of these samples seven were found to be positive by PCR assay (three DENV-2 and 4 DENV-3), 27 were IgM positive (three of which were also PCR positive), and 22 were NS1 positive (four PCR positive and 10 IgM positive).

Although the methods used to detect DENV in the CNS are evolving, the diagnosis of DENV infection in the CNS remains difficult because the clinical manifestations of the infection are not well-characterized and the markers typically used to diagnose the virus (e.g., IgM and viral RNA) are not well-correlated with these clinical manifestations. A summary of how the results from the main diagnostic tests that are used to identify DENV infection in the CNS are interpreted is presented in Table 1. Cases of DENV infection in the CNS that produced neurological manifestations in the absence of other classical symptoms of dengue have also been described. Thus, additional diagnostic tools such as magnetic resonance imaging (MRI) may be necessary to confirm the clinical signs and diagnosis of the virus (Hegde et al. 2015).

As more sensitive tools are being used to detect DENV in the CNS (via analysis of the CNS) the number of detected DENV cases is rising. This enhances the epidemiological data that are available to describe the relationship of this virus to CNS disease.

Table 1 Interpretation of the results produced by the different methods that are used for DENV identification in CSF samples (based on the data of Carod-Artal et al. 2013 and of Puccioni-Sohler et al. 2013)

PCR (cDNA detection), CSF		IgM detection, CSF		Virus isolation	
Positive	Negative	Positive	Negative	Positive	Negative
Confirms neuroinvasion, but does not confirm active infection	Does not rule out neuroinvasion or active infection	Confirms neuroinvasion, but does not confirm active infection	Does not rule out neuroinvasion or active infection	Confirms neuroinvasion and confirms active infection	Does not rule out neuroinvasion or active infection

Treatment and Prophylaxis

There is no specific antiviral treatment for DENV infection. The clinical management of patients is mainly supportive and has been extensively described by the WHO (2012). For the supportive management of patients with neurological manifestations, it is necessary to rule out alternative underlying causes such as intracranial bleeding, liver failure, hyponatremia, hypokalemia, or metabolic acidosis. Symptomatic seizures should be treated with non-hepato-toxic anticonvulsants (Carod-Artal et al. 2013).

The prevention of dengue is currently centered on vector control and personal protection from the bites of infected mosquitoes; however, several dengue vaccine candidates are in development (Lam 2013). A recombinant, live, attenuated, tetravalent dengue vaccine was evaluated in phase 3 clinical trials conducted in Asia and Latin America. The vaccine was found to be efficacious against both dengue and severe dengue and led to fewer dengue-related hospitalizations in five Latin American countries where dengue is endemic (Villar et al. 2015).

Animal Models and Evidence of Neurological Involvement

Over the past six decades, researches across the world have sought ideal models to study the virulence and/or pathogenesis of DENV. These efforts have led to little progress, however, because even non-human primates that are closely related to humans show no signs of disease following inoculation with the virus. A suitable animal model is particularly important for testing the efficacy of vaccines and specific antiviral drugs, as well as for the identification of both the methods to inhibit viral replication and clinical symptoms (Bente and Rico-Hesse 2006; Rico-Hesse 2007; Zellweger and Shresta 2014; Smith et al. 2014).

Although several murine models have been described, none have been able to recreate the entire spectrum of the disease (FD and DHF/DSS). Three different approaches have been taken to try to develop a murine model, which is focused on inducing a human-like disease in either (1) immunocompetent mice, (2) knockout mice in which specific genes that are important to the immune response have been removed, or (3) immunodeficient mice that have had human cells implanted. We are of the opinion that it is advantageous to have numerous murine models of dengue, as they facilitate a better understanding of the disease. For a more detailed review of the above-discussed murine models, please see Bente and Rico-Hesse 2006; Yauch and Shresta 2008; Smith et al. 2014; Zellweger and Shresta 2014, and Plummer and Shresta 2014.

In regions where the virus is endemic, DENV infection can cause both encephalitis and other neurological manifestations. Despite this, the immune mechanisms that drive neurovirulence remain poorly understood.

Nitric oxide (NO) is an important paracrine and autocrine signaling molecule that is used and produced by a variety of cells in the body. NO has a wide range of functions, including blood vessels dilation, platelets aggregation, combating infections and tumors, mediating inflammation and macrophage cytotoxic activity, and serving to transmit messages between nerve cells. Many cells are able to synthesize NO using hemeproteins called NO synthases (NOS). Three isoenzymes of NOS have been isolated and cloned, two of which constitutive, cNOS and eNOS, and one that is inducible, iNOS/Nos2 (D'Ávila et al. 2008; Queiroz and Batista 1999). iNOS/Nos2 is produced in response to viral infection by two mechanisms: these include either direct induction during viral replication or indirect induction by cytokines (Blais and Rivest 2004).

In a study conducted by Barcelos Figueiredo et al. (2008) in Belo Horizonte (MG, Brazil), genotype I of DENV-3 was isolated in 2004 from the serum of a lethal dengue case that had CNS involvement. This virus isolate may be a useful tool in studying DENV pathogenicity and the host immune response that develops during neurological infection with it, which could in turn aid in the design of new dengue vaccine formulations.

In 2010, Ferreira et al. described an immune-competent, adult mouse model that was inoculated with genotype I of DENV-3 via an intracranial route (called the i.c. model), which led the virus to infect and replicate within the brain and ultimately caused the deaths of affected animals. Conversely, other genotypes of DENV-1, DENV-2, DENV-3, and DENV-4 were not infectious following i.c. inoculation in the above-described mouse model. In these cases, the mice were asymptomatic and all of them survived. The primary symptoms that developed in the animals infected with genotype I of DENV-3 included hunched posture, pilo-erection, loss of balance, and paralysis (Fig. 2). The identification of these symptoms during initial experimentation is important, as they can be used to set parameters for when to euthanize animals in subsequent experiments aimed at studying the immune response (e.g., the development of encephalitis).

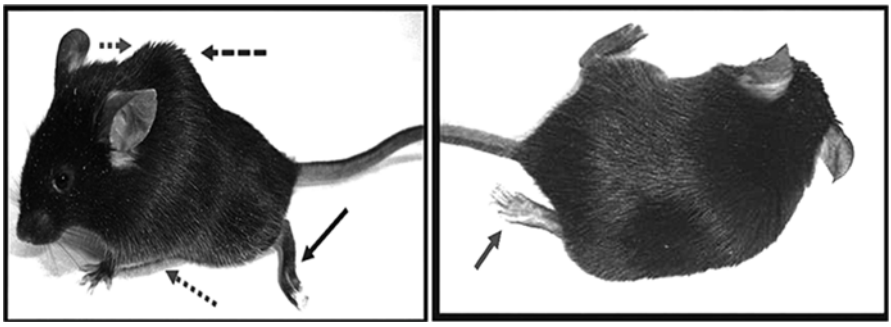

Fig. 2 Neurological symptoms of mice infected with DENV-3 genotype I. Following i.c. infection of DENV-3 genotype I, the mouse on the *left* exhibited a hunched posture (shown by an *interrupted arrow*), pilo-erection (shown by *arrows with points*), and hind limb paralysis (shown by a *full arrow*). The mouse on the *right* experienced a loss of balance (i.e., it had fallen on its side in the cage) in addition to hind limb paralysis (shown by a *full arrow*)

In 2013, Souza et al. demonstrated that the same clinical signs and mortality that were observed in mice following infection with DENV-3 genotype I were also observed in mice infected with the Mochizuki strain of DENV-1 (Hotta 1951), a classical mouse model of neurovirulence. In these experiments C57BL/6 mice were intracranially infected with DENV-3 genotype I. Following the infection, an increase in *Nos2* and cytokine expression was noted in the brain, in addition to neuronal degeneration and necrosis, worsening clinical signs, viral load peak in the brain, tissue damage, and subsequent death. Conversely, *Nos2* knockout mice subjected to intracranial infection with the same neurovirulent DENV, despite evidence of high viral titers in the brain, were found to have low expression levels of pro-inflammatory cytokines (except for interferon (IFN) gamma) and a 100 % rate of survival. At 5 days post infection (d.p.i.) with DENV-3 genotype I, evidence of inflammatory infiltrates and edema in the brain were found in both C57BL/6 and *Nos2* knockout mice. On the eighth d.p.i., the DENV-infected C57BL/6 mice exhibited an intense endothelial response in the brain, which included perivascular polymorphonuclear cell infiltration, mononuclear leukocyte cell migration, meningitis, and intense vacuolization suggestive of neuronal degeneration, necrosis, and apoptosis. Interestingly, by contrast, the DENV-infected *Nos2* knockout mice showed only mild histopathological changes in the brain at 8 d.p.i. At later time points, just before the death of the animals, the infected C57BL/6 mice became lethargic and had reduced motility. Associated with TNF alpha and IFN gamma, NO production in the brain can cause cerebral damage: this damage may increase the expression of genes encoding pro-inflammatory molecules (Blais and Rivest 2004). NO has a protective and regulatory function at optimal doses and when acting on the cells of an organ that can quickly regenerate; however, NO has toxic effects at higher concentrations (Blais and Rivest 2004) and also, in our opinion, when acting on cells of an organ that cannot easily regenerate, such as the brain. Therefore, we conclude that NOS2 has a deleterious role in neurological dengue, likely because of its destructive action toward brain cells, which do not regenerate as quickly and easily as other cell types.

Involvement of the Host Immune Response

In addition to the deleterious action of NOS2 on host immune defense, C57BL/6 mice infected with DENV-3 genotype I (Souza et al. 2013) exhibited similar resultant levels of cytokine and viral gene transcript expression, both of which peaked at 7–8 d.p.i. Concomitant with this finding was an increase in viral replication, neural tissue damage, and paralysis, ultimately culminating in death. These findings once again demonstrate the role of the immune system in the pathogenesis of DENV. Furthermore, IFN gamma and TNF alpha appear to be markers of dengue disease severity and prognosis (Lin et al. 2005; Bozza et al. 2008). IFN-gamma knockout mice exposed to DENV-3 genotype I (Souza et al. 2013) were susceptible to infection, exhibiting 100 % mortality rate and presenting the same clinical signs of infection as infected C57BL/6 mice. Moreover, in addition to discovering the

deleterious role of NO in the host immune response to neurological dengue, Souza et al. (2013) were able to use this i.c., mouse model to show that IFN gamma has a possible protective role in neurological dengue.

Although $CD4^+$ T cells, $CD8^+$ T-cells, and macrophages are all present in the brain tissues of DENV-virus infected mice (Souza et al. 2013), only the $CD4^+$ T-cells are responsible for producing IFN gamma, which subsequently leads to the production of NOS2 by C57BL/6 mouse macrophages in response to DENV infection. Beyond that, *Nos2*-deficient animals that are infected with DENV do not exhibit increased cytokine levels in the brain, with the exception of IFN gamma, which was expressed at a higher level than in uninfected mice. *Nos2* knockout mice also had fewer histopathological alterations following infection, confirming the importance of NOS2 in the neuropathogenesis of neurovirulent DENV. Conversely, *Nos2*-deficient mice had a 100 % rate of mortality following intraperitoneal injection of mouse-adapted DENV-3 (Costa et al. 2012), indicating that both the isolate of virus (DENV-3 genotype I vs. adapted DENV-3) and the route of inoculation (intracranial vs. intraperitoneal) are distinct variables that can cause contrasting results. Thus, each of the DENV mouse models serve as unique tools for studying the various pathological patterns of the virus.

The intracranial infection of mice with a DENV isolate known to cause human CNS disease is an additional method of studying the immune response, immunopathological manifestations, and neurological manifestations of DENV infection, which are increasingly being reported in regions where dengue is endemic.

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Japanese Encephalitis Virus: Molecular Biology to Pathology

Sunit K. Singh

Introduction

Flaviviridae family can be classified into three genera, namely: flaviviruses, hepaciviruses, and pestiviruses. Japanese encephalitis virus (JEV), West Nile virus (WNV), Dengue virus (DENV), Yellow fever virus (YFV), and Tick-Borne Encephalitis Virus (TBEV) are the most common flaviviruses. Most of the flaviviruses are transmitted through ticks and mosquitoes, therefore termed as arthropod-borne viral infections or arboviral infections. To be evolutionarily successful, arboviruses need immunological naïve hosts, therefore most of the arboviruses have evolved to use rapidly reproducing animals as their reservoir hosts.

Japanese encephalitis (JE) is caused by JEV. The worldwide annual incidence is 45,000 human cases of infection and 10,000 deaths (van den Hurk et al. 2009). Approximately 25 % of encephalitis patients die while about 50 % of the survivors suffer from permanent neurologic sequelae, such as memory loss, impaired cognition, behavioral disturbances, convulsions, motor weakness or paralysis, and abnormalities of tone and coordination. (Campbell et al. 2011). Since 1974–2013, 62 cases have been reported of travel-associated JE from non-endemic regions (Langevin et al. 2012; Tappe et al. 2012). In India, the state of Uttar Pradesh and adjoining areas like foothills of Himalayas, Nepal, West Bengal, or areas prone to floods and extensive rice cultivation are affected by JE due to breeding of mosquitoes.

JEV is maintained in a zoonotic cycle, which can be both enzootic and epizootic. This cycle involves pigs as the major reservoir/amplifying host, water birds as carriers, and mosquitoes as vectors (van den Hurk et al. 2009). Pigs act as amplifying hosts because of the high natural infection rate (98–100 %), high viremia, to infect

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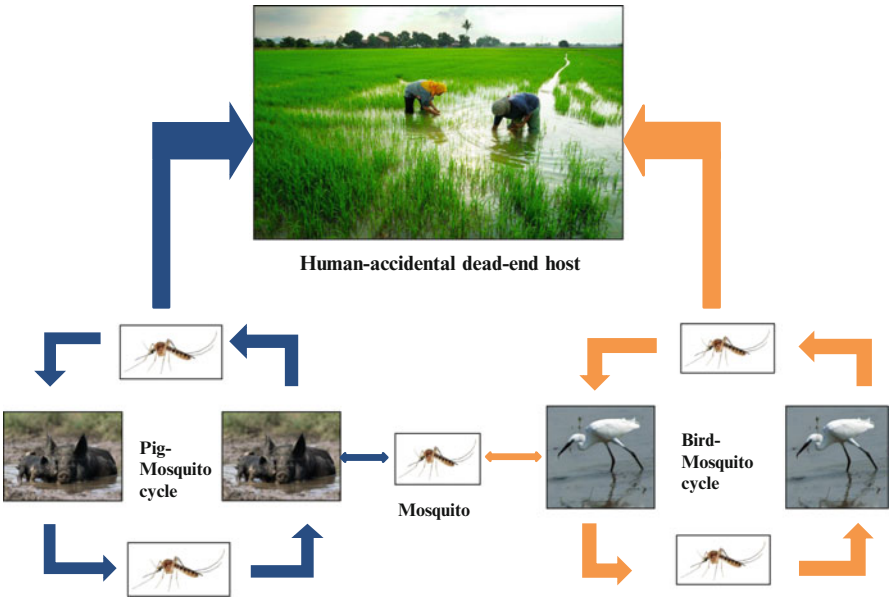

Fig. 1 JEV life cycle

mosquitoes, propensity of vector mosquitoes to feed on swine, and high birth rate in pigs, which provide adequate number of pigs for infection every year (van den Hurk et al. 2009; Gresser et al. 1958; Scherer et al. 1959a, 1959b). Humans are known as accidental or dead end hosts of JEV due to the low viremia levels that are not sufficient to infect feeding mosquitoes (Solomon et al. 2000) (Fig. 1). Thus, human to human transmission of virus has not been reported so far but the vertical transmission from the JEV infected mother to the developing fetus has been reported in the literature (Chaturvedi et al. 1980; Mathur et al. 1981, 1982). *Culex tritaeniorhynchus* belonging to the *Culex vishnui* subgroup of mosquitoes is the primary vector for JEV (Karunaratne and Hemingway 2000), while *Culex gelidus*, *Culex fuscocephala,* and *Culex annulirostris* are considered as secondary/regional vectors (van den Hurk et al. 2009). Mosquito-borne flaviviruses can persistently infect the mosquito without causing any pathology in the central nervous system (CNS) of mosquitoes similar to JE in humans. Recently Xiao et al. (2015) reported an *Aedes aegypti* homologue of the neural factor Hikaru genki (AaHig) in the CNS of mosquitoes, which efficiently restricts flavivirus infection of the CNS of mosquitoes. AaHig predominantly expresses in the mosquito nervous system and remains localized to the plasma membrane of neural cells. Therefore, this evolutionarily conserved antiviral mechanism prevents lethal flaviviral infections in the CNS of mosquitoes, and thus may facilitate flaviviral transmission of mosquito-borne viruses in nature (Xiao et al. 2015). The first clinical case of JEV infection was reported in Japan in 1871 (van den Hurk et al. 2009). The Nakayama strain of JEV was first isolated in 1935 from the postmortem brain sample of a person died with

JEV infection. Based on mosquito activities, two different seasonal prevalence patterns have been reported for JE outbreaks. In the tropical regions, human JEV infections are observed throughout the year, although peak prevalence has been reported after the start of the rainy season. In subtropical and temperate regions, JE epidemics are observed from May/June to September/October due to increase in the mosquito density during rice cultivation (Ishikawa et al. 2014). The variations in the JEV genotypes and differences in temperatures have been considered as important factors for the differences in the epidemiological patterns of JE outbreaks (Solomon et al. 2000). In addition to socioeconomic conditions, the geographical conditions play an important role in the spread of the JEV infections.

Genome Organization

JEV is an enveloped virus about 50 nm in diameter with a single stranded (ss), plus sense, RNA genome of ~11 Kb in length (Unni et al. 2011). The virion comprises viral envelope and membrane proteins. Membrane proteins are arranged into head to tail manner in the form of heterodimers, embedded in a host cell-derived lipid bilayer, which surrounds a nucleocapsid core. The genome is organized into a nucleocapsid composed of capsid (C) protein surrounding and anchoring RNA genome (Kuhn et al. 2002). Genome is represented by one open reading frame (ORF) encoding for a single polyprotein, which is further cleaved into 3 structural—capsid (C), precursor to membrane (prM), envelope (E) and 7 nonstructural proteins—NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5, by viral proteases and host signalases, post-translationally (Fig. 2). The structural genes are found within the N′ terminal of the polyprotein (780 residues) (Rice et al. 1985). The ORF is flanked on the sides by 5′ and 3′ non-coding regions (NCRs). NCRs have crucial *cis*-acting elements for replication, transcription, and translation of JEV (Unni et al. 2011) (Fig. 2). The genomic RNA of JEV has a type I cap at the 5′ end (m7GpppAmp) but lacks a 3′-terminal poly (A) tail. Nucleotide sequencing of C, prM, E genes and phylogenetic analyses have revealed the existence of 5 genotypes (genotype I, II, III, IV, and V) of JEV worldwide (Solomon et al. 2003; Nitatpattana et al. 2008; Uchil and Satchidanandam 2001). Hemagglutination inhibition (HI) and neutralization assays using monoclonal antibodies (MAbs) and polyclonal antibodies can be used to identify different isolates within the same genotype (Hasegawa et al. 1995; Saito et al. 2007).

Structural Proteins

C protein consists of ~120 amino acids, in the form of homodimers. It is involved in packaging of the viral genome and formation of the nucleocapsid (Mukhopadhyay et al. 2005). prM (~165 aa) and E (~495 aa) are glycoproteins having two

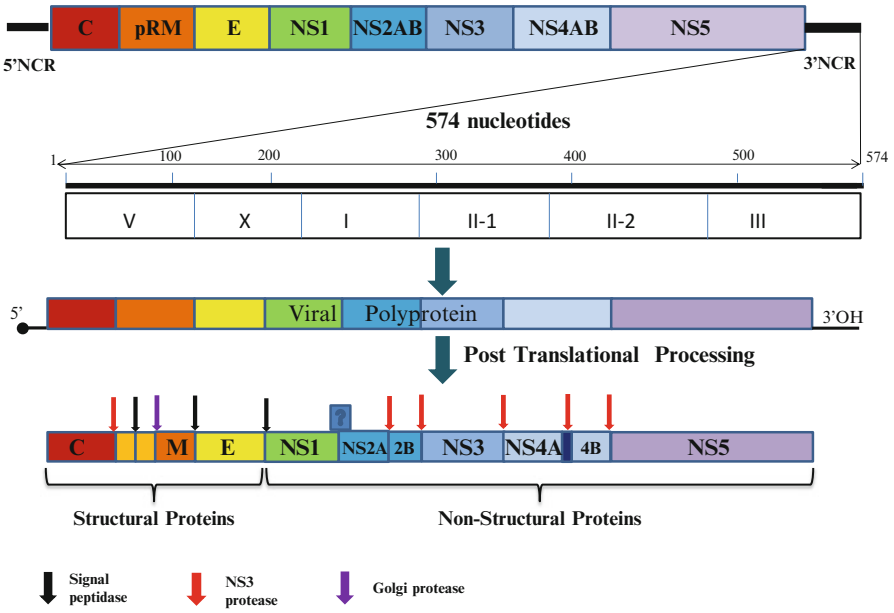

Fig. 2 Genome of JEV

transmembrane helices. These two glycoproteins are released from the nascent polyprotein following co-translational cleavage by signal peptidases (Unni et al. 2011). The "signal sequence" present at N′ terminal of prM protein helps in the insertion of this protein into the ER membrane, where it gets cleaved off by the host enzymes into ER lumen. In the immature virions, prM protein serves as a chaperone for folding and assembly of the E protein. The prM gets cleaved by cellular furin-like protease to form M (~75 aa) and the peptide during the maturation of the flaviviruses in the Golgi complex. E protein composed of 90 homodimers remains present in the host-derived lipid bilayer and forms the major part of the mature virion component (Mukhopadhyay et al. 2005). The E protein (53 kDa) helps in the JEV attachment, membrane fusion, hemagglutination, cell tropism, etc., therefore the E protein is the main target of neutralizing antibodies (Pierson et al. 2008). E protein contains a cell receptor binding protein and the mediator of the membrane fusion and cell entry (Ding et al. 2003). E proteins are believed to interact with various cellular receptors such as DC-SIGN and DC-SIGNR. E protein (~495 aa) is the major component of the surface projections of the virion, and also determines the virulence. The single amino acid substitutions in E protein are sufficient enough to cause loss of neurovirulence or neuroinvasiveness (Cecilia and Gould 1991; Hasegawa et al. 1992; Ni and Barrett 1996). Despite the lack of proof reading in RNA viruses during replication, the envelope gene is one of the most slowly evolving sites, which might be due to the selective pressure of infection and replication of JEV in both vertebrate and arthropod cells.

Nonstructural Proteins

NS1, a ~48 kDa protein, exists as homodimer in cellular and hexadimer in secreted forms. It is capable of eliciting protective antibody response in the host (Liao et al. 1998). NS1 is also known as complement fixing antigen of JEV (Smith and Wright 1985). NS1′ is an NS1 extension protein (53 kDa), which has been reported in JEV infected cells (Mason 1989). NS1′ translates from C′ terminal of NS2A through alternate splicing mechanism (Fan and Mason 1990). This happens due to -1 Programed Ribosomal Frameshift (-1PRF), the new reading frame stops at 46th amino acid downstream of heptapeptide leading to an addition of 52 amino acids sequence (Melian et al. 2010). NS2A is a small hydrophobic, membrane-associated protein involved in RNA replication. It acts in a *cis* fashion to cleave the NS1–NS2A junction after translation and plays functional role in viral replicase complex, viral assembly, and secretion. NS2A modulates the antiviral response of the host by inhibiting interferon (IFN) signaling pathway (Leung et al. 2008). NS2B remains as a heterodimer along with NS3, which helps in anchoring of this heterodimeric complex to the ER membrane. NS2B acts as a cofactor for the serine protease, which cleaves the viral polyprotein at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions (Shiryaev et al. 2007; Bera et al. 2007; Arias et al. 1993; Jan et al. 1995; Bessaud et al. 2006). FRET (Fluorescence Resonance Energy Transfer) and BiFC (Biologic Fluorescence Complementation) based data demonstrated that NS2B plays an important role in bringing all the transmembrane non-structural proteins (NS2B, NS4A, and NS4B) together with NS3 and NS5 proteins for efficient JEV replication (Yu et al. 2013). NS3 protein possesses RNA helicase, serine protease, and NTPase activity, which help in JEV replication and contribute in RNA capping and pathogenesis (Utama et al. 2000; Luo et al. 2008). NS3 protein is dispersed throughout the cytoplasm in infected cells including nuclear envelope and targets microtubules and golgi-associated tumor susceptible gene 101 (TSG101), which is involved in intracellular trafficking of viral components (Wang et al. 1998; Chiou et al. 2003). NS4A and NS4B are small hydrophobic transmembrane proteins, involved in viral replication and formation of replication complex along with NS2A and NS2B proteins. In addition, NS4A acts as an IFN antagonist to counteract the antiviral machinery of the host cell (Lin et al. 2008). NS5 of JEV has methyltransferase activity (MTase) on N-terminal and RNA-dependent RNA polymerase (RdRp) activity on C-terminal motifs (Mukhopadhyay et al. 2005; Dong et al. 2014; Lu and Gong 2013), which is important for RNA replication and transcription. NS5 is multifunctional heterodimer, which localizes along with other non-structural JEV proteins in the perinuclear ER of the infected cells (Welsch et al. 2009). It also acts as an IFN antagonist by blocking IFN induced JAK-STAT signaling cascade by preventing Tyk2 tyrosine phosphorylation and STAT activation (Lin et al. 2006).

Cellular Tropism and Replication

Many different molecules have been reported to interact with JEV at the cell surface. The glycosaminoglycans, such as heparan sulfate, have been reported to play role in JEV entry and infectivity in BHK-21 cells (Unni et al. 2011; Chien et al. 2008). A 74-KDa heat shock cognate protein 70 (HSP70) has been reported to interact with JEV E protein in C6/36 mosquito cell lines (Ren et al. 2007). Low density lipoprotein like receptor may play a role in attachment of non-heparan sulfate adapted JEV strains in mammalian cells (Kaufmann and Rossmann 2011). Many different host cellular proteins are believed to bind with viral attachment proteins (VAP) rather than a single receptor.

The entry of virus is carried out via receptor mediated endocytosis in clathrin coated vesicles (Nawa et al. 2003; Lee et al. 2008) and the acidic pH of the endosome leads to acid-catalyzed conformational changes in E protein of JEV, which results in the membrane fusion, uncoating, and release of genome into the cytoplasm (Unni et al. 2011). A viral replicase is assembled from viral non-structural proteins and host factors after the translation into the cytoplasm. Viral replication complex (RC) is formed by the NS proteins (NS3 and NS5) along with other host factors (Chen et al. 1997). Cytoplasm is the major site for the presence of JEV RC. 60 % of the RdRp activity has been reported to be associated with cytoplasmic membranes, 20 % with outer nuclear envelope membrane, and the remaining 20 % with the nucleus (Uchil et al. 2006). RC is surrounded by a membrane, which protects RC from extensive protease treatment (Kumar et al. 2003). The presence of cis-acting RNA elements in NCRs helps in JEV replication (Chen et al. 1997). The 3′NCR of JEV has 6 domains namely: V, X, I, II-1, II-2, and III in the 5′-to-3′ direction. The domains II-2 and III have been reported to be sufficient for replication, but other domains increase the replication efficiency (Yun et al. 2009). The formation of RC is followed by cyclization of viral genome, where a double stranded replicative form (dsRF) is formed by the RC using viral RNA. This involves long range RNA interactions mediated by invert complementarity of the 5′ and 3′ NCRs. Cyclization in flavivirus genomes ensures that replicated viral RNA molecules are of full length (Hahn et al. 1987). The dsRF initiates the formation of new RNA strands complementary to the parent strand (Uchil and Satchidanandam 2003). This mode of JEV replication is asymmetric and semiconservative forming about 10–100 folds more plus strands than the minus strands (Unni et al. 2011).

The protein after translation moves out from the ER to *trans*-golgi network for assembly and maturation. Virion assembly takes place in association with intracellular membranes of endoplasmic reticulum (Chambers et al. 1990). However, the immature virions assemble in membrane bound vesicles. Virions are accumulated in the rough ER and transported to the cell surface in acidic vesicles through the host secretory pathway (Chambers et al. 1990). The virions fuse with the plasma membrane and release by exocytosis after the cleavage of membrane protein from the precursor membrane protein in the golgi bodies by golgi protease (Unni et al. 2011).

Mechanism of Pathogenesis

JEV infection starts with the bite of infected mosquito. Mosquitoes are relatively short lived and should feed frequently in order to complete their reproductive cycles. Therefore mosquito-borne viruses have a relatively brief opportunity for transmission. Mosquito-borne viruses have 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. Mutations may accumulate relatively faster in the mosquito-borne RNA viruses due to multiple replication cycles in the vector, which results into the generation of many different genetic lineages. Mosquito saliva contains immunosuppressive bioactive substances, which suppress the local immune response at the site of mosquito bite and ultimately help JEV to establish in host (Edwards et al. 1998; Limesand et al. 2003; Schneider et al. 2004). In order to achieve successful JEV transmission through mosquitoes into hosts, the following criteria should be met: (1) Mosquito must feed on viremic host, (2) an infectious dose of virus (above minimum threshold) must enter into 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 into cell cytoplasm and replicate to produce infectious virions, (5) virions must disseminate from midgut epithelial cells and enter the hemocoel, (6) virions must infect salivary glands, and (7) virions must be secreted in saliva while mosquito feeds upon a host. In natural human infection, the infected mosquito inoculates the JEV into the skin along with saliva during the bite. Initial virus infection occurs in the skin cells at the site of inoculation (Johnston et al. 2000). Virus is then transported in migrating dendritic langerhans cells to the lymph nodes and then to the bloodstream. Primary viremia leads to the infection of connective tissues, skeletal muscles, etc. The primary viremia continues for several days due to the release of the virus from these tissues back into bloodstream. JEV might also have a peripheral replication cycle in monocytes and macrophages (Sapkal et al. 2007). An immune response mounted at this point clears the viral infection in most of the cases, which might explain the absence of JEV in peripheral blood. When the infection progresses to encephalitis, the virions are thought to disseminate to secondary sites through blood, which generates a rapid inflammatory response with mononuclear and polymorphonuclear cell infiltration. JEV leads to functional impairment of DCs through both MyD88 dependent and independent pathways (Aleyas et al. 2009). Splenic DC population has also been reported to be altered in JEV infection with preferential depletion of $CD8\alpha^{+}CD11c^{+}$DCs (Aleyas et al. 2010). As DCs express various co-stimulatory and adhesion molecules, which can activate naive T cells, their functional impairment and depletion could augment viral dissemination by downregulating $CD8^{+}$ and $CD4^{+}$ T cell responses (Aleyas et al. 2010) (Unni et al. 2011).

The invasion of JEV into brain may occur via antipodal transport of virions or through the brain microvascular endothelial cells. Replication of JEV in endothelial cells is still a debatable issue (Johnson et al. 1985; Liou and Hsu 1998). However,

blood brain barrier (BBB) breaching does occur during JEV infection (Liou and Hsu 1998). Chen et al. (2014) reported that the infection of pericytes with JEV leads to the production of interleukin-6 (IL-6), which contributed to the disruption in the endothelial barrier integrity in cultured brain microvascular endothelial cells. They demonstrated that ubiquitin-protein ligase E3 component n-recognin-1 (Ubr 1) as a key upstream regulator led to the proteasomal degradation of ZO-1 downstream of IL-6 signaling (Chen et al. 2014). Recently Li et al. (2015) demonstrated that JEV gains entry into the CNS prior to BBB disruption. They reported that it is not JEV infection, but the inflammatory cytokines/chemokines induced by JEV infection inhibit the expression of Tight Junction proteins (TJPs), which compromises the BBB permeability (Li et al. 2015). JEV primarily infects the neurons in the CNS, which results into neuronal loss (Yasui 2002), by apoptosis via the ER stress pathway (Su et al. 2002). Reactive oxygen species (ROS) mediated neuronal cell death has also been observed in vitro by replication-incompetent JEV (Lin et al. 2004). Apoptosis is very specific to neuronal cells in JEV infection and suggests an unidentified receptor mediated death signaling pathway. It has been demonstrated that JEV replicates exclusively in the rough endoplasmic reticulum (RER) of the neurons (Hase 1993). It is expected that JEV can also induce death in neighboring uninfected cells in a bystander fashion along with JEV infected neurons (Unni et al. 2011). Efficient replication of JEV has been reported in human neuroblast derived (NB) cells as compared to non-NB cells (Yang et al. 2004). Neuronal maturity has been closely associated with the infectivity of the virus. Viral tropism for immature neurons has been reported in experimental models of JEV infection (Ogata et al. 1991; Kimura-Kuroda et al. 1993). Mature neurons become more resistant to the JEV induced apoptosis and this resistance might be due to the neuronal expression of cellular inhibitors of apoptosis such as bcl-2 and bcl-x (Levine et al. 1993; Griffin 1995). However, aggressive neurovirulent viruses might cause neuronal death in mature neurons by suppressing the inhibitors of apoptosis (Ubol et al. 1994). Neural progenitor stem cells (NPSCs) are also infected by JEV leading to their loss along with damage of subventricular zone (SVZ). JEV infected NPSCs show reduction in proliferative properties, impairment in the repair and regeneration of damaged neurons, which contributes to the neurological sequelae in the survivors of JE (Das and Basu 2008).

Astrocytes and microglial cells can also get productively infected by JEV in addition to neurons (Thongtan et al. 2010). Astrocytes form a part of BBB and play multiple roles in the CNS. In general, astrocytes maintain the homeostasis in CNS by storing energy in the form of glycogen and produce enzymes for detoxification activities (Unni et al. 2011). Prominent astrocyte activation has been specifically reported in the areas of neuronal damage (German et al. 2006). Microglial cells are the resident macrophages of the CNS and can be productively infected by JEV and might serve as a reservoir for the virus (Thongtan et al. 2010). Ghosal et al. reported that activation of microglial cells may play significant role in inducing neuronal cell death due to the production of the proinflammatory mediators by microglial cells (Ghoshal et al. 2007). Both virus induced apoptosis and necrosis mediated through

overactivation of microglia and release of ROS, TNF-α, and nitric oxide, leading towards "bystander" damage to neuronal cells, have been demonstrated in vitro (Su et al. 2002; Raung et al. 2001). We have recently reported that JEV JaOArS982 strain induces the expression of miR-146a in human microglial cells to suppress the NF-κB activity and disruption of antiviral Jak-STAT signaling, which helps the virus to evade the cellular immune response (Sharma et al. 2015).

JEV latently infected cells have been reported in in vitro culture systems and mouse models. Sharma et al. (1991) described about the JEV latency and recurrence of JE in children many months after the initial infection (Sharma et al. 1991). However, this does not seem to be a common feature of JEV infection, but the monocytes might remain infected for the long time even after the decline of apparent viremia (Yang et al. 2004). Persistent infection of JEV has been reported in the nervous system of ~5 % of the human survivors of JE (Ravi et al. 1993).

Host and Japanese Encephalitis Virus interactions

Humoral Immune Response

Antiviral antibodies have been reported as an important player in dealing with the arbovirus induced encephalitis. Cell free virus is usually cleared by antibodies through neutralization of the virus and phagocytic clearance of the virions (Griffin 1995). In case of neuronal infection, antibodies are supposed to act at the surface of infected neurons to alter intracellular replication of viruses in order to follow the noncytolytic mechanism (Griffin 1995). After infection most patients produce IgM, both in serum and cerebrospinal fluid (CSF) (Unni et al. 2011). IgM in CSF is detected as early as day 1 after the onset of the symptoms, while it is not detected in serum until after 9–10 days. The presence of JEV specific IgM antibodies in the serum or CSF is necessary for laboratory confirmation of JEV infection (Solomon et al. 2008). JEV specific IgM has been used for clinical diagnosis of JEV infected patients through IgM capture ELISA. Class switching to IgG occurs few days later after the onset of the symptoms (Burke et al. 1985a). However, if a person has been infected with dengue virus (DENV) prior to JEV infection, high titers of IgGs have been reported (Innis et al. 1989) due to the presence of cross reactive antibodies of JEV with other flaviviruses. NS1 specific antibodies are detected in sera of JE patients (Krishna et al. 2009). Passive transfer of monoclonal antibody (mAb) against JEV has shown to protect mice from JEV infection (Kimura-Kuroda and Yasui 1988; Zhang et al. 1989).

E glycoprotein is the major target of neutralizing antibodies in the host and recombinant E protein has also shown immunogenicity (Xu et al. 2010). Various studies have proved the efficiency of different epitopes of E protein in eliciting an immune response among hosts during JEV infection (Verma et al. 2009a; Feng et al. 2007).

Cell Mediated Immune Response

Clearance of the JEV from the infected cells (especially from neurons) is more challenging task. $CD8^+$ cytotoxic T cells (CTLs) are known to eliminate the infected cells through major histocompatibility complex 1 (MHC class I) recognition. Neurons are deficient in expression of class I and class II MHC antigens (Joly et al. 1991; Daar et al. 1984a, 1984b). Therefore CTLs can't directly recognize the infected neurons in various neuroviral infections. However, most of the studies have been conducted on animal models for understanding the mechanism of cell mediated immunity against JEV infections. Only few studies have been conducted in infected humans. These studies have shown that memory T cells are helpful in protecting a person from secondary infections with the same or related viruses. JEV infection induces the expression of classical and nonclassical MHC-I through NF-κB pathway (Abraham et al. 2010). NS3 protein of JEV has been reported to elicit $CD4^+$ and $CD8^+$ T cell responses. The presence of NS3 specific memory T cells has been reported in majority of people living in JEV endemic regions (Kumar et al. 2003).

T helper 1 (Th1) and cytotoxic T (Tc) cells infiltrate into mice brain following JEV infection (Fujii et al. 2008). Th1 responses have also been reported in mice model by the envelope protein domain III of JEV (Verma et al. 2009b). Infiltrated T cell repertoire analysis showed the presence of unique T cell receptors (TCRs). Production of JEV specific IgM and IgG1 antibodies along with a simultaneous increase in Th2 cytokines has been reported in JEV challenged mice (Biswas et al. 2009).

Soluble Factors in Progression of Japanese Encephalitis

JEV activates microglia, which in turn produces proinflammatory cytokines, i.e., COX-2, iNOS, MCP-1, IL-6, and TNF-α (Ghoshal et al. 2007). Neurons die due to the bystander effect of cytokines produced by microglia rather than by JEV infection itself. Different regions of the brains show different pattern of expression of the proinflammatory cytokines after JEV infection. Hippocampus, the region of the brain associated with memory and learning, has the highest amount of these proinflammatory cytokines. This might explain the neurological sequelae in the JE survivors (Ghoshal et al. 2007). Increased levels of TNF-α have been found in both serum and CSF samples of JE patients and correlated with increasing mortality (Ravi et al. 1997). Tumor necrosis factor receptor (TNFR-1) complex is activated during JEV infection, specifically in the neurons, initiating the apoptotic cascade through p38 mitogen activated protein kinase (MAPK) and c-Jun N-Terminal Kinase (pJNK) pathway (Swarup et al. 2007a). It leads to mitochondria mediated apoptosis in the neurons. Infected NPSCs secrete INF-γ and IL-6, which activate the microglia and astrocytes. Infected NPSCs also produce TNF-α and CCL-2, which induce the expression of cell adhesion molecules on the endothelial cells of BBB and help in the recruitment of activated T cells and monocytes into CNS (Das et al. 2009). A striking inverse association between IFN-γ levels and the severity of postencephalitic sequelae has been observed in JEV infected patients (Kumar et al. 2004).

The reduced expression of anti-inflammatory cytokines, IL-10 and IL-4, has been reported in microglial cells of the JEV infected mouse (Swarup et al. 2007b). This was associated with increased viral load and tissue pathology. Pretreatment of cells with IL-10 reduces IL-1β and TNF-α mediated microglial COX-2 and ROS production and also inhibits neuronal death following microglial activation (Swarup et al. 2007b). Protein tyrosine kinase (PTK) inhibitors were found to be effective in controlling JEV induced neurotoxicity by suppressing JEV induced IL-1β and TNF-α production though PTK inhibitors were not successful in suppressing the JEV replication (Raung et al. 2005). This finding demonstrates that proinflammatory cytokines play major role in JEV induced neurotoxicity.

JEV infection induces the expression of chemokines RANTES, IP-10, and IL-8 in CNS (Chen et al. 2004; Singh et al. 2000). The IP-10 production of astrocytes is stimulated by INF-γ during JEV infection, and progressively increases in the brain during JEV infection. Though expressed as a protective response, IP-10 might cause bystander death of neurons by causing excessive inflammation along with reactive gliosis (Bhowmick et al. 2007). JEV-induced RANTES production by astrocytes and microglia contributes to recruitment of immune cells (Chen et al. 2004). In CNS, higher levels of IL-8 have been reported to be associated with severe illness and fatality among JE patients (Singh et al. 2000).

Clinical Manifestations

Most of the JEV infections are asymptomatic. JEV infection primarily occurs in children less than 15 years old, but it has also been reported in adults. JE is mostly a disease of children in India, whereas WNV and St.LNV are more likely to affect adults in the USA. Such differences depend on the variations in the intensity of transmission and acquired immunity. The incubation time for JE ranges from 5 to 15 days (Fischer et al. 2010). Typical febrile illness of JE manifests with the onset of fever, headache, backache, myalgia/muscle pain, anorexia, and lasts for a week. This is followed by change in mental status, disturbances in speech, gait and other motor functions in advance stages. The neurological manifestations depend on the part of the nervous system affected—the meninges (meningitis), the parenchyma of the brain (encephalitis), or the spinal cord (myelitis) (Solomon and Vaughn 2002; Solomon 2004).

In children, the initial presentation of disease includes gastrointestinal symptoms, like abdominal pain, nausea, etc. The disease progression can be classified into three distinct stages, namely a prodromal stage before CNS disease manifestation, an encephalitic stage, and a late stage in which the patient recovers completely, or the signs of neuronal injury may persist (Tiroumourougane et al. 2002).

Histopathological examination shows a characteristic pattern of microglial proliferation with the formation of microglial nodules. Viral antigens have been reported in dead or degenerating neurons by immunohistochemical staining (Johnson et al. 1985; Li et al. 1988). Gliomesenchymal nodules are distributed in the superficial and

deep grey matter, including the brain stem, thalamus, basal ganglia, hippocampus, and anterior horn cells of the spinal cord. Patients with residual neurologic impairment show scarred ramified foci in the thalamus, substantia nigra, and hippocampus (Ishii et al. 1977). Cerebral edema and congested leptomeninges have been reported in gross examination of the brain (Li et al. 1988). Basal ganglia, midbrain, and thalamus have been reported to show characteristic patterns of mixed intensities or hypodense lesions in MRI scans in patients (Kalita and Misra 2000).

Due to the brief transient viremia and cross reactivity of elicited antibodies to other flavivirus antigens in serological assays, identification of the specific flavivirus presents a challenge. Such complexity increases in the secondary flavivirus infections, where the immune response may be greater to the primary infecting flavivirus compared to the most recent infection (Porterfield 1986; Johnson et al. 2005). The isolation of JEV from the blood of JE patients is mostly unsuccessful due to transient viremia and low viral titer. JEV has been occasionally isolated from the CSF of patients who do not yet have antibody (Burke et al. 1985b) and postmortem brain tissue (Solomon 2004; Burke et al. 1985b). Viral RNA can be detected in CSF by the polymerase chain reaction (Igarashi et al. 1994). However, the accepted standard for flavivirus encephalitis diagnosis is IgM ELISA. IgM antibody is produced early in the infection, rises rapidly to the detectable levels, and is less cross reactive than IgG antibodies. This assay is often performed on CSF or serum samples. CSF is a preferable diagnostic specimen in neuroinvasive disease, as an anti-flavivirus IgM antibody may be present in serum, in the cases of inapparent or mild infections or following a flavivirus vaccination, but may not be cause of encephalitis (Burke et al. 1982, 1985a; Burke and Nisalak 1982). Anti-JE IgM antibody has been shown to be detectable in serum of the vaccinees as much as 6 months after vaccination with live attenuated vaccine (Roehrig et al. 2003). IgM antibodies elicited in the serum of non-neuroinvasive flavivirus infections or following flavivirus vaccination do not enter CSF (Johnson et al. 2000). IgM antibody mostly appears in CSF with the onset of illness or within few days of infection, except in very acute, sudden onset of encephalitis, when the IgM antibodies may not have reached detectable levels at hospital admission, in which case the IgM ELISA may result in false negative (Johnson et al. 2000). There is no established antiviral treatment for JEV infection other than the most common compound interferon-alpha. A notable breakthrough in anti-flaviviral drug research is the discovery of minocycline, a member of the broad spectrum antibiotic tetracycline group, as an antiviral drug (Michaelis et al. 2007). Minocycline has been reported to significantly reduce the neuronal apoptosis, microglial activation, active caspase activity, proinflammatory mediators, and viral titer in an in-vivo study (Ghosh and Basu 2009).

Preventive and Therapeutic Measures

There is need to control the spread of the flaviviral infections in new areas. India, Nepal, Thailand, and Vietnam mostly have genotype 1 and genotype 3. The genotype 1 became the dominant genotype in these areas in due course of time

(Nitatpattana et al. 2008). In India, phylogenetic analysis of the JEVs isolated from human CSF in 2009–2010 showed that genotype 1 and genotype 3 were co-circulating (Fulmali et al. 2011; Sarkar et al. 2012). The existing preventive measures include mosquito control (spraying of pesticides, impregnated mosquito nets), pig control (segregation, slaughtering, and vaccination), and human vaccination. Pyrethroids, organophosphates, and carbamates are common insecticides used in vector control program. The spraying can break the transmission cycle during the outbreak of JE for the short time. However, rising levels of insecticide resistance have compromised the effectiveness of such vector control program. Indeed, JE vectors prefer man-made habitats, such as irrigated rice fields, and are often heavily exposed to pesticide selection pressure. Although JE vectors are prone to develop insecticide resistance, usually this issue arises with insecticides that are not directly targeted to JE control, but rather are targeted to control of other pests (Karunaratne and Hemingway 2000). The vaccination of pigs represents another potential strategy to control JE, but this program is having few limitations. High turnover rate in pig populations requires annual vaccinations of newborn pigs, which would be costly. Second, the effectiveness of live attenuated vaccines is decreased in young pigs due to the presence of maternal antibodies (Erlanger et al. 2009). The first JEV vaccine, a mouse brain-derived inactivated vaccine using the Nakayama strain, was developed in Japan in year 1954 (Ishikawa et al. 2014). Currently, three types of vaccines (mouse brain-derived inactivated, cell culture-derived inactivated, and cell culture-derived live attenuated SA 14-14-2 JE vaccine) are being used in many parts of Asia (Table 1). JE-VAX vaccine with good protective efficacy was developed by Biken, Japan, using formalin-inactivated Nakayama strain of JEV, propagated in mouse brains. Case–control field trials revealed that the mouse brain-derived inactivated vaccine (JE-VAX) exhibited 81–95 % efficacy but it was discontinued in year 2005 because a 14-year-old girl developed severe ADEM (acute disseminated encephalomyelitis) (Paulke-Korinek and Kollaritsch 2008; Tauber et al. 2007) (Table 1).

Because of a potential problem in the use of mouse brains for a vaccine antigen source, efforts to develop cell culture-derived vaccines have been made. A new inactivated vaccine IC-51 (IXIARO®), which uses SA-14-14-2 strain of JEV propagated in *Vero* cells, was launched by Intercell AG, Austria and JEBIKV®, using the Beijing-1 strain, was launched by BIKEN, Japan (Ishikawa et al. 2014; Jelinek 2009) (Table 1). In Japan, JEBIKV® was launched by BIKEN in 2009 (Kikukawa et al. 2012). In addition, another *Vero* cell-derived inactivated vaccine ENCEVAC® was produced by Kaketsuken in year 2011 (Kuzuhara et al. 2003) (Table 1). These two vaccines contained the formalin inactivated *Vero* cell-grown Beijing-1 strain (Kikukawa et al. 2012). Live attenuated vaccine based on SA-14-14-2 JEV strain was introduced in China in the year 1988 and has been in use since then (Ding et al. 2003). It has been recently licensed to be used in India, Nepal, Sri Lanka, and South Korea due to its low cost of production and high efficacy under the trade name CD.JEVAX™. WHO prequalified CD.JEVAX™ for the pediatric use in the year 2013.

The YFV-17D vaccine has been used to generate a chimeric live attenuated JEV vaccine, IMOJEV(®) (JE-CV and previously known as ChimeriVax™-JE) (Table 1). It has the prM and E coding sequences of the JEV SA-14-14-2 strain inserted into the

Table 1 List of currently licensed JEV vaccines

Vaccine Type	Manufacturer(s)	Strain	Status
Mouse brain-derived inactivated vaccine	Green Cross (South Korea), Central Research Institute (India), Adimmune corp (Taiwan), Government Pharmaceutical Organization (Thailand), Vabiotech (Vietnam)	Nakayama Beijing-1	Shifting toward cell culture-derived vaccines in many countries
Vero cell-derived inactivated vaccine	Valneva SE (France), Biological E (India)	SA14-14-2	IXIARO® (Valneva) was approved in Europe, USA, Canada, Hong Kong, Singapore, and Israel, and JESPECT® in Australia and New Zealand for adult use. For pediatric use, IXIARO® was approved in USA, European Union, Norway, Liechtenstein, and Iceland. JEEV® (Biological E) was prequalified by the WHO for adult use in July 2013
PHK cell-derived inactivated vaccine	Biken (Japan)	Beijing-1	JEBIKV® (Biken) and ENCEVAC® (Kaketsuken) were approved in Japan. Replaced by a live attenuated vaccine
	Kaketsuken (Japan)	Beijing-P3	
	Beijing, Shanghai, Wuhan and Changchun Institute of Biological Products (China)		
Live attenuated vaccine	Chengdu Institute of Biological Products (China)	SA14-14-2	CD, JEVAX™ was approved in China, Cambodia, North Korea, India, Laos, Myanmar, Nepal, South Korea, Sri Lanka, and Thailand
			CD, JEVAX™ was prequalified by the WHO for pediatric use in Oct 2013
Live attenuated chimeric vaccine	Sanofi Pasteur (France)	prM/E genes replaced YFV-17D genes.	IMOJEV® and THAIJEV® are approved in Australia and Thailand, respectively

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Yellow fever virus (YFV) vaccine vector YFV17D by replacing the cDNA encoding the envelope proteins of YFV (Guy et al. 2010). The resulting virus cultivated on *vero* cells has proved to be highly immunogenic in rhesus monkeys against intracerebral and intranasal challenges with wild-type JEV. IMOJEV was found to be safe, highly immunogenic, and capable of inducing long-lasting immunity in both preclinical and

clinical trials. Though the vaccine was developed from genotype III virus, it was able to stimulate protective antibodies against other genotypes of JEV as well (Beasley et al. 2004).

Other attempts at developing new JE vaccines have focused on DNA vaccines. Studies in mice have shown that plasmid encoding NS1 of JEV could protect 90 % of mice after lethal infection with JEV (Lin et al. 1998). A single intramuscular immunization of DNA carrying the prM and E coding sequences from JEV or WNV protected mice from virus infection (Lin et al. 1998). Intranasal/mucosal vaccination approach using mouse brain-derived formalin-inactivated JEV has shown its potential but effective adjuvant has to be used for achieving better immunogenicity (Harakuni et al. 2009). India launched its first indigenous Vero cell-derived purified inactivated JE vaccine "JENVAC" to protect children from JE. The results proved that JENVAC can be administered as a single dose during epidemics for mass vaccination campaigns and also as a two-dose schedule during routine immunization as part of the National immunization programme of India in JE endemic regions of India. Out of five distinct genotypes of JEV, JEV genotype I has been reported to gradually dominate other genotypes in many countries. All current vaccine strains belong to genotype III, therefore the change in genotype dominance could be a challenge for the currently available JEV vaccines.

Conclusion

JEV is a threat to human health because JE outbreaks take place almost every year in Asia and other parts of the world. The knowledge of the interaction of the JEV with various components of the host immune system is required to understand the molecular mechanism of JEV pathogenesis. Effective strategies have to be developed in order to implement the preventive measures for the control of JE spread. Though some vaccines have been licensed to be used in JEV endemic regions and for travelers, more research work needs to be done for the development of new vaccines with having low cost of production and improved safety and efficacy. To have a better control over spread of JE, one should have a better mosquito control programs, coupled with improved animal husbandry and agricultural practices. Effective control of JE outbreaks needs multitasking efforts with combination of preventive measures, disease surveillance strategies, and the mass immunization of the population inhabiting in the JE endemic areas through effective safer and inexpensive vaccines.

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Neurotropic Influenza Virus Infections

Nobuko Yamashita

Introduction

In humans, the influenza virus (IV) usually infects only the respiratory epithelium. After an incubation period of 1–5 days, an infected individual exhibits symptoms of upper airway infection with systematic manifestations such as high fever and myalgia. These symptoms generally resolve after 3–7 days in normal individuals. However, psychic disturbances associated with influenza were first reported in connection with the 1385 epidemic in Germany (Menninger 1919). The epidemic of 1890 was followed by many cases of mental illness, described in many reports. An outbreak of reversible schizophrenic syndrome followed the 1918/1919 pandemic (Menninger 1926). Since then, several neurological complications of influenza virus infection have been documented, including Economo's encephalitis lethargica (EL), postencephalitic Parkinsonism, Reye's syndrome (RS), increased febrile seizures, and influenza-associated encephalopathy. Virus–host interactions are the main causes of these complications; however, some neurotropic influenza viruses such as the highly pathogenic avian influenza virus can also infect humans. In this chapter, we discuss neurotropic influenza viruses (highly pathogenic avian influenza virus and a laboratory strain A/WSN/33) and the neurological complications associated with seasonal influenza virus infection.

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Virology

Structure of the Influenza Virus

The influenza virus (IV) is an enveloped negative-strand RNA virus of the Orthomyxoviridae family (Knipe and Howley 2013), which is composed of five genera (*Influenza virus* A; *Influenza virus* B; *Influenza virus* C; *Thogotovirus*; and *Isavirus*) that differ in host range and the severity of the disease they cause. Influenza A viruses (IAVs) and influenza B viruses cause respiratory illness in humans. IAV infects a wide variety of mammals (including humans, horses, pigs, ferrets, cats, and dogs) as well as avian species, while influenza B and C viruses predominantly infect the human population.

IAVs are enveloped by a host cell-derived lipid membrane. The genome consists of eight RNA segments that encode nine structural proteins [hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix 1 (M1), M2, nonstructural protein (NS) 2, polymerase acidic protein (PA), polymerase basic (PB) 1, and PB2] and two nonstructural proteins (NS 1 and PB1-F2). IAVs are covered with spikes of three proteins, HA, NA, and M2 (Fig. 1); subtypes are identified by the serotype of HA and NA expressed on the virion surface. There are 17 HA types and 9 NA subtypes.

HA functions as the viral receptor-binding protein and as a fusion protein. The HA precursor HA0 is made up of two subunits: HA1, which contains the receptor-binding domain, and HA2, which contains the fusion peptide (Lazarowitz and Choppin 1975) (Fig. 2). After binding HA spikes to sialic acid residues on the host

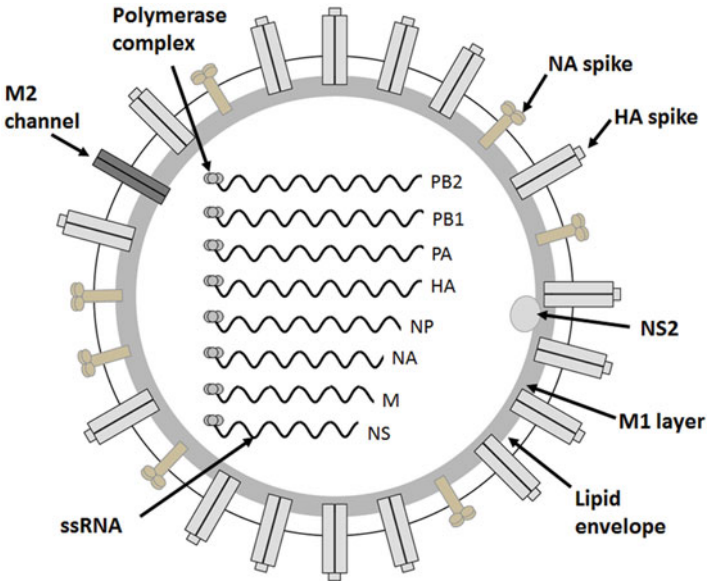

Fig. 1 Schematic of influenza virus particles. Influenza A genome is single-stranded, eight-segmented RNA. The HA and NA spikes are two large glycoproteins on the outside of the particles, derived from host lipid envelope. HA exists as a trimer and NA exists as a tetramer

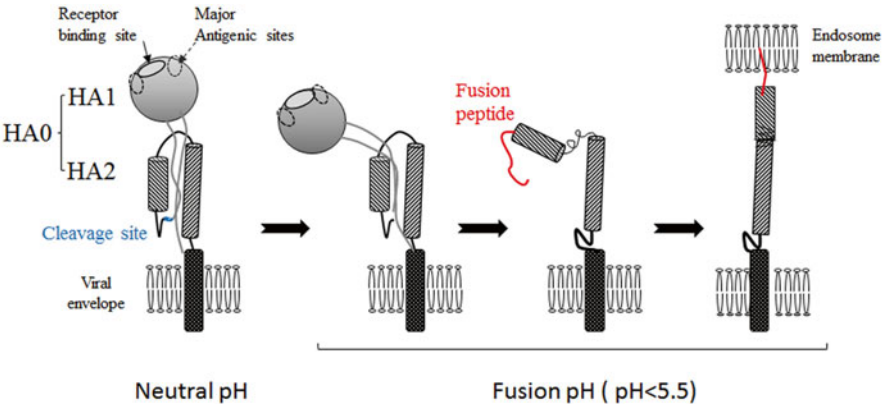

Fig. 2 The structural changes that occur in HA spike upon virus entry. The leftmost panel shows a single monomer of trimeric native HA (HA0) at neutral pH. HA0 is cleaved by host cellular protease to two subunits (HA1 and HA2; second panel from the left). The fusion peptide HA2 is exposed at fusion pH (second panel from the right) and it inserts itself into the target endosome membrane (rightmost panel). This structural change in HA protein induces membrane infusion between viral envelope and the target endosome membrane

cell, receptor-mediated endocytosis occurs, and the virus enters the host cell in an endosome (virus adsorption). The endosome has a pH of around 5–6, which induces conformational changes in HA0, maintaining the HA1 receptor-binding protein but exposing the HA2 fusion peptide. HA2 fusion peptide inserts itself into the endosomal membranes, bringing the viral and endosomal membranes in contact (virus entry) (Fig. 2). The acidic environment of the endosome is also important for opening the M2 ion channel, which acidifies the viral core. The acidic environment in the virion releases the viral RNP from M1, and viral RNP enters the host cell cytoplasm (virus uncoating). The viral RNP consists of NP, PA, PB1, and PB2.

After being released into the cytoplasm, the viral RNP enters the nucleus. RNA synthesis of the influenza virus occurs in the nucleus, where the genomic ssRNA of negative polarity (viral RNA) is used as a template for the syntheses of two classes of positive ssRNAs: messenger RNAs (transcription) and full-length complementary copies (replication of the viral RNA genome). The next step in replication of the influenza genomic segment is the copying of viral RNA on the template of positive strand cRNAs. This process also generates full-length products that assemble with NP and polymerase subunits to form progeny RNP complexes, which are exported from the nucleus into the cytoplasm. Assembly of viral components occurs preferentially in lipid rafts of the host cells. Since influenza is an enveloped virus, it uses the host cell's plasma membrane to form the viral particles that leave the cell.

Viral Proteins Associated with Pathogenicity

Three of the 11 IV viral proteins (HA, PB2, and NS-1) were associated with pathogenicity (Table 1).

Table 1 Differences in pathogenic viral proteins between human and avian influenza virus (IV)

		Human IV	Avian IV
HA	Host cell receptor	α2,6-linked sialic acid	α2,3-linked sialic acid[a]
	The cleavage site → Host HA0 protease	Localized protease to airway epithelia (e.g., Tryptase clara)	LPAIV cleavage site has single basic amino acid (Arg or Lys) sequence → Localized protease to airway and digestive tract epithelia
			HPAIV cleavage site has contiguous basic amino acid (Arg or Lys) sequence → Ubiquitous cellular proteases (furin and PC6)
PB2	Residue 627 → optimum temperature for viral replication	Lysine → 33 °C.	Glutamic acid → 42 °C
NS1	Host CPSF30[b]	Suppression host IFN-β mRNA synthesis	Strengthening of CPSF30 binding of H5N1 → Systemic spread of the virus in mice
	Residue 92	Aspartic acid	Glutamic acid → Increased antiviral cytokine resistance

[a]H7N9 virus can recognize α2,6-linked sialic acid

[b]CPSF30; cleavage and polyadenylation specificity factor, 30-kDa subunit

HA

The HA glycoprotein exists as a precursor molecule (HA0) that must be cleaved by cellular protease to become biologically active (HA1 and HA2). Proteolytic activation of HA is essential for viral infectivity, dissemination, and pathogenicity.

IAV can infect a wide variety of hosts (i.e., mammals and birds); however, the spread of infection between species is unusual, in part, because the preferential binding of HA to sialic acid on the cell surface varies with HA subtype. HA from human viruses selectively binds sialic acid residues with an α2,6 linkage, while HA molecules from avian viruses selectively bind sialic acid residues with an α2,3 linkage (Connor et al. 1994) (Table 1). Human bronchial epithelial cells contain a much higher ratio of α2,6-linked sialic acid residues, while the epithelium of the duck gut contains mostly α2,3-linked sialic acid residues (Couceiro et al. 1993). In addition, because of anatomical differences with respect to the distribution through the human airway, some alveolar cells of the human lung contain α2,3-linked sialic acid residues (Shinya et al. 2006). This is why avian IV rarely infects and spreads between humans, and more frequently causes severe pneumonia rather than upper respiratory infection.

The pathogenicity of avian IV also correlates with host HA0 protease distribution in the body (Horimoto and Kawaoka 1994). That is, the host protease of LPI HA0 is localized to the respiratory and digestive tract epithelia, but the host proteases of HPI HA0, i.e., furin and PC6, exist all over the body (Stieneke-Grober et al.

1992; Horimoto et al. 1994) and lead to systemic HPI infection. The difference of HA0 cleavability between LPI and HPI is derived from differences in the amino acid sequence of HA0 cleavage site. The cleavage site of low-pathogenic avian influenza virus (LPAIV) HA consists of a single basic amino acid (lysine or arginine). In contrast, the cleavage site of the highly pathogenic avian influenza virus (HPAIV) HA includes contiguous arginine and/or lysine sequences and is recognized by furin and PC6 (Table 1). Thus, a combination of the differences in terms of the cleavage site amino acid sequence and the protease determines the pathogenicity of a given IV. The HA cleavage site of H1 or H3 type IV, which are infective in humans, consists of arginine alone, and its protease is expressed only in the respiratory system.

PB2

IAV expresses three viral polymerase (P) subunits—PB1, PB2, and PA—all of which are essential for RNA replication and transcription. This viral RNA polymerase complex, especially the PB2 segment, also determines viral host range and replication. Most avian IAVs have a glutamic acid at residue 627 of the PB2 subunit (E-627); they replicate in the intestinal tract of infected birds at close to 42 °C, and the infection is usually asymptomatic. However, human IAVs have a lysine at this position (K-627) and replicate in the human respiratory tract at lower temperature (33 °C) (Table 1). The presence of lysine at residue 627 of PB2 enhances polymerase activity and viral replication, and determines host specificity. Many HPAI isolated from human patients have this Lys/Glu disparity (E627K), not only in H5N1 (Hatta et al. 2001; Maines et al. 2005) but also in H7N9 (Liu et al. 2013) and H10N8 (Chen et al. 2014). In addition, a change in residue 701 of PB2 from aspartic acid to asparagine has also been implicated in the expansion of the host range to mammals (Li et al. 2005; Steel et al. 2009).

NS1

NS1 is designated as a nonstructural protein because it is synthesized in infected cells but is not incorporated into the virion. NS1A is a virulence factor of IAV, conferring resistance to the antiviral effects of the host interferon (IFN) system (Garcia-Sastre 2001; Krug et al. 2003) by several mechanisms. (1) NS1A inhibits host cell mRNA processing and blocks nuclear export of polyadenylated cellular transcripts. The NS1 protein of human IAV binds the 30-kDa subunit of the cleavage and polyadenylation specificity factor (CPSF30), which is a protein required for 3′-end processing of cellular pre-mRNAs. It also associates with and suppresses production of IFN-β mRNA. The strengthening of CPSF30 binding by the NS1 protein of H5N1 viruses enhances virulence in mice by increasing the systemic spread of the virus from the lungs, particularly to the brain (Spesock et al. 2011) (Table 1). (2) Viral single-stranded and double-stranded RNAs activate a variety of signaling pathways

by activating IFN regulatory factors (IRFs) (Sato et al. 2000; Iwamura et al. 2001) and NF-kβ (Iwamura et al. 2001). NS1 functions an anti-host IFN-α/β system (Kochs et al. 2007; Hale et al. 2008), blocking activation of IRF3 (Kochs et al. 2007), and of the IFN-β promoter gene (Kochs et al. 2007). In addition, NS-1 directly blocks the function of two cytoplasmic antiviral proteins: 2′–5′ oligoadenylate synthetase (OAS) (Min and Krug 2006) and the ds RNA-dependent serine-threonine protein kinase R (PKR) (Min et al. 2007). (3) NS1 is also associated with TNF-α induction in H5N1 infection (Seo et al. 2002; Cheung et al. 2002). The change in residue 92 of NS1 from aspartic acid to glutamic acid brings cytokine (IFN-α, IFN-γ, and TNF-α) resistance to H5N1 virus (Seo et al. 2002) (Table 1).

Antigenic Shift Induces New Pandemics in Humans

Numbering of the IAV subtypes is based on the immunologic specificities of their HAs and NAs. There are 17 HA antigens (H1 to H17) and nine N antigens (N1 to N9). As for HA antigens, human seasonal IVs usually have H1or H3 antigen. However, human cases naturally infected with avian IV spanned hemagglutinin subtypes H5, H6, H7, H9, and H10 (Fig. 3) (Freidl et al. 2014).

The multigenic IAV undergoes antigenic changes through the accumulation of point mutations during propagation in the human host (antigenic drift). These changes provide a means for immunologic escape and propagative success in partially and variably immune populations. Genetic recombination between viral strains may also be a source of antigenic alternation and induce new pandemics in human (antigenic shift). The most abrupt changes in viral antigens occur through HA and NA gene reassortment. Antigenic shift has been shown to occur during dual infection with different influenza virus in single cells in culture.

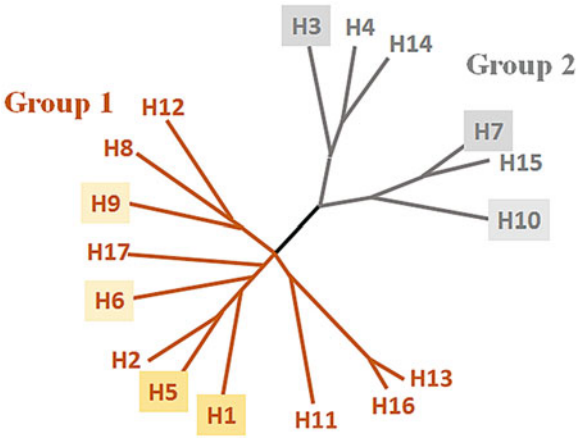

Fig. 3 Phylogenic tree of influenza A HAs (Adapted from Russell et al. (2008)). HAs are mainly divided into two groups. It is possible that the acquired immunity that affects disease severity is partially related within each group. The HA number with hatched square has been reported in human infection

Neurotropic Influenza Viruses

H5N1 Virus

Overview

There are two types of avian H5N1 IV, classified by their pathogenicity: LPAIV and HPAIV. LPAIV occurs naturally in wild birds and can spread to domestic birds. In most cases, it causes no signs of infection or only minor symptoms in birds. HPAIV causes systemic infection and is often fatal in terrestrial poultry (chickens and turkeys). HPAIV spreads rapidly, has a high death rate in birds in comparison to LPAIV, and can be considered a serious pandemic threat.

The factors that may cause H5N1 epidemics include sialic acid (α2,6- or α2,3-linked), the host site of HA cleavage protease expression, and the PB2 mutation of the influenza virus itself. HPAIV H5N1 influenza infection in humans was first described in 1997 in Hong Kong in an epidemic among poultry (Claas et al. 1998; Subbarao 1998). During this event, death from respiratory failure occurred in six cases, but the H5N1 IV genome was detected in the cerebrospinal fluid. Detection of the virus in the cerebrospinal fluid and the development of acute encephalitis in isolated cases of severe H5N1 infection in humans suggest a possible neurological involvement in H5N1 pathogenesis (de Jong et al. 2005; Abdel-Ghafar et al. 2008).

Neurotropism of the Highly Pathogenic Avian H5N1 Influenza Virus

Usually, seasonal H1N1 and H3N2 viruses are not detected in human brain tissue and cerebrospinal fluid because viral replication is localized to the respiratory system. However, neurotropism of the H5N1 virus has been confirmed and studied in animal models, including mice, ferrets, cats, martens (Klopfleisch et al. 2007), and swans (Teifke et al. 2007). Here, we discuss the pathogenesis of H5N1 CNS infection in mouse and ferret models.

Mouse Model of HPAIV

Avian IV can replicate effectively in the mouse respiratory epithelium without adaptation because there are fewer α2,3-linked sialic acid residues. Depending on the strain, H5N1 viruses generally fall into either a high or low virulence phenotype in mice after intranasal infection. Low-pathogenic H5N1 virus produces only modest weight loss and infected mice recover 7–9 days post-infection (p.i.). In contrast, infection with highly pathogenic viruses leads to pronounced morbidity, systemic spread of the virus, cytokine dysregulation, severe tissue pathology, and death (Maines et al. 2005). The mouse model of highly pathogenic H5N1 virus infection is used to study the pathogenesis of CNS infection by H5N1 (Tanaka et al. 2003; Iwasaki et al. 2004; Jang et al. 2009).

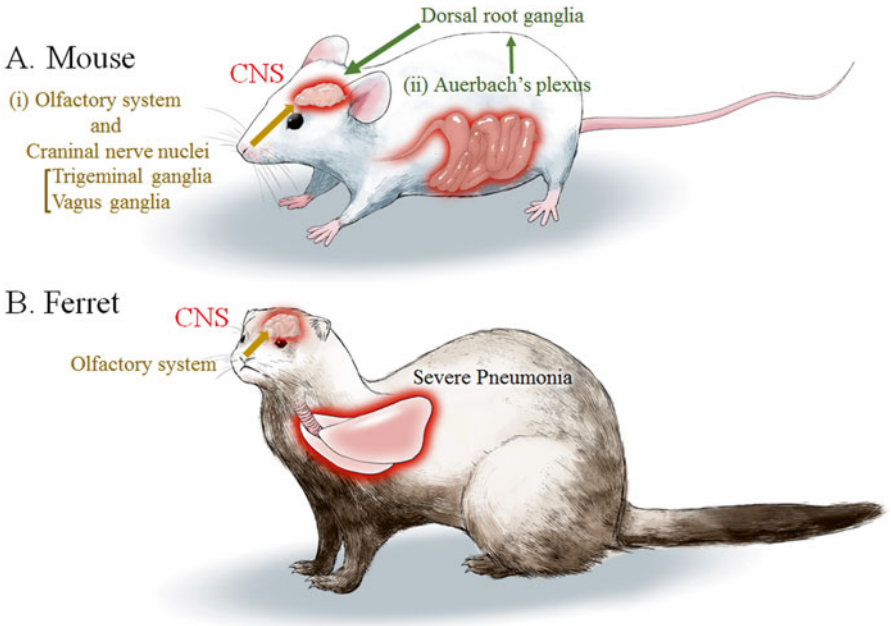

Fig. 4 Virus entry route into the CNS of an animal model with highly pathogenic H5N1 infection. (**a**) Intranasal infection proceeds by two routes in mice: via the olfactory system and cranial nerve nuclei adjacent to the brain, or from the mesenteric and myenteric (Auerbach's) plexi, via dorsal root ganglia of the peripheral nervous system, to the brain stem and midbrain. (**b**) After intranasal infection of ferrets with highly pathogenic H5N1, the virus spreads from the olfactory system to the CNS. In intratracheal infection, ferrets die from severe pneumonia without neurological symptoms

H5N1 virus invades the mouse CNS by two different pathways (Fig. 4).

One route of entry is through the cranial nerve (Tanaka et al. 2003; Iwasaki et al. 2004). When the H5N1 virus (A/Hong Kong/483/97) is introduced intranasally, infectious viruses and/or viral antigens are recognized first in the mouse respiratory epithelial cells, and then in the olfactory system and cranial nerve nuclei (trigeminal and vagus ganglia) (Tanaka et al. 2003; Iwasaki et al. 2004), but it is never detected in the blood (Tanaka et al. 2003). Thus, the A/Hong Kong/483/97 virus is principally transmitted from the nasal cavity to the CNS through the cranial nerves.

Another viral entry route is via the intestinal peripheral nerve. In C57BL/6J mice intranasally infected with the A/Vietnam/1203/04 H5N1virus and showing neurological signs, the virus had traveled from the peripheral nervous system into the CNS to higher levels of the neuraxis (Jang et al. 2009). H5N1 virus was first detected 2–3 days p.i. in the mesenteric and myenteric (Auerbach's) plexi of the enteric nervous system and in neurons within the dorsal root ganglia of the peripheral nervous system (Fig. 4) (Jang et al. 2009). In the CNS, H5N1 virus was first detected in the brain stem solitary nucleus, and then in the midbrain, the olfactory bulb and spinal cord, and finally the central neuraxis (Jang et al. 2009). In addition, neurodegenerative processes, such as loss of dopaminergic neurons and alpha-synuclein aggregation,

were observed in CNS regions of the H5N1 mouse model, long after resolution of the infection (Jang et al. 2009). Alpha-synuclein aggregation is observed as characteristic Lewy bodies in Parkinson's disease. H5N1 virus infection may affect the pathogenesis of neurodegenerative diseases such as Parkinson's disease, viral Parkinsonism, and Alzheimer's disease (Jang et al. 2009).

In addition to the virus invasion route, an amino acid mutation of HA position 222 is associated with neurotropism. In mice infected with the H5N1 virus (KAN-1), growth of the HA_{222E}-encoding virus is mainly confined to the lung, but reversion to HA_{222K} allows the virus to spread to the brain (Manz et al. 2010). The higher receptor-binding affinity of HA_{222K} mediates efficient infection of the CNS (Manz et al. 2010).

Ferret Model of HPAIV

A ferret model of HPAIV infection is often used because, as in humans, the ferret's upper respiratory epithelium mainly has α2,6-linked sialic acid residues and infection of the epithelium with LPAIV produces fever and rhinorrhea (Maher and DeStefano 2004; van Riel et al. 2006, 2007).

Ferret infection with H5N1 viruses of low pathogenicity is typically characterized by fever, mild to modest weight loss (<15 % of pre-inoculation body weight), relatively mild lethargy, and minimal clinical signs (Maines et al. 2005; Lu et al. 2003; Govorkova et al. 2005; Yen et al. 2007; Imai et al. 2010; Belser and Tumpey 2013). Neurological complications and death are rare (Maines et al. 2005), and ferrets return to pre-inoculation body weight and activity levels by 14 days p.i. Low pathogenicity H5N1 viruses in the ferret model are capable of efficient replication throughout the respiratory tract, with infectious viruses frequently being detected in the trachea and lungs by day 3 p.i.(Maines et al. 2005). Following intranasal inoculation, H5N1 viruses of low virulence in ferrets generally do not spread to extrapulmonary tissues, except in the olfactory bulb of the brain, which is adjacent to the nasal cavity (Maines et al. 2005; Zitzow et al. 2002; Govorkova et al. 2005).

Infection with highly pathogenic H5N1 viruses causes severe and fatal disease in ferrets, with demonstrated dissemination to multiple organs, including the CNS (Zitzow et al. 2002). Infected ferrets die during the acute phase of infection (typically 5–7 days p.i.) in the absence of neurological dysfunction or they develop neurological symptoms (typically 7–13 days p.i.) (Maines et al. 2005).

Infectious viral particles and viral antigens are detected in the olfactory bulb and brain of ferrets infected with highly pathogenic H5N1 viruses, with neurons representing the major infected cell type in the CNS (Maines et al. 2005; Rowe et al. 2003; Bodewes et al. 2011; Peng et al. 2012). Viral load in the ferret brain has been correlated with the severity of meningoencephalitis; high viral titers and inflammatory lesions in the brain can be detected by 3–4 days p.i. with widely distributed lesions present by 5–6 days p.i. (Peng et al. 2012). Viral antigens were detected predominantly in neurons, correlating with inflammatory lesions, and less frequently in astrocytes and ependymal cells during active infection (Peng et al. 2012).

High titers of viral particles (Plourde et al. 2012) or viral antigens (Bodewes et al. 2011) were frequently observed in the olfactory bulb, cerebral cortex, and brain stem of ferrets after intranasal inoculation with highly virulent H5N1 viruses.

While the precise route of virus entry to the brain has yet to be elucidated, the olfactory system is a likely source of viruses for brain invasion in ferrets infected with highly virulent H5N1 viruses (Fig. 4). However, previous reports suggest it is most likely that H5N1 virus spreads from the nasal cavity, which is located near the brain. When the H5N1 virus infects the ferret intranasally, it spreads from the olfactory bulb to the brain (Bodewes et al. 2011; Shinya et al. 2011; Yamada et al. 2012), but is not detected in the liver, spleen, or duodenum (Bodewes et al. 2011). In addition, there has been no evidence for hematogenous dissemination in the form of antigens in the brain parenchyma surrounding blood vessels (Yamada et al. 2012). In the ferret intratracheal model, ferrets died of pneumonia without neurological symptoms (Bodewes et al. 2011). H5N1 virus strains may enter the CNS via cranial pathways (Yamada et al. 2012). The efficient growth of virus in the upper respiratory tract may facilitate viral brain invasion (Shinya et al. 2011).

Antigenic similarity explains why H1N1pdm09, but not H3N2, virus infection protects ferrets from H5N1 encephalitis (Bissel et al. 2014). Ferrets previously infected with H1N1 survive H5N1 challenge, while those previously infected with H3N2 die of encephalitis. Phylogenetically, IAV can be divided into two groups (Russell et al. 2008), each of which can be further subdivided into three clades (H8, H9, and H12; H1, H2, H5, H6, and H17; H11, H13, and H16) and two clades (H3, H4, and H14; H7, H10, and H15) (Fig. 3). H1N1 and H5N1 virus subtypes belong to the same clade, while H3N2 strains belong to another group (Russell et al. 2008). Prior H1N1 virus infection may protect the host from lethal H5N1 infection via antigenic similarities between the HA1 and HA5 stalks, soliciting cross-protective humoral immunity (Bissel et al. 2014).

The widespread dissemination in animal models has not been recognized in the limited number of human autopsies performed on H5N1 patients, and whether these species represent appropriate models for the pulmonary disease and secondary multiple organ failure observed in man remains to be determined (Mansfield 2007).

Other Types of HPAIV

The H7N7 virus causes brain stem encephalitis in mice (Shinya et al. 2005). H7N9 influenza-infected patients showed severe and fatal respiratory disease but not CNS infection (Gao et al. 2013) because H7N9 viruses showed a greater tropism for respiratory epithelium than did H5N1 viruses (Meliopoulos et al. 2014). The H7N1 virus isolated from ostrich induced severe pneumonia and encephalitis after intranasal infection of mice, while the strain isolated from chickens did not induce severe clinical effects (Rigoni et al. 2007).

Laboratory Strain: A/WSN33 (H1N1)

The only human IV from which variants expressing neurovirulence in mice have ever been derived directly is the 1918 H1N1 progeny strain A/WS/33 (strain A/Wilson-Smith/1933, H1N1) (Smith et al. 1933), which has pneumotropism. As laboratory neurotropic IAV strains, one WS variant A/NWS/33 (Stuart-Harris 1939) was established after passage in chicken embryonated egg and mouse brain, and another WS variant A/WSN/33 (Francis and Moore 1940) was established after passage in normal saline solution containing chicken embryonic brain tissue. Since the late 1940s, these neurotropic strains have been maintained in many laboratories and have retained their unique pathogenic properties.

A/WSN/33 is characterized by the fact that its NA segment is important for the cleavage of HA segment. The NA segment of A/WSN/33 with a significant effect on pathogenicity through pantropism was first reported in the 1970s (Schulman and Palese 1977; Sugiura and Ueda 1980; Castrucci and Kawaoka 1993). Mutations in the NA genes have been identified at a single codon (N146R or N146Y); they lead to loss of a glycosylation site and allow the virus to escape to cause systemic infection outside the respiratory tract (Li et al. 1993). This mutation was not observed in the NA gene of the 1918 H1N1 virus (Reid et al. 2000).

The NA segment of IV is a neuraminidase (Air and Laver 1989), but the HA segment of A/WSN/33 NA cannot be cleaved by the neuraminidase. The A/WSN/33 NA segment activates plasminogen to plasmin (Goto and Kawaoka 1998), which cleaves the single arginine of the HA segment of IV (Lazarowitz et al. 1973; Tashiro et al. 1987).

Neurological Complications of Seasonal IV Infection

Seasonal human IV infection typically causes only respiratory infection; however, many CNS complications have been associated with seasonal IV infection. Today, these CNS complications are mainly due to the host response to infection, according to studies of influenza-associated encephalopathy dating from the 1980s. Here, we outline the history of CNS complications associated with seasonal IV infection.

Economo's Encephalitis Lethargica and Postencephalitic Parkinsonism

Economo's encephalitis lethargica (EL) was prevalent mainly from 1915 to 1920 and was the first major reported neurological complication of IV infection (Ravenholt and Foege 1982; Foley 2009). It is also representative of postencephalitic Parkinsonism.

EL was first reported in central Europe in 1917, and then spread worldwide until 1930. The cause of EL remains unknown, but is thought to be associated with a viral infection, based on clinicopathological findings. The etiology of EL has remained obscure, although the EL epidemic overlapped with the 1918 influenza epidemic. The characterization of EL was largely performed by von Economo (von Economo 1917; von Economo and vom Wiesner 1918; Sak and Grzybowski 2012), who described a typical case as a "somnolent-ophthalmoplegic form" that occurred several days after the prodromal symptoms of general malaise, pharyngitis, and fever (von Economo and Newman 1931). Extensive inflammation centered on the mesencephalic tegmentum lesion was described; this pathological change would lead to sleep dysfunction and oculomotor ataxia (von Economo 1917). Thereafter, the cause of EL was linked to virus-associated encephalopathy (Howard and Lees 1987) or an autoimmune mechanism (Dale et al. 2004), after observing a positive-oligoclonal band in the spinal fluid of a patient with postencephalitic Parkinsonism) (Williams et al. 1979).

Reye's Syndrome

Reye's syndrome (RS) was defined as an acute encephalopathy with fatty degeneration of the viscera, most commonly occurring in children (Reye et al. 1963). The conditions present as vomiting, seizures, disturbed consciousness, and altered personality following an antecedent infection with fever. Characteristic laboratory findings include severe brain edema, liver dysfunction, and pathologically fatty degeneration of the viscera, including the liver.

Cases resembling RS were first reported in 1929 (Russell Brain and Hunter 1929); Reye and others described it as a distinct clinical entity in 1963 (Reye et al. 1963), when 16 cases of encephalitis-like disease were reported during epidemics of influenza B in the USA (Johnson et al. 1963). The viruses responsible for RS are IV and varicella virus (Belay et al. 1999; Hurwitz et al. 1987). RS rates have fallen significantly since the 1980s (Belay et al. 1999), mainly due to the contraindication of aspirin administration during influenza and varicella infection (Hurwitz et al. 1985, 1987; Starko et al. 1980; Waldman et al. 1982). In recent years, because of hyperammonemia, abnormal and transient metabolic processes of mitochondria have come to be considered the primary causal factor of RS (Lemasters et al. 1998). Indeed, historic cases diagnosed as RS may include patient with acute exacerbation of a metabolic disorder. Improved diagnostic accuracy of metabolic disorders has accounted for some of the reduction in the frequency of RS diagnoses (Orlowski 1999).

IV-Associated Febrile Seizure

Febrile seizure is common in infants and children with influenza and is an important cause of hospitalization (Chiu et al. 2001; Kwong et al. 2006; Hara et al. 2007). In hospitalized children, in comparison to adenovirus or parainfluenza infections, IAV

infection is associated with a higher incidence of febrile seizures and of repeated seizures in the same febrile episode (Chiu et al. 2001). Children who developed febrile seizures in IAV infection had a significantly higher maximum body temperature, shorter duration of fever before seizure onset, and more frequent occurrence of partial seizures (Kwong et al. 2006). In addition, IAV infection was independently associated with 30-min or longer prolonged postictal impairment of consciousness (Hara et al. 2007).

IV-Associated Encephalopathy

To the best of our knowledge, IV-associated encephalopathy was first described in 1979 in a report of six patients (Delorme and Middleton 1979). It then became difficult to distinguish IV-associated encephalopathy from RS. RS usually occurs during recovery from IV infection, is associated with aspirin use, is characterized biochemically by hyperammonemia and hypoglycemia, and has characteristic liver histopathology (Surtees 2006). None of these are found in IV-associated encephalopathy (Surtees 2006). Encephalopathy and encephalitis are distinguished by the absence of brain inflammation or meningitis in patients with encephalopathy, although both groups exhibit an altered mental status or personality change lasting >24 h within 5 days of laboratory-confirmed influenza (Newland et al. 2007). Clinically, these conditions may be difficult to distinguish without a CSF examination or brain biopsy (Davis 2000).

In 1996–2000, influenza-associated encephalopathy was widely recognized in Japan, where its incidence was estimated to be 100–500 cases per year (Mizuguchi 2013; Gu et al. 2013). Multiple-organ failure sometimes developed. The mortality is approximately 30 %, and the risk of neurological sequelae is high (Morishima et al. 2002; Sugaya 2002). It is most frequently reported in children (Kasai et al. 2000), although adult cases have also been reported. The condition develops mainly in children age <5 years, either on the day on which influenza signs appear or the following day (Morishima et al. 2002; Yoshikawa et al. 2001). The major signs of encephalopathy include altered consciousness or loss of consciousness, convulsions, and delirium. Inappropriate emotional outbursts of laughter or fear, visual hallucinations, and incoherent speech are sometimes observed. CSF examination in most patients shows no pleocytosis and normal protein and glucose levels. EEG findings often demonstrate diffuse slowing, focal areas of slowing, and epileptiform spikes.

Today, IV-associated encephalopathy is classified into four main types based on clinical course and MRI findings: (1) acute necrotizing encephalopathy, (2) acute encephalopathy with biphasic seizures and late reduced diffusion, (3) clinically mild encephalopathy with reversible splenial lesion, and (iv) hemorrhagic shock and encephalopathy syndrome (Mizuguchi 2013; Mizuguchi et al. 2007) (Table 2).

A major cause of IV-associated encephalopathy is excess production and activity of pro-inflammatory cytokines such as tumor necrosis factor-α and interleukin-6, which induce vascular epithelial injury and apoptosis in brain edema and systemic organ damage (Mizuguchi et al. 2007). Excitotoxicity may also trigger intractable

Table 2 Classification and characteristics of influenza-associated encephalopathy

	Acute necrotizing encephalopathy	Acute encephalopathy with biphasic seizures and late reduced diffusion	Clinically mild encephalopathy with reversible splenial lesion	Hemorrhagic shock and encephalopathy syndrome
Pathomechanism	Hypercytokinemia	Excitotoxicity	Unclear	Hypercytokinemia
Frequency[a]	6 %	10 %	20 %	<1 %
MRI finding	Acute	Subacute	Acute	Acute
	Bilateral thalamic lesion	Localized brain edema	Reversible, splenium of corpus callosum lesion	Diffuse brain edema
Blood examination	Multiple organ failure	Non-specific	Hyponatremia	Multiple organ failure
	DIC			DIC
Mortality	High	Low (frequently recover with neurologic sequelae)	Low (recover without sequel)	High
Exacerbating factor	NSAIDs	Theophylline		NSAIDs

[a]Unclassified mild encephalopathy consisted about 60 % in Japan study (Mizuguchi 2013)

seizures based on local brain edema and slow-onset neuronal death. IV-associated encephalopathy in Japan is treated with antiviral agents, m-PSL pulse therapy to suppress pro-inflammatory cytokines, supportive therapy to manage coagulation disorder, blood circulation, respiration, body temperature and intracranial pressure, and anticonvulsant medication (Morishima et al. 2009). It is also important to prohibit the use of NSAIDs and theophylline due to exacerbating factors, in patients with IV infection.

Conclusions

An HPAIV pandemic is likely to occur in the future, and could be an important public health concern. If human IV mutates and becomes neurotropic, high mortality is expected in the absence of effective antivirals for IV meningitis/encephalitis. Currently, early detection and rapid cure prior to the onset of meningitis/encephalitis is considered the only way to control the occurrence of neurotropic IV infection. Studies of viral pathogenesis and therapeutic strategies for new strains of IV infection are expected.

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Borna Disease Virus

Mady Hornig

Introduction

The neurotropic virus, Borna disease virus (BDV), infects a wide range of warm-blooded animal species (Hornig et al. 2003). BDV is the prototype genus (bornavirus) of the family Bornaviridae, a member of a group of nonsegmented, negative strand (NNS) ribonucleic acid (RNA) viruses (order Mononegavirales) (Briese et al. 1994); however, recent identification of divergent bornaviruses in birds and reptiles is challenging established virus taxonomy (Kuhn et al. 2015). Infection may be asymptomatic, cause mild neurobehavioral changes, or result in fatal meningoencephalitis (Lipkin et al. 2011). It is unique among animal viruses in the order Mononegavirales in its nuclear localization of replication and transcription (Briese et al. 1992) and distinctive in its low levels of viral replication and capacity to establish persistent infection of the peripheral and central nervous systems (CNS) (Schneemann et al. 1994; Siemetzki et al. 2009; Bonnaud et al. 2015; Cubitt and de la Torre 1994; Walker et al. 2000). The name Borna derives from an equine epidemic in the late 1800s that crippled the Prussian cavalry in the town of Borna, Germany (Lipkin et al. 2011). Natural infection, long described in horses and sheep, has more recently been recognized to extend to parrots and other bird species in association with a related virus, avian bornavirus (ABV), manifesting as a fatal syndrome involving failure of digestive system motility, inflammation of the enteric nervous system as well as encephalomyelitis (Honkavuori et al. 2008; Staeheli et al. 2010). Nonhuman primates can be experimentally infected; however, natural infection of

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humans and nonhuman primates appears to be quite rare. Decades-long controversy over a role for BDV infection in human neuropsychiatric illnesses has more recently waned; however, the recent discovery that the genomes of humans as well as other vertebrate lineages contain remnants of BDV sequences—estimated to have been inserted into ancestral human DNA at least 40 million years ago (Horie et al. 2010; Belyi et al. 2010)—has raised questions regarding its potential evolutionary implications. Experimental BDV infections of rodents have built upon the unusual characteristics of the virus and its ability to establish non-cytolytic, persistent infections of the CNS, providing tools for exploring the interplay of neurotropic agents, immune responses, neurotransmitter signaling pathways, and maturational factors in the neuropsychiatric disorders potentially linked to infection (Hornig et al. 2001; Lipkin et al. 2001). Analysis of rodent models of infection has yielded insights into mechanisms by which neurotropic agents and host responses may impact upon developing or mature CNS circuitry to effect complex disturbances in behavior.

History and Classification

One of the earliest descriptions of Borna disease (BD), dating to 1766, notes altered emotional behavior of horses at the onset of illness ("In the beginning the horse is sad") (Gellert 1995). Early work on BD ('Borna'sche Krankheit' in German (Gensert 1896)) initially classified the disease as a meningoencephalitis of horses (Trichtern 1716). The identification by Joest and Degen of characteristic intranuclear inclusion bodies in the hippocampus of animals with BD provided the first diagnostic marker for disease (Joest and Degen 1909). Its infectious nature was established by studies of experimental transmission from naturally infected horses or sheep to rabbits, guinea pigs, rats, monkeys, and chickens (Zwick et al. 1926, 1929; Zwick and Seifried 1925; Beck and Frohböse 1926; Nicolau and Galloway 1928; Pette and Környey 1935).

Interest in BD and its causative agent accelerated again in the early 1970s with a focus on mechanisms of neuropathogenesis in rabbit, rat, and tree shrew models (Ludwig et al. 1977; Sprankel et al. 1978). Observations in the early 1980s of a biphasic disease in adult-infected rats, characterized initially by hyperactivity and excitability and followed by decreased locomotor behavior, led some investigators to consider the potential parallel of adult BD with human bipolar disorder (Narayan et al. 1983a, b). Tropism of the virus for limbic circuitry strengthened the hypothesis of a connection with human neuropsychiatric illness (Ludwig and Bode 1997). This in turn prompted efforts to determine whether humans were infected with a related agent. Although BDV still remained uncharacterized, it had already been propagated in primary tissue culture and established in permanent cell lines through cocultivation (Mayr and Danner 1972, 1974; Ludwig et al. 1973; Danner et al. 1978; Herzog and Rott 1980). These advances facilitated the development of an immunofluorescence assay for serology (Wagner et al. 1968; Danner and Mayr 1973; Ludwig et al. 1973). Reports in 1985 that sera from patients with bipolar disorder were immunoreactive with infected cell lines (Rott et al. 1985; Amsterdam

et al. 1985) stimulated further research focused on identifying and characterizing the BD agent. Efforts in several laboratories to isolate virus for biochemical characterization or visualize particles by electron microscopy were unsuccessful. In the first application of purely genetic methods in pathogen discovery, complementary DNAs (cDNAs) derived through subtractive hybridization were used in in situ hybridization experiments with rat brain to demonstrate the relationship of the virus to disease (Lipkin et al. 1990). Demonstration of the nuclear localization of transcription, RNA splicing, and determination of the complete genomic sequence led to classification of BDV in 1997 (Pringle 1997) as the first member of a new family *Bornaviridae* in the order *Mononegavirales* (Schneider et al. 1994b; Schneemann et al. 1994; Lipkin et al. 1990; Briese et al. 1992, 1994; Cubitt and de la Torre 1994; de la Torre 1994).

Identification of BDV sequences dovetailed with development of PCR as a tool for molecular epidemiology (Lipkin and Hornig 2015). Application of PCR, as well as serologic surveys, led to reports of BDV infection in association with a wide range of neuropsychiatric diseases (Lipkin et al. 2011). The recent publication of a case series of fatal encephalitis among breeders of variegated squirrels in Germany, wherein investigators reported detecting a novel bornavirus in the blood and other tissues of a squirrel to which one of the breeders was exposed as well as in the brains of the patients, lent support to the concept that bornaviruses may be correlated with isolated cases of fatal human encephalitis, however rarely (Hoffmann et al. 2015). To address the question of the relationship of BDV exposure to the pathogenesis of neuropsychiatric disorders (schizophrenia, bipolar disorder, or unipolar major depressive disorder), this author and an international team of collaborators applied sensitive molecular and serologic assays for BDV to blood samples from 198 matched pairs of neuropsychiatric patients and healthy controls in a blinded, tightly controlled, prospective multi-center study; no evidence was found to link BDV to these neuropsychiatric illnesses (Hornig et al. 2012).

Genomic organization: BDV is similar in genomic organization to other nonsegmented, (-) RNA viruses, however, its ~9 kb genome is smaller than among Rhabdoviridae (~11–15 kb), Paramyxoviridae (~16 kb), or Filoviridae (~19 kB) (Briese et al. 1994). The remarkably compact BDV genome encodes six major open reading frames (ORFs) within three transcription units (Schneemann et al. 1995a, b). BDV is distinctive in its localization of replication and transcription to the nucleus (Schwemmle et al. 1999b). Although this feature is shared with plant nucleorhabdoviruses, it is unique amongst NNS animal RNA viruses. The first transcription unit contains a single coded protein (nucleoprotein, N, p40). The second transcription unit encodes, in overlapping ORFs, the proteins X (p10) and P (phosphoprotein, p23). The third transcription unit contains coding sequence for the atypical glycoprotein/matrix protein (M, gp18), type I membrane glycoprotein (G, p57, gp94), and polymerase (L, p190). A variety of mechanisms for transcriptional, post-transcriptional, and translational control of expression, including alternative transcriptional initiation, readthrough of termination signals, alternative splicing, and leaky ribosomal scanning, regulate the expression of these proteins. Splicing, although also found in Orthomyxoviridae (segmented, negative strand RNA viruses), is unprecedented among Mononegavirales (Jordan and Lipkin 2001).

Sequence conservation: Sequences of BDV diverge less than 6 % at the nucleotide (nt) level, a remarkable degree of conservation for an RNA virus (Kilbourne 1991; Schneider et al. 1994a). The extent to which such a degree of sequence conservation represents selective pressure or fidelity of the BDV RNA-dependent RNA polymerase is unclear. Intriguingly, replication and spread of BDV are inhibited by a nucleoside analogue that specifically inhibits DNA polymerase enzymes, Ara-C (1-β-D-Arabinofuranosylcytosine). The mechanism of action remains uncertain, but is postulated to reflect direct inhibition of the viral polymerase rather than an indirect effect mediated by host cell factors (Bajramovic et al. 2002).

This high level of sequence conservation among bornavirus isolates, including laboratory strains, poses a serious challenge for epidemiologic studies of BDV. Diagnosis of BDV infection frequently depends on detection of transcripts in clinical materials (peripheral blood mononuclear cells (PBMC) or other tissues) following amplification by nested reverse transcription-polymerase chain reaction (nRT-PCR). Although nested PCR methods are sensitive, they are also prone to artifact through inadvertent introduction of template from laboratory isolates or cross-contamination of samples (Schwemmle et al. 1999a; Schneider et al. 1994a). Whereas specific signatures can readily facilitate the establishment of provenance in other viral systems, for BDV, similarities in sequence between putative new isolates and confirmed BDV isolates impede the ability to exclude contamination as a potential confound (Durrwald et al. 2007).

Sequence variations that may be unique to a particular host species, timepoint of isolation, or geographic origin are as yet incompletely defined (Binz et al. 1994; Schneider et al. 1994a; Zimmermann et al. 1994). One isolate from a horse in Austria was found to have a higher level of divergence at the nt level than others (strain No/98), but protein sequence was highly conserved (93–96 % over the genome) (Nowotny et al. 2000; Pleschka et al. 2001). Passage history influences the virulence of laboratory isolates in animal models (Hirano et al. 1983; Kao et al. 1984); the molecular basis for such differences appears to stem from two amino acid changes each in the G protein and the polymerase genes (Nishino et al. 2002). Sensitivity to amantadine sulfate is reported for some human isolates (Bode et al. 1997; Ferszt et al. 1999; Dietrich et al. 2000) and may represent an additional strain-dependent phenotypic difference. Other isolates appear to be resistant to amantadine sulfate in vitro and in vivo (Cubitt and de la Torre 1997; Hallensleben et al. 1997; Stitz et al. 1998). Amantadine sensitivity has not been reported for other human isolates (Planz et al. 1999; Nakamura et al. 2000).

Epidemiology

Animals

"Classical" natural mammalian BD is a fatal disorder of movement and behavior of horses and sheep long recognized as endemic to certain areas of Central Europe (Ludwig et al. 1988). Severe disease outbreaks, largely restricted to this region and

these species, had appeared only infrequently. More recently, larger host as well as geographic ranges for BDV infection have been suspected (Kao et al. 1993; Lundgren et al. 1993; Malkinson et al. 1993; Caplazi et al. 1994; Nakamura et al. 1995, 1996; Hagiwara et al. 1996, 1997a, b, 2001; Bahmani et al. 1996; Reeves et al. 1998; Galabru et al. 2000; Dauphin et al. 2001; Helps et al. 2001; Horii et al. 2001; Yilmaz et al. 2002). However, comparisons of archived specimens with more recently collected ones are required to resolve the issue of virus dissemination. In addition, reports of BDV infection in new regions or host species are rarely accompanied by investigations that isolate and fully sequence virus, detail the neuropathologic changes, or infect experimental animals. Notable exceptions include reports from Japan of disease in horses (Hagiwara et al. 2000), domestic cats (Nakamura et al. 1999), and dogs (Okamoto et al. 2002), and from Austria and France in dogs (Weissenbock et al. 1998) and lynx (Degiorgis et al. 2000). The absence of comprehensive investigations limits our understanding of the epidemiology and natural history of BDV in animals. More recently, molecular investigation of proventricular dilatation disease (PDD), a disorder recognized since the 1970s in psittacine species (Gregory et al. 1994), led in 2008 to the recognition of ABV, a virus genetically related to BDV (Kistler et al. 2008; Honkavuori et al. 2008). ABV is associated with nonsuppurative encephalitis and enteric ganglioneuritis and appears to be globally distributed (Rinder et al. 2009; Lierz et al. 2009; Weissenbock et al. 2009; Ogawa et al. 2011; Heffels-Redmann et al. 2011). The recent discovery of sequences distantly related to BDV L, M, and N genes in the genomes of bats, elephants, fish, lemurs, rodents, squirrels, primates, and humans (Horie et al. 2010; Belyi et al. 2010) indicates that at least historically, bornaviruses infected a wide range of vertebrate species.

There is increasing evidence to support a variant course after BDV infection in lieu of the classical and typically fatal BD. Emerging epidemiologic data indicate not only that the host range and geographic distribution of BoDV are larger than previously appreciated; reports of naturally infected animals further suggest that infection may also result in asymptomatic carrier status or subtle disturbances in learning and memory, movement, and behavior (Nobach et al. 2015; Lutz et al. 2015; Durrwald et al. 2014; Bourg et al. 2013; Encarnacao et al. 2013; Puorger et al. 2010). Although Central Europe has the highest reported prevalence of BD, natural infection without disease has been described throughout Europe, in Asia, and in North America. It is unclear whether the apparent increase in host and geographic range of BDV is due to spread of the virus or enhanced case ascertainment. Natural infection of horses and sheep is typically sporadic and peaks in spring months; epidemics of disease are infrequent (Encarnacao et al. 2013).

Neither the reservoir nor the mode for transmission of natural infection is known. Although experimental animals are most frequently infected by either intracranial or intranasal inoculation, infection can be achieved by virtually any method of parenteral inoculation. Based on the efficiency of intranasal infection, and findings of inflammation and edema in the olfactory bulbs of horses early in the course of BD, an olfactory route for transmission has been proposed (Ludwig et al. 1988). The possibility of hematogenous transmission has also been raised based on reports of

the presence of BDV nucleic acid and proteins in PBMC (Sierra-Honigmann et al. 1993; Rubin et al. 1995). A recent case series investigating fatal encephalitis in breeders of variegated squirrels lends some tentative support to the potential for zoonotic blood-borne transmission from rodents. The same novel mammalian bornavirus (variegated squirrel 1 bornavirus [VSBV-1]) found in the brains of all three encephalitis patients was also found in the blood of one of the healthy variegated squirrels that had been bred in captivity and had reportedly been in direct contact with one of the breeders; two of the three breeders had been reported to have bites or scratches from the squirrels prior to illness onset (Hoffmann et al. 2015). Another report suggests the potential for vertical transmission of BDV in mammals (Hagiwara et al. 2000).

Although persistence of virus in saliva, urine, and feces after experimental infection of neonatal Lewis rats also suggested that rodents may be able to serve as reservoirs for mammalian (Sierra-Honigmann et al. 1993), one study examining natural infection of wild rodents did not support this hypothesis (Tsujimura et al. 1999). Nonetheless, the presence of viral gene products in saliva, urine, and other secreta/excreta is important in aerosol transmission of other pathogenic viruses (e.g., lymphocytic choriomeningitis virus, hantaviruses). Normal adult rats housed in cages separate but adjacent to those of neonatally infected rats can become infected, suggesting that aerosol transmission of BDV is plausible. The observations that rodents can be persistently infected with mammalian and excrete virus suggest that they have the potential to serve both as natural reservoirs and as vectors for virus dissemination; the finding of VSBV-1 in the brains of fatal human encephalitis cases and in the blood and other tissues of variegated squirrels they had bred in captivity lends further support to the hypothesis that zoonotic transmission may be possible through some rodent species (Hoffmann et al. 2015). However, the overall significance of rodents for transmission of BDV to domesticated animals and humans remains unresolved, particularly for the less florid, non-encephalitic presentations that are anticipated in most neuropsychiatric illnesses. Reports of BDV nucleic acids and proteins in PBMC support the potential for hematogenous transmission.

Other small mammals may play a role as vectors or serve as reservoirs of the virus. Mammalian BDV has recently been shown to be present in the tissues of apparently healthy bicolored white-toothed shrews (*Crocidura leucodon*)—a species within the subfamily Crocidurinae, which is more closely related to moles than to rodents—in BDV-endemic regions of Switzerland (Puorger et al. 2010) and Germany (Bourg et al. 2013; Durrwald et al. 2014). In a study of wild *C. leucodon*, trapped in Germany without apparent clinical disease and followed for up to 600 days, 6 of 11 were found to be naturally infected with neurotropic BDV (BoDV-1); infectious virus and viral RNA were shed for up to 250 days in captivity in saliva, urine, skin swabs, lacrimal fluid, and feces (Nobach et al. 2015). Ecological models also show high rates of colocalization of equine BD cases with the distribution of *C. leucodon* in BDV-endemic areas of Bavaria, Germany (Encarnacao et al. 2013). Taken together, these data suggest that studies of BoDV-1 in *C. leucodon* may help to elucidate important aspects of virus–host interactions and their relationship to disease and may assist in resolving whether *C. leucodon* is a potential reservoir for

BDV transmission. It is intriguing to consider the possibility that endogenous bornavirus-like nucleoprotein (EBLN) elements that might be present in the genome could inhibit the replication of exogenous BDV strains. When EBLNs were present in the genomes of ground squirrels, the BDV polymerase was incorporated into the viral ribonucleoprotein, inhibiting its activity, an effect not observed with human EBLNs (Fujino et al. 2014). BDV has also been reported in bird excrement, suggesting the alternative of an avian reservoir (Berg et al. 2001), although the bornaviruses more commonly identified in association with disease in psittacine and other birds, the avian bornaviruses, are highly divergent from mammalian bornavirus strains. Rigorous epidemiologic investigation of the global distribution and ecology of BDV should be emphasized in future research.

Humans

Considerable controversy has long surrounded the question of whether humans can be infected with BDV. Although most reports of an association between infection and disease have focused on unipolar depression, bipolar disorder, or schizophrenia, BDV has also been implicated in an improbably wide range of disorders, including chronic fatigue syndrome, acquired immune deficiency syndrome (AIDS) encephalopathy, multiple sclerosis, motor neuron disease, and aggressive brain tumors (Hatalski et al. 1997). Isolation of infectious virus from humans is only rarely reported (Salvatore et al. 1997; Haga et al. 1997; Hoffmann et al. 2015). Instead, infection is more frequently established by detecting BDV transcripts in clinical materials such as PBMC or tissues following amplification by nRT-PCR). This method, although sensitive, is prone to artifact due to inadvertent introduction of template from laboratory isolates or cross-contamination of samples. Unlike other NNS RNA viruses, wherein the inherent low fidelity of viral RNA-dependent RNA polymerases results in sequence divergence of 10^3–10^4 per site in each round of replication, BDV is characterized by an extraordinary degree of sequence conservation; sequencing, therefore, cannot readily distinguish between products representing *bona fide* isolates and those reflecting amplification of low level contaminants. Methods used for serological diagnosis of infection include indirect immunofluorescence with infected cells, western immunoblotting, or enzyme-linked immunosorbent assays (ELISAs) relying on extracts of infected cells or recombinant proteins. The performance of an immune complex assay, described as more sensitive and specific than other immunoassays (Bode et al. 2001), has not been independently validated (Wolff et al. 2006). However, infection of human oligodendrocyte cells with strains of BDV isolated from humans (Hu-H1, isolated from a bipolar patient) inhibited proliferation and promoted apoptosis through upregulation of Bax and downregulation of Bcl2, whereas the laboratory-derived strain (Strain V) had opposite effects. While leaving questions about the role of BDV in human disease unresolved, these results suggest that BDV strains differ functionally and that Hu-H1 was unlikely to be a Strain V-derived contaminant.

Naturally infected horses, sheep, cattle, cats, and birds could serve as reservoirs for the virus; however, there are few epidemiological studies in animal populations and no studies that demonstrate transmission from domestic animals to humans. BDV is transmitted efficiently through contact with nerve terminals (e.g., olfactory infection); however, the presence of BDV in PBMC suggests that the source of infection could be hematogenous. One study revealed 4–5 % of random blood donors in Japan to be harboring BDV nucleic acids in PBMC (Kishi et al. 1995). The only study reporting historical infection at a higher rate in healthy blood donors (30 %) relied on a sandwich enzyme immunoassay for detection of circulating immune complexes (Bode et al. 2001), a method that does not discriminate the presence of BDV antigen from nonspecific reactivity (Wolff et al. 2006). These limitations in our understanding of BDV pathogenesis have constrained our capacity to tailor study designs best suited to the selection of the optimal sampling compartment(s) or timing of sample collection relative to illness onset or exacerbation, or the most reliable diagnostic markers for the identification of infection (Hornig et al. 2003; Lipkin et al. 2001).

To definitively address the question of the association of BDV infection with neuropsychiatric disorders, study design controls—ranging from well-defined criteria for recruitment of cases and controls to strict procedures averting contamination during sample processing and assay setup—were incorporated into a large, blinded multi-center epidemiologic investigation (Hornig et al. 2012). Unlike prior studies of human BDV infection, rigorous clinical characterization was pursued using standardized instruments not only for patient groups (schizophrenia, bipolar disorder, and unipolar major depressive disorder) but also for their healthy matched controls. Controls were individually matched to patients within each diagnostic group on the basis of age, sex, geographic residence, and socioeconomic status, as well as seasonal and circadian timing of sample collection. To maximize the possibility that evidence of current or past BDV infection could be detected, serum, plasma, and white blood cell samples were collected both at the time of an acute onset or exacerbation of existing illness and 6 weeks later, to allow for capture of a potential anamnestic response to BDV. Sensitive molecular and serologic measures (real-time RT-PCR, and ELISA and IFA, respectively) were applied. Samples were processed in a setting without any known exposure to BDV to guard against the potential for laboratory contamination. Strict blinding was maintained at all laboratory sites, with specific, predetermined criteria for designating samples as positive or negative, until a predesignated endpoint was reached for breaking the study blind. Using real-time RT-PCR or ELISA, we found no evidence of infection in any sample at either the acute sampling time point (study entry) or 6 weeks after the acute new episode of illness or its exacerbation (Hornig et al. 2012). IFA results revealed that four of 396 baseline samples contained high-avidity antibodies targeting BDV (1 %); however, we found no relationship to psychiatric diagnosis (two positive samples were from subjects with bipolar disorder, one derived from a bipolar disorder group control, and the fourth was from a schizophrenia group control) and the IFA pattern for these samples was demonstrated to be nonspecific in follow-up western immunoblotting experiments.

Bornavirus Tropism

Although cells of many lineages and species can be experimentally infected with BDV, virus production is more efficient in neural than nonneural cells. BDV is also neurotropic in vivo, with a particular predilection for neurons of the limbic system (Ludwig et al. 1988). Neurons of the hippocampus and amygdala are among the cells first targeted in natural infection of horses and experimental infection of rats. Infection in vitro is not associated with cytopathic effect. Tropism for limbic system may relate to regional distribution or activity of host phosphorylating kinases such as PKC. Early in the viral life cycle of BDV, its proteins are phosphorylated by PKCε, an isoform of the host enzyme that is highly expressed within limbic regions (Schwemmle et al. 1997). The virus ultimately spreads throughout the central, peripheral, and autonomic nervous systems infecting astrocytes, Schwann cells, and ependymal cells in the CNS; sensory and autonomic ganglia; and nerves to organs. Viral transport is presumed to be axonal and transsynaptic. After intranasal infection, viral antigen is sequentially detected in olfactory receptor cells, olfactory nerve fibers and cells of the olfactory bulb followed by olfactory cortex. Within the hippocampus, viral antigen is localized in axon terminals that first form synapses with CA1 pyramidal cell dendrites prior to appearing in pyramidal cell bodies. As with rabies virus, it is likely that the spread of BDV infection within the CNS is not primarily mediated by enveloped virions but rather by ribonucleoprotein particles (Gosztonyi et al. 1993; Clemente and de la Torre 2007).

Experimental Rodent Models of Bornavirus Infection

Depending on the integrity, intensity, and maturational status of the host immune response at the time of infection, clinical signs of BDV infection may be dramatic, subtle, or inapparent. The autoimmune disease-susceptible Lewis rat has been the most common model system for interrogating the pathobiological consequences of experimental BDV exposure. Adult, immunocompetent animals develop an immune-mediated syndrome that is multiphasic, including stereotyped motor behaviors and hyperactivity, dyskinesias, dystonias, ataxia, and paresis (Narayan et al. 1983a). Disturbances in adult-infected Lewis rats include altered brain levels of catecholamine neurotransmitters, unusual sensitivity to dopamine agonists, and decreased levels of dopamine receptors in caudate-putamen and nucleus accumbens (Solbrig et al. 1996b). In contrast to the robust disease observed in adult-infected rats, rats infected as neonates demonstrate tolerance and do not mount a cellular immune response to the virus; neonatally infected rats manifest a different syndrome characterized by stunted growth, hyperactivity, subtle learning disturbances, altered taste preferences, and abnormal responses to novel environments (ranging from excessive inhibition to excessive exploratory behavior). CNS dysfunction in neonatally infected animals may reflect direct effects of the virus on the

morphogenesis of the hippocampus and cerebellum, two structures in rodents that continue to mature postnatally. Although overall architecture is maintained, granule cells of dentate gyrus and Purkinje cells of cerebellum are lost through apoptosis.

Adult rat model: Disorders of movement and behavior in adult-infected rats correlate with dysfunction in dopamine (DA) circuits (Solbrig et al. 1994, 1995, 1996a, c, 1998), as seen in many neuropsychiatric disorders (Anderson 1994; Ernst et al. 1997; Kelsoe et al. 1996; Partonen 1996), as well as to serotonin (5HT) abnormalities (Solbrig et al. 1995). Disturbed brain levels of catecholamine neurotransmitters, exaggerated sensitivity to dopamine agonists, and altered levels of dopamine receptors in caudate-putamen and nucleus accumbens (D2 and D2 and D3 receptors, respectively) are found in adult-infected BD rats. Enhanced sensitivity of central DA systems of adult-infected BD animals to DA agonists, antagonists, and DA reuptake inhibitors is observed. Administration to adult-infected rats of dextroamphetamine, a mixed-acting DA agonist, or cocaine, a DA reuptake inhibitor, elicits increased locomotor and stereotypic behavior, indicating dose-dependent potentiation of DA neurotransmission (Solbrig et al. 1994, 1998). Furthermore, the administration of psychotropic drugs active in dopamine circuits suppresses some behavioral disturbances in these animals (e.g., hyperactivity, self-mutilation). Both pre- and postsynaptic sites of the DA transmitter system appear to be damaged in striatum: DA reuptake sites are reduced in caudate-putamen and nucleus accumbens; postsynaptic D2, but not D1, receptor binding is markedly reduced in caudate-putamen; D2 and D3 receptor binding are reduced in nucleus accumbens. In contrast, postsynaptic DA receptors (D1, D2) remain intact in prefrontal cortex. Further support for D2-selective losses and resultant D1 hypersensitivity as mediators of neurobehavioral disturbances in adult BD is found in the ability to reverse locomotor hyperactivity through administration of D1 receptor blocking agents, such as the D1 antagonist, SCH23390, but not through the administration of D2-selective antagonists, haloperidol or raclopride (Solbrig et al. 1996a, b, 1998).

Dysfunction in serotonin and noradrenergic systems also accompanies the behavioral syndrome. The atypical neuroleptic clozapine, an agent with multiple pharmacologic effects, including antagonism of dopaminergic, serotonergic, and adrenergic receptors, reduces stereotypies and hyperactivity. Pharmacologic and neurotransmitter-specific molecular probes have also been used to characterize endogenous opioid systems in the adult rat model. Infected animals respond abnormally to the opiate antagonist, naloxone, with hyperkinesis and seizures, and also demonstrate increases in striatal preproenkephalin mRNA (Solbrig et al. 2002; 1996d). Induction of the enkephalin system may relate to increased levels of phosphorylated cyclic AMP response element binding (phosphoCREB) protein through activation by BDV of the mitogen-activated protein (MAP) kinase pathway, thus stimulating transcription factors that regulate enkephalin expression in striatum. A decrease in choline acetyltransferase-positive fibers is found early in infection (Gies et al. 2001), progressing to nearly complete loss of these fibers in hippocampus and neocortex, and anticholinergics such as scopolamine worsen stereotypies and dys-

kinesias. The expression of genes for other neuromodulatory substances and their associated synthesizing enzymes, including somatostatin, cholecystokinin, and glutamic acid decarboxylase, is also greatly reduced during the acute phase and recovers toward normal in the chronic phase of adult BD (Lipkin et al. 1988).

Neonatal rat model: The neonatal rat model does not show overt immunopathology. Virus load is high in brain and persists over a lifetime. Humoral immune responses to BDV in neonatally infected animals are also diminished, with anti-BDV antibody titers remaining <1:10 through at least 4 months postinfection (Carbone et al. 1991). Animals infected within the first 12 h of life appear to remain tolerant to BDV, developing a mild behavioral syndrome with restricted neuropathologic features that provides a more intriguing model for neuropsychiatric illnesses—disorders not typically accompanied by frank encephalitis. The dysgenesis of cerebellum and hippocampus found in neonatally infected animals is consistent with the more subtle neurodevelopmental abnormalities reported in some studies of autism (Kemper and Bauman 1993), schizophrenia (Altshuler et al. 1987; Fish et al. 1992), and affective illness (Soares and Mann 1997). Neonatally infected animals display a wide range of physiologic and neurobehavioral disturbances. They are smaller than uninfected littermates (Carbone et al. 1991; Bautista et al. 1994), without demonstrable alteration of glucose, growth hormone, or insulin-like growth factor-1 (Bautista et al. 1994) or amount of food ingested (Bautista et al. 1995); display an enhanced preference for salt solutions; and exhibit altered circadian rhythms (Bautista et al. 1994). Behavioral and cognitive changes in rats infected in the neonatal period include abnormal early locomotor development (Hornig et al. 1999), spatial and aversive learning deficits (Dittrich et al. 1989; Rubin et al. 1999), increased motor activity (Bautista et al. 1994; Hornig et al. 1999), abnormal anxiety responses (Dittrich et al. 1989; Hornig et al. 1999; Pletnikov et al. 1999a), stereotypic behaviors (Hornig et al. 1999), and reduced initiation of and response to nondominance-related play interactions (Pletnikov et al. 1999b). Thus, the neuropathologic, physiologic, and neurobehavioral features of BDV infection of neonates indicate that it not only provides a useful model for exploring the mechanisms by which viral and immune factors may damage developing neurocircuitry, but also has significant links to the range of biologic, neurostructural, locomotor, cognitive, and social deficits observed in a wide range of human neuropsychiatric illnesses, including the neurodevelopmental disorder, autism.

Although cellular inflammatory response to BDV following neonatal infection is restricted, a phenomenon ascribed to the immaturity of rat postnatal immune function, mononuclear cell infiltrates (Hornig et al. 1999) and gene expression associated with proinflammatory cytokines (Hornig et al. 1999; Sauder and de la Torre 1999), chemokines (Sauder et al. 2000), and chemokine receptors (Rauer et al. 2002) briefly surge. This transient immune response, however, does not colocalize with regions of neuropathologic damage (Weissenbock et al. 2000). Instead, histologic changes parallel the regions and timecourse for proliferation of microglia and the expression of MHC class I and class II, ICAM, CD4, and CD8 molecules (Weissenbock et al. 2000). In contrast to the immune mechanisms postulated to

underlie damage in adult BD rats, then, CNS dysfunction in neonatally infected animals has been proposed to stem from direct viral effects on the morphogenesis of two structures that continue to mature postnatally in rodents, the hippocampus and the cerebellum. Although overall architecture of these brain regions is maintained, dentate gyrus granule cells (Hornig et al. 1999; Rubin et al. 1999) and cerebellar Purkinje cells (Hornig et al. 1999; Eisenman et al. 1999) are lost through apoptosis (Hornig et al. 1999). Neuronal losses in the dentate gyrus of neonatally infected Lewis rats correlate with the severity of their deficits in spatial learning and memory (Rubin et al. 1999). Subtle cerebellar tests demonstrate functional deficits in motor coordination and postural stability, consistent with Purkinje cell losses (Hornig et al. 1999; Pletnikov et al. 2001). Host genetic background appears to influence degeneration in dentate gyrus granule cell neuronal cultures, potentially through the presence or absence of soluble protective factors produced by a disease-resistant rat strain (Sprague–Dawley), despite similar efficiency of viral replication in Sprague–Dawley rats and in susceptible Lewis rats (Wu et al. 2013). Additionally, the entorhinal axonal afferents, which target granule cells, were curiously found to persist without any apparent disruption of cytoarchitecture, despite nearly complete loss of dentate gyrus granule cells in neonatally infected susceptible Lewis rats (Heimrich et al. 2009). The differential cellular and regional patterns of the expression of genes associated with individual BDV proteins may also play a role in these observed patterns in CNS. BDV glycoprotein gene expression was at lower levels than that of the N protein, and was largely restricted to cortex, hippocampus, amygdala, and thalamus as well as to larger neurons, whereas BDV N gene expression also occurred in astrocytes, oligodendrocytes, and ependymal cells (Werner-Keiss et al. 2008).

Levels of neurotrophic factor mRNAs are decreased in the neonatal rat model (Hornig et al. 1999; Zocher et al. 2000); however, they are found only in hippocampus and thus unlikely to account for neuropathologic changes in cerebellum, including Purkinje cell losses. Alternatively, abnormal regulation of apoptosis—either failure of normal apoptotic sequences to be curtailed with age or excess activation of apoptotic cell programs—might contribute to abnormal CNS development. Excitotoxic stimulation, particularly through activation of glutamatergic circuitry, may trigger neuronal apoptosis. In vitro studies indicate that infection of glial cells impairs glutamate reuptake. Altered glutamate receptor expression is found in vivo. BDV P protein also has been reported to shift the localization of gamma-aminobutyric acid receptor-associated protein (GABARAP) from the cytoplasm to the nucleus; this shift in GABARAP localization inhibits the ability of the receptors for GABA, a major inhibitory neurotransmitter, to traffic to cell membranes, where they play an important role in GABA-induced inhibitory neural transmission. These changes are postulated to promote hyperactivity and anxiety by reducing the inhibitory inputs of GABA currents (Peng et al. 2008). Complex dysregulation of gene expression of apoptosis mediators is noted in hippocampus, amygdala, prefrontal cortex, nucleus accumbens, and cerebellum, including increased levels of mRNAs for FAS and ICE (caspase-1), two promoters of apoptosis, and decreased mRNA for bcl-x, a factor that inhibits apoptosis. These findings are consistent with an overall

promotion of apoptosis throughout the brains of rats neonatally infected with BDV (Hornig et al. 1999).

Altered developmental gene expression and cell signaling: Disturbances in cytoarchitecture following experimental neonatal rat infections have been shown to be linked to alterations in expression of tissue factors, cytokines, neurotrophins, and apoptosis-related products during critical periods of neural development (Hornig et al. 1999). In vitro studies may also provide insights into the neuropathogenesis of neonatal BDV infection. Cell-to-cell spread of BDV can be inhibited in cell culture with the addition of a MAPK/ERK kinase (MEK) inhibitor (Planz et al. 2001). Studies of neuronal differentiation of PC12 cells (Hans et al. 2001) also show that BDV interacts with cellular MAP kinase signaling pathways. Although constitutive phosphorylation of MEK, ERK, and the transcriptional activator Elk-1 (Hans et al. 2001) has been demonstrated in infected PC12 cells, these cells fail to differentiate upon exposure to NGF. Neurite outgrowth is also inhibited in other infected cell lines (Kamitani et al. 2001); this effect has been ascribed to P protein-mediated interference with the normal interaction between amphoterin, a neurite outgrowth factor (Zhang et al. 2003), and its receptor, RAGE (*R*eceptor for *A*dvanced *G*lycation *E*nd-products). BDV infection alters intracellular distribution of amphoterin, such that amphoterin and RAGE activation levels are found to be decreased at the growth cones of cells undergoing extension (Kamitani et al. 2001). PKC-dependent phosphorylation of P protein also appears to mediate the inhibitory effects of BDV on neuronal plasticity. Mutations introduced at the PKC phosphorylation site of BDV P protein abolish the capacity of BDV to interfere with phosphorylation of endogenous substrates of PKC (MARCKS, SNAP-25) and reverse its ability to downregulate activity-dependent synaptic modulation (Prat et al. 2009). These results suggest that BDV-induced dysfunction of neuronal signaling may arise during the phosphorylation of BDV P protein as a result of competition for components of the PKC signaling pathway.

In addition to infection of neurons, BDV also infects astrocytes. Behavioral abnormalities reminiscent of neonatal rat infection have been found in a transgenic mouse model in which the BDV P protein was expressed in glial cells (Kamitani et al. 2003). Animals expressing high brain levels of BDV P had decreased levels of brain-derived neurotropic factor and serotonin receptors as well as reduced synaptic density, in the absence of astrocytosis. These findings are consistent with the hypothesis that BDV gene products can directly interfere with neuronal function without inducing gross neurodegeneration (Volmer et al. 2006; Prat et al. 2009). Interactions of neurons with other resident cell subsets of the CNS are likely to play an important role in pathogenesis. Astrocytes are reportedly required for activation of microglia early in the course of BDV infection. Astrocytes may be activated by BDV-infected neurons without becoming infected themselves. Notably, activated microglial cells appear before the onset of apoptosis-mediated neuronal losses in the dentate gyrus of hippocampus (Ovanesov et al. 2008).

Recent work indicates that BDV impairs neurogenesis in infected human neural progenitor cells (hNPCs) through a BDV P-restricted mechanism. Specific impair-

ment in GABAergic neurogenesis as well as in neuronal factors that play a role in neuronal differentiation (e.g., ApoE, Noggin, TH, and Scg10/Stathmin2) were found with expression of P protein, but not with BDV X. These findings suggest that BDV infection impairs specific components of the molecular programs regulating neurogenesis (Scordel et al. 2015). Infection of rat cortical neurons by a BDV strain isolated from humans (Hu-H1) led to a different metabolomic profile than infection with a laboratory isolate of BDV (Strain V), with key differences in energy-related and amino acid metabolites (Liu et al. 2015). Infection of human oligodendroglial cells with Hu-H1 strain also led to alterations in proteomic and acetylomic profiles, with prominent differences in proteins representing metabolic pathways, immune responses, DNA replication and repair and transcriptional regulation, and in patterns of histone lysine acetylation. BDV infection of cerebral cortical neurons was associated with P protein-dependent inhibition of histone acetyltransferase activity, with effects restricted to selected lysine residues; pharmacologic inhibition of acetyltransferases also reduced viral replication (Bonnaud et al. 2015). In a related infection system, distinctive changes in proteins were found in infected cortical neurons, particularly in proteins implicated in neurotransmission, neurogenesis, cytoskeleton dynamics, regulation of gene expression, and chromatin remodeling (Suberbielle et al. 2008).

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Reovirus

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Neurotropic Reoviruses

The *Reoviridae* are a family of non enveloped, double-stranded RNA viruses that infect a wide range of hosts including plants, mollusks, crustaceans, arthropods, reptiles, birds, fish, and mammals. Reoviridae viruses linked to encephalitis in mammals are found in the genera *Rotavirus* and *Orthoreovirus*. Rotaviruses usually cause diarrhea in infants and young children and are occasionally detected in cerebrospinal fluid obtained from children with neurologic disease. However, a causal relationship between rotaviruses and viral encephalitis has not been demonstrated (Lynch et al. 2001; Schumacher and Forster 1999). The genus *Orthoreovirus* is subdivided into the fusogenic and non-fusogenic orthoreoviruses. One member of the fusogenic group, baboon reovirus (BRV), has been associated with outbreaks of viral meningoencephalitis in colonies of captive baboons (Chua et al. 2007; Leland et al. 2000). Non-fusogenic mammalian orthoreoviruses (called reoviruses here) were isolated from children in the 1950s and are classified into three serotypes (1, 2, and 3) based on serology. Each of the serotypes is represented by prototype strains, type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Abney (T3A) and Dearing (T3D) (Rosen 1962; Rosen et al. 1960; Sabin 1959). While a small number of clinical reports link reovirus to neurologic disease (Johansson et al. 1996; Hermann et al. 2004; Tyler et al. 2004), infection of adults rarely leads to clinical symptoms, hence the name *reo*virus: *r*espiratory *e*nteric *o*rphan virus. The discovery that type

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3 strains of reovirus have a cytopathic effect on malignant cells has led to the development of T3D as a cancer therapeutic. Pelareorep (Reolysin®) is currently in phase I–III clinical trials for the treatment of solid tumors and hematologic malignancies (Hingorani et al. 2011; Gollamudi et al. 2009). In contrast to human adults, newborn mice are highly susceptible to infection by reoviruses. In these animals, reoviruses cause a variety of diseases including pneumonia, myocarditis, hepatitis, hydrocephalus, and encephalitis depending on the viral strain. The availability of a tractable model for the study of viral pathogenesis and a reverse genetics system for the manipulation of reovirus genomes has yielded a wealth of knowledge about virus and host determinants of reovirus disease (Dermody et al. 2013). Mammalian reoviruses are the focus of this chapter.

Primary Replication and Dissemination

Reovirus has a broad host range in nature and infects most mammals via either the respiratory or oral route. Following inoculation of mice into the respiratory tract, reovirus first infects specialized microfold (M) cells in the bronchial epithelium. From there, reovirus spreads to the bronchial lymphoid tissue, causing a viral pneumonia characterized by a prominent neutrophil influx (Morin et al. 1994, 1996). Analogously, inoculation of mice into the gastrointestinal tract is initiated by traversing M cells that overly the Peyer's patches (PPs) in the intestine (Wolf et al. 1981). In adult mice, primary replication takes place in the epithelial cells overlying the PPs and the lamina propria and in the crypts of Lieberkuhn (Rubin 1987; Bass et al. 1988). In newborn mice, viral antigen is primarily detected in the tips of the villi and the mononuclear cells within the PPs (Antar et al. 2009). Productive infection of the intestine requires proteolytic processing of viral outer-capsid proteins by proteases residing in the intestinal lumen (Bass et al. 1990; Bodkin et al. 1989) or the endocytic compartment (Borsa et al. 1981; Silverstein et al. 1972; Sturzenbecker et al. 1987; Chang and Zweerink 1971; Ebert et al. 2002; Johnson et al. 2009). Following exposure of reovirus to host proteases, outer-capsid protein σ3 is removed and an additional outer-capsid protein μ1 is cleaved internally to form the δ and φ fragments (Nibert et al. 2005; Odegard et al. 2004; Nibert and Fields 1992; Chandran et al. 2003) (Fig. 1). The resulting infectious subvirion particles (ISVPs) are the predominant infectious form of the virus in the intestine (Bodkin et al. 1989). T1L produces higher titers in the intestine than does T3D (Bodkin and Fields 1989; Keroack and Fields 1986). This difference in viral load in the intestine is genetically linked to the viral S1 and L2 gene segments, which encode viral attachment protein σ1 and core spike protein λ2, respectively (Bodkin and Fields 1989). The T3D σ1 protein is susceptible to cleavage by proteases in the intestinal lumen, resulting in reduced infectivity of T3D relative to T1L at that site (Chappell et al. 1998). Following primary replication in the intestine, reovirus disseminates systemically to infect virtually all major organs including the mesenteric lymph nodes (MLN),

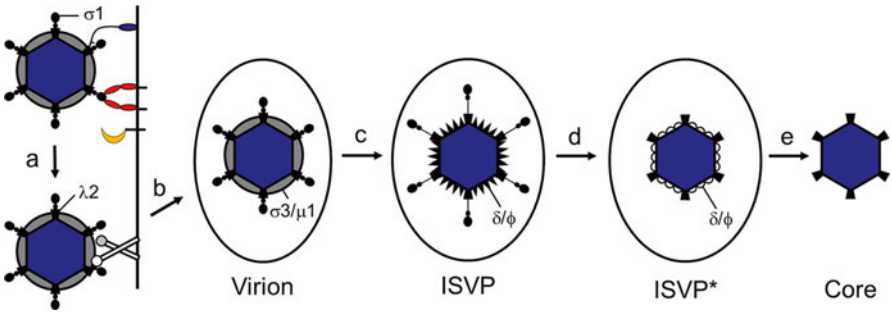

Fig. 1 Reovirus cell entry and disassembly. (**a**) Reovirus cell entry is initiated by attachment of virions to cell-surface carbohydrate (GM2 for type 1, GM3 [and other] for type 3 strains; *blue*) and proteinaceous receptor JAM-A (nonneuronal cells; *red*) and NgR1 (neuronal cells; *orange*). (**b**) Virions are internalized by β1-integrin-mediated endocytosis. Within the endocytic compartment, the viral outer capsid undergoes a series of acid-dependent proteolytic cleavages. (**c**) Protein σ3 is lost and μ1C is cleaved into particle-associated fragments μ1N, *δ*, and *φ* to form the ISVP. (**d**) Conformational rearrangements of the μ1 fragments expose hydrophobic residues, and μ1N, *φ*, and attachment protein σ1 are released, resulting in the formation of ISVP*s. (**e**) The μ1 cleavage fragments mediate endosomal membrane penetration and release of the transcriptionally active core into the cytoplasm. Figure and legend modified from Danthi et al. (2010b)

liver, spleen, lungs, heart, and central nervous system (CNS). Systemic spread of serotype 1 strains occurs exclusively via hematogenous routes, whereas serotype 3 strains spread via hematogenous and neural routes (Tyler et al. 1986; Morrison et al. 1991; Antar et al. 2009). Studies using mutant viruses that do not express σ1s, a nonstructural protein required for hematogenous spread, identified the hematogenous route as the primary mode of virus delivery to the brain and a key viral determinant of viral neuropathogenesis (Boehme et al. 2009, 2011).

Reovirus Receptors and Neuronal Targeting

Type 1 and 3 reoviruses differ markedly in CNS tropism and pathology. Upon arrival to the CNS, type 1 strains infect ependymal cells, leading to a nonlethal hydrocephalus characterized by disruption of the ependymal layer, inflammation of the ventricles, and an increase in ventricular volume (Tyler et al. 1986; Weiner et al. 1977, 1980; Stencel-Baerenwald et al. 2015). In contrast, type 3 strains infect in neurons located in specific layers of the frontoparietal cortex, the CA1 to CA4 region of the hippocampus, the cingulate gyrus, thalamus, and the Purkinje neurons in the cerebellum (Tyler 1998) (Fig. 2). Infection of neurons leads to apoptosis followed by an influx of inflammatory infiltrates, culminating in a lethal meningoencephalitis (Tyler et al. 1986; Weiner et al. 1977, 1980). The molecular basis for these differences in reovirus tropism has not been elucidated. However, divergent receptor usage may dictate the serotype-dependent differences in disease.

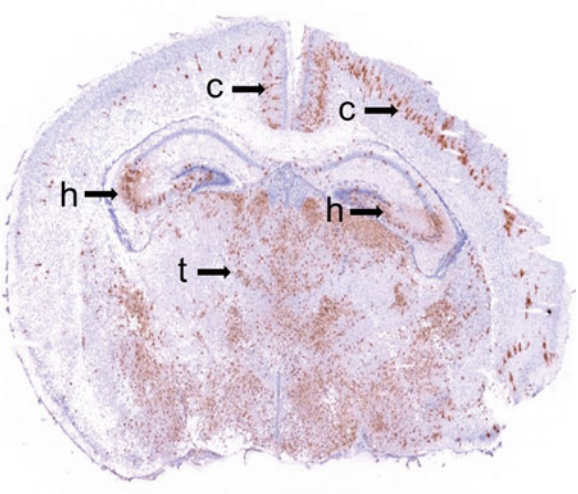

Fig. 2 Reovirus antigen localization in the newborn mouse brain. Two-day-old C57BL/6 mice were inoculated intracranially with 10^4 PFU of reovirus strain T3SA+. At 8 days post-inoculation, brains were resected, flash-frozen, and immunostained using polyclonal reovirus antiserum. Reovirus antigen (in *brown*) localizes to distinct regions including the cortex (c), hippocampus (h), and thalamus (t)

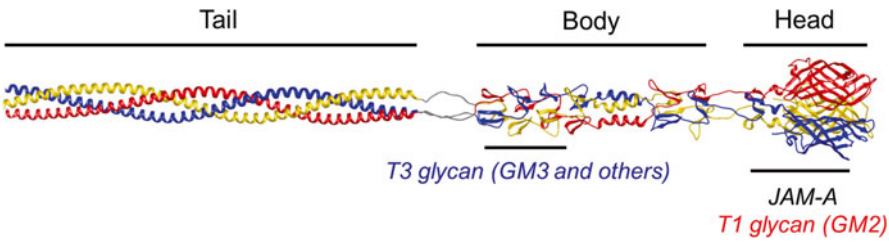

Fig. 3 Crystal structure of σ1. The σ1 protein is a trimer comprised of a C-terminal globular head domain, a β-spiral body domain, and a long, filamentous α-helical tail that inserts into the virion capsid. Individual trimers are shown in yellow, red, and blue. Regions containing known receptor binding sites are indicated. Figure modified from Reiter et al. (2011)

Reovirus cell entry is initiated by low-affinity binding of the virion to abundantly expressed glycans on the surface of target cells followed by high-affinity interactions with proteinaceous receptors present in lower abundance via an adhesion strengthening mechanism (Barton et al. 2001a). Binding to cells is mediated by attachment protein σ1. The σ1 protein is a trimer comprised of a C-terminal globular head domain, a β-spiral body domain, and a long, filamentous α-helical tail that inserts into the viral capsid (Fraser et al. 1990; Furlong et al. 1988) (Fig. 3). Two proteinaceous receptors have been identified for reovirus: junctional adhesion molecule-A (JAM-A) and Nogo receptor 1 (NgR1) (Barton et al. 2001b; Konopka-Anstadt et al. 2014). JAM-A is an immunoglobulin (Ig) superfamily member expressed on leukocytes and platelets (Kornecki et al. 1990; Martin-Padura et al. 1998) as well as endothelial and epithelial cells (Martin-Padura et al. 1998; Liu et al. 2000). JAM-A is located in tight junctions and functions in formation and maintenance of endothelial and epithelial barriers (Martin-Padura et al. 1998; Bazzoni 2003). During an inflammatory response, JAM-A is upregulated and redistributes to

the apical surface of endothelial cells, where it serves in the recruitment of leukocytes (Weber et al. 2007). The σ1 head domain binds to the membrane-distal Ig-like domain of JAM-A (Barton et al. 2001b; Kirchner et al. 2008; Guglielmi et al. 2007). Binding to JAM-A on endothelial but not hematopoietic cells is required for establishment of viremia and viral dissemination from the intestine to sites of secondary replication (Antar et al. 2009; Lai et al. 2014). However, JAM-A is dispensable for reovirus infection of the CNS (Antar et al. 2009).

NgR1, a glycosylphosphatidylinositol (GPI)-anchored molecule that belongs to the leucine-rich repeat (LRR) domain-containing family of proteins, is a proteinaceous receptor mediating reovirus infection of neurons (Konopka-Anstadt et al. 2014; Barton et al. 2003a; Fournier et al. 2001). NgR1 was originally identified as the receptor for myelin-associated glycoprotein (MAG) and Nogo66, two negative regulators of axon regeneration and myelination expressed by oligodendrocytes in the CNS (Qiu et al. 2000; Huber and Schwab 2000; Fournier et al. 2002). NgR1 mRNA can be detected in neurons located in areas targeted by reovirus, including cerebral cortical neurons, hippocampal neurons, thalamic neurons, and cerebellar Purkinje cells (Hunt et al. 2002). Ectopic expression of NgR1 in Chinese hamster ovary (CHO) cells, which are not naturally susceptible to reovirus infection, confers susceptibility to both type 1 and type 3 reovirus strains. It is unclear which reovirus capsid component interacts with NgR1. Intact reovirus virions can engage NgR1, whereas ISVP disassembly intermediates cannot (Konopka-Anstadt et al. 2014). ISVPs differ structurally from virions in that they lack the outer-capsid protein σ3, the μ1 protein has been proteolytically cleaved to form δ and φ, and a conformational change in the σ1 fiber yields a more extended version of the protein (Borsa et al. 1981; Sturzenbecker et al. 1987; Nibert and Fields 1992; Furlong et al. 1988; Dryden et al. 1993). The observation that NgR1 mediates entry of virions but not ISVPs could be explained by a model in which NgR1 engages the more compact form of σ1. Length and flexibility of σ1 are required for efficient infection (Bokiej et al. 2012), providing plausibility for this idea. Alternatively, NgR1 may engage σ3 instead of σ1 (Konopka-Anstadt et al. 2014). A σ1-independent attachment mechanism has been described for BRV, a fusogenic reovirus that does not express an attachment fiber (Yan et al. 2011). BRV encodes σ3 homolog σB and causes meningoencephalomyelitis in young baboons, indicating that an attachment fiber is not required for neurovirulence of some reovirus strains (Leland et al. 2000; Kumar et al. 2014).

While binding of proteinaceous receptors is essential for reovirus infection of the CNS, low-affinity interactions with glycans dictate infection efficiency and influence viral tropism. Initial evidence supporting a role for carbohydrates in reovirus tropism and disease was obtained by comparing isogenic strains T3SA+ and T3SA-, which differ only in the capacity to bind sialic acid, in infected mice (Barton et al. 2003b). Following oral inoculation, both T3SA+ and T3SA- cause encephalitis, but only T3SA+ leads to obstructive liver disease. Both type 1 and 3 σ1 molecules bind carbohydrates, but the binding sites are structurally distinct and engage different carbohydrates (Chappell et al. 2000, 2002; Reiter et al. 2011). Type 1 σ1 binds to the GM2 glycan, whereas type 3 σ1 engages a broader range of glycans that terminate in α-linked sialic acid (Chappell et al. 1997; Reiss et al. 2012). High-resolution

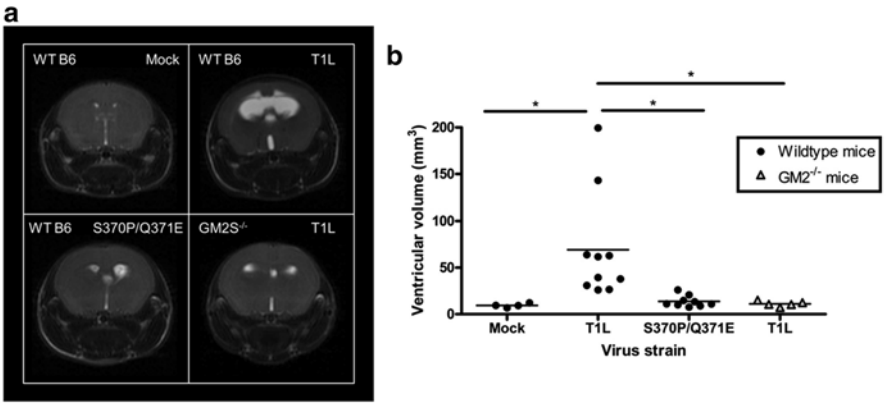

Fig. 4 T1 reovirus glycan-binding capacity influences hydrocephalus induction. Two-day-old wild-type C57BL/6 and GM2$^{-/-}$ mice were inoculated intracranially with PBS or 10^8 PFU of either wild-type T1L or a GM2-blind T1L mutant (S370P/Q371E). T2-weighted magnetic resonance images were obtained 21 days post-inoculation. (**a**) Coronal images from representative wild-type mice inoculated with PBS (*top left*), wild-type mice inoculated with T1L (*top right*), wild-type mice inoculated with the S370P/Q371E mutant (*bottom left*), and GM2$^{-/-}$ mice inoculated with T1L (*bottom right*) are shown. Cerebrospinal fluid appears white, allowing ventricular volume to be quantified. The images shown were obtained from mice with the median ventricular volume for each virus and mouse strain ($n = 4$–10 mice per group). (**b**) Ventricular volume of reovirus-infected mice. Each symbol represents the ventricular volume from a single mouse. Mean ventricular volume is indicated by a horizontal bar. *$P < 0.05$, as quantified by one-way ANOVA followed by Bonferroni's correction for multiple tests. Figure and legend modified from Stencel-Baerenwald et al. (2015)

crystal structures of type 1 and 3 σ1 have informed mutagenesis studies, which identified a number of residues required for carbohydrate engagement (Reiter et al. 2011; Reiss et al. 2012). Replacement of residues in the GM2-binding domain of T1L σ1 resulted in diminished infectivity in cultured ependymal cells, while mutagenesis of the sialic acid binding site in T3D yielded reduced infectivity in cultured neurons. Infection of newborn mice with a T1L mutant incapable of binding GM2 resulted in diminished hydrocephalus and modestly reduced titers in the brain (Stencel-Baerenwald et al. 2015). Furthermore, mice lacking an enzyme required for the synthesis of GM2 do not develop hydrocephalus following infection with type 1 reovirus (Fig. 4). Analogously, a T3D mutant incapable of binding sialic acid resulted in diminished encephalitis, reduced titers in the brain, and increased rates of survival (Barton et al. 2003b; Frierson et al. 2012). No differences were observed in the CNS tropism of carbohydrate binding mutants compared with wild-type strains (Stencel-Baerenwald et al. 2015; Frierson et al. 2012). These findings suggest that the low-affinity interactions with glycans on the cell surface enhance the efficiency of reovirus infection in vitro but do not independently explain serotype-specific differences in reovirus tropism in the CNS. The identification of additional receptors may shed light on the molecular basis of serotype-specific CNS tropism and disease.

Reovirus-Induced Neuronal Injury

Apoptosis is the chief mechanism of neuronal cell death induced by type 3 reovirus strains (Oberhaus et al. 1997). In areas of the brain infected by reovirus, neurons display condensed nuclei, stain positive for terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL), and are immunoreactive to antiserum detecting the cleaved (activated) form of the executioner caspase, caspase-3 (Oberhaus et al. 1997; Richardson-Burns et al. 2002; Richardson-Burns and Tyler 2004; Danthi et al. 2008a, 2010a; Pruijssers et al. 2013; O'Donnell et al. 2005) (Fig. 5). Neuronal apoptosis is a consequence of viral infection and is observed at early times postinfection in the absence of inflammation (Tyler and Fields 1996). An influx of inflammatory cells is observed at later time points and coincides with an exacerbation of neurologic injury (Danthi et al. 2010a; O'Donnell et al. 2005).

Cultures of primary cortical neurons established from embryonic mice provide a tractable system for studies of reovirus infection of neurons. These cultures recapitulate infection of neurons in vivo in several ways. First, neurons are susceptible to infection with type 3 but not type 1 reovirus (Antar et al. 2009). Second, infection efficiency depends on glycan binding, as neuraminidase treatment or alteration of the σ1 sialic acid binding site reduces infectivity (Antar et al. 2009; Frierson et al. 2012). Third, cortical neurons undergo apoptosis following type 3 reovirus infection as detected by DNA fragmentation, cell membrane integrity and chromatin condensation (EB/AO), TUNEL, increased translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane, and increased levels of activated caspase-3 (Richardson-Burns et al. 2002; Pruijssers et al. 2013). Infection of cultured neurons is considerably more efficient when virions are digested to ISVPs in vitro prior to adsorption (Antar et al. 2009; Pruijssers et al. 2013). The molecular basis for this observation is not well understood, but it is possible that sorting in the endocytic compartment or cathepsin activity is not as efficient in neurons relative to other types of cells.

Although reovirus primarily infects CNS neurons in vivo, CNS infection activates pro-inflammatory cytokine and chemokine-producing nonneuronal cells that likely augment neuronal apoptosis and mediate recruitment of leukocytes into the CNS (Tyler 1998; Tardieu et al. 1983). In the CNS, neurons are embedded in a matrix of nonneuronal cells including astrocytes, oligodendrocytes, and microglia. Astrocytes, the predominant glial cell type in the brain, control cerebral blood flow and provide structural support and nutrients to neurons (Rossi and Volterra 2009; Tower and Young 1973). Oligodendrocytes form layers around neuronal axons and produce myelin, which insulates axons and enhances action potential transmission. Microglia are the resident macrophages of the brain and carry out surveillance of the brain parenchyma. Detection of pathogens or other types of damage activates the microglia, which leads to upregulation of the antigen presentation machinery, induction of nitric oxide, production of pro-inflammatory cytokines, and activation of other microglia and astrocytes. Activated microglia also phagocytize apoptotic neurons and promote repair of damaged tissue (Brown and Neher 2010). The inducible

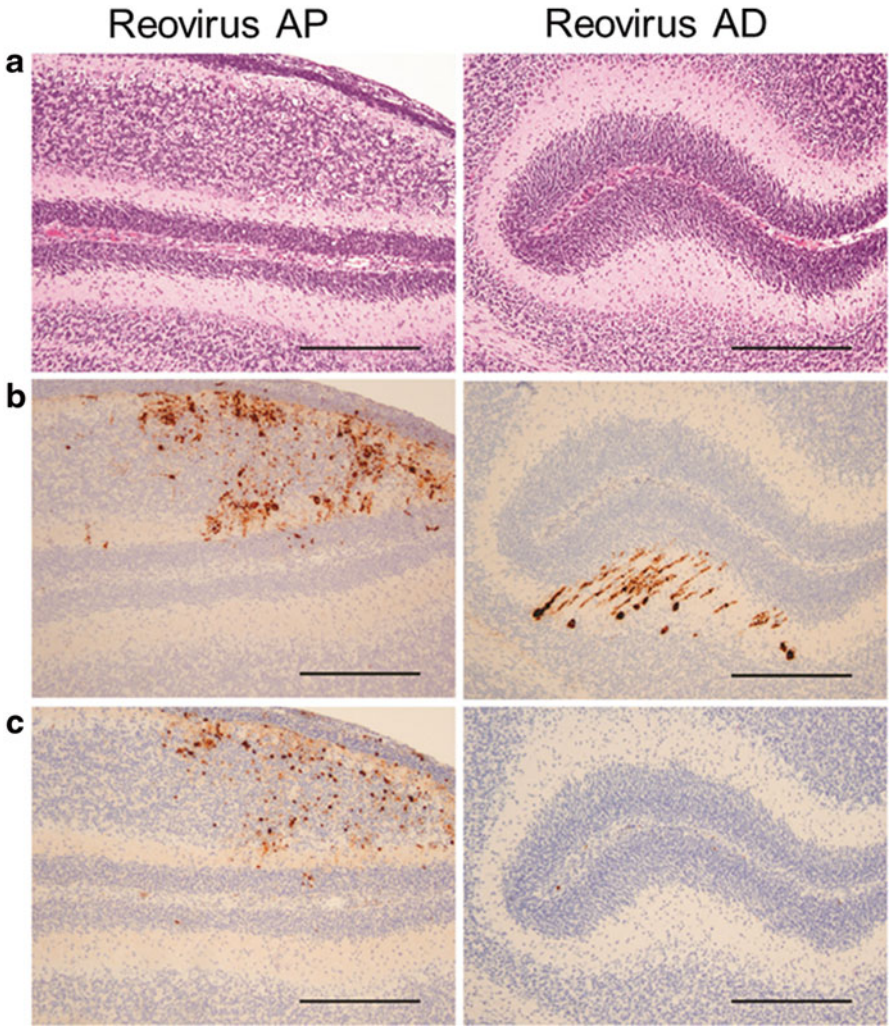

Fig. 5 Reovirus induces apoptosis of CNS neurons. Two-day-old wild-type C57BL/6 mice were inoculated intracranially with 40 PFU of apoptosis-proficient strain AP (*left* column) or apoptosis-deficient strain AD (*right* column). At 8 days post-inoculation, brains were removed, and the right hemispheres were processed for immunohistochemistry. Consecutive coronal sections of the brain were stained with (**a**) hematoxylin and eosin, (**b**) polyclonal reovirus antiserum, and (**c**) an antibody specific for the cleaved (active) form of caspase-3 as a marker for apoptosis. Shown is a 10× magnification of a region of cerebellum (scale bars, 500 μm). Figure and legend modified from Pruijssers et al. (2013)

form of nitric oxide (iNOS) serves an antiviral function in brains infected with type 3 reovirus. iNOS also is detected following infection of neuronal and microglial cultures. Reovirus infects neurons and microglia but not astrocytes in vitro, yet only neurons undergo apoptosis (Richardson-Burns and Tyler 2004; Goody et al. 2005).

The precise role of microglia and astrocytes in the response to reovirus infection is unknown. However, the detection of pro-inflammatory cytokines at early times postinfection when inflammatory infiltrates are absent suggests that these cells function in the early response to reovirus infection (Derrien and Fields 1999).

Inflammatory cytokines contribute to reovirus neuropathogenesis. A significant increase in interleukin-1alpha (IL-1α) is detected in the brains of newborn mice inoculated with a lethal dose of type 3 strain T3C9. Survival is enhanced following injection of neutralizing monoclonal antibodies specific for IL-1α into brain of infected mice, suggesting that this pro-inflammatory cytokine mediates lethal encephalitis caused by reovirus (Derrien and Fields 1999). Pro-inflammatory cytokine IL-6 and chemokines CXCL10, KC, and RANTES are detected following reovirus infection of brain-slice cultures (BSCs), an ex vivo model of infection (Dionne et al. 2011).

Gene-expression screens conducted to identify effectors of reovirus apoptosis and CNS injury have yielded a large number of additional potential mediators of reovirus neuropathogenesis. Comparison of expression profiles after infection of cultured cells with T3A, which induces apoptosis, and T1L, which does not, identified several genes involved in apoptosis and DNA repair (DeBiasi et al. 2003). An RNA microarray screen comparing reovirus-induced gene expression in the presence or absence of an intact nuclear factor kappa B (NF-κB) pathway identified a number of NF-κB-dependent genes involved in the antiviral innate immune response and apoptosis pathways (O'Donnell et al. 2006). Another microarray screen assessing the influence of host translation shutoff revealed a role for the integrated cellular stress response in reovirus pathogenesis (Smith et al. 2006). A fourth microarray study comparing RNA extracted from infected and uninfected brain tissue at 3, 6, and 8 days after inoculation revealed upregulation of mainly innate immune signaling pathway components at the early time post-inoculation, followed by the upregulation of additional pathways, including apoptosis and cytokine signaling pathways at later times post-inoculation (Tyler et al. 2010). The specific genes identified in these microarray screens share little in common. However, genetic networks connected to innate immune signaling, DNA repair, and apoptosis were identified in all. Validation of the candidates identified in these screens could lead to the identification of drug targets for the treatment of viral encephalitis.

Mechanisms of Reovirus Apoptosis

Consistent with observations made in studies of reovirus infection in the murine CNS, reovirus causes apoptosis in many types of cultured cells. Experiments using primary and immortalized cell cultures along with in vivo pathogenesis studies have yielded detailed insights into mechanisms underlying reovirus-induced apoptosis and identified components of the host innate immune system and viral factors required for this process.

Innate Immune Response to Reovirus Infection

Early events during reovirus infection trigger activation of several key innate immune signaling pathways including NF-kB, interferon regulatory factor-3 (IRF-3), and mitogen-activated protein kinase (MAPK), culminating in inhibition of viral replication and protection or induction of apoptosis, depending on the viral strain and cellular context (O'Donnell et al. 2005; Dionne et al. 2011; Connolly et al. 2000; Hansberger et al. 2007; Holm et al. 2007; Clarke et al. 2001a; Goody et al. 2007).

Activation of the classical NF-kB signaling pathway plays a prominent role in apoptosis induction and reovirus encephalitis. NF-κB activation is triggered by the φ fragment of μ1 protein following viral penetration of the endosomal membrane and release of the transcriptionally active core into the cytoplasm. These viral entry events lead to activation of an inhibitor kappa B kinase (IKK) complex comprised of IKKα and IKKγ/NEMO, which in turn phosphorylates inhibitor kappa B alpha (IκBα) (Connolly et al. 2000; Hansberger et al. 2007). Phosphorylation and ubiquitylation of IκBα targets it for proteasomal degradation, allowing heterodimers of p50 and p65/RelA to translocate into the nucleus and activate expression of NF-kB-dependent genes. Nuclear translocation of the NF-κB p50 and p65/RelA heterodimer occurs in a biphasic pattern, starting as early as 4 h postinfection. New complexes containing p50, p65/RelA, and c-Rel appear in the nucleus at 16 h postinfection (Hansberger et al. 2007). NF-κB is activated following infection with both type 1 and type 3 reovirus strains. However, following infection with type 1 strains, the activation of NF-κB in the first phase is reduced relative to type 3 strains, and the second phase of NF-κB activation is inhibited (Clarke et al. 2005). Diminished expression of components of the NF-κB pathway in mouse embryonic fibroblasts (MEFs) by genetic depletion or RNA interference or expression of a dominant-negative form of IkB in HeLa cells diminishes reovirus-induced apoptosis (Connolly et al. 2000; Hansberger et al. 2007), indicating an important function for NF-κB-dependent genes in reovirus-induced apoptotic injur. NF-κB p50-null mice, which do not express a functional p50 subunit in any cell type or tissue, are protected from reovirus-induced encephalitis following infection. While viral loads are comparable in brains of infected wild-type and p50-null mice, brains of infected p50-null mice display a marked decrease in the number of apoptotic neurons compared with brains of wild-type mice. These data provide evidence that NF-κB signaling is required for neuronal apoptosis and resultant reovirus encephalitis (O'Donnell et al. 2005).

In addition to NF-κB, reovirus-dependent activation of the MAPK pathway is observed in both cultured cells and the infected host. Infection of cultured cells induces the phosphorylation and activation of c-Jun N-terminal kinase (JNK) and recruitment of Fas-associated death domain protein (FADD), leading to the nuclear translocation of transcription factor c-Jun. JNK activation is required for the upregulation of Fas, which initiates caspase-8-dependent apoptosis (Clarke et al. 2009). Inhibition of JNK in the CNS reduces apoptosis of neurons and increases survival following reovirus infection (Beckham et al. 2007).

Reovirus infection stimulates transcription factor IRF-3 by activating RNA helicase retinoic acid-inducible gene 1 (RIG-I) in the cytosol. Activated RIG-I signals via adaptor protein interferon-β promoter stimulator 1 (IPS-1) to induce the nuclear translocation of IRF-3 homodimers and promote expression and secretion of type 1 interferons (IFNs) and activation of interferon-stimulated genes (ISGs) (Holm et al. 2007). Binding of type 1 IFNs to the IFNα/β receptor (IFNAR) on the cell surface induces the phosphorylation of signal transducer and activator of transcription (STAT) 1 and STAT 2, which couple with IRF-9 to form the interferon-stimulated gene factor 3 (ISGF3) complex. ISGF3 translocates into the nucleus and promotes expression of antiviral ISGs including the antiviral protein kinase R (PKR), ribonuclease latent (RNAseL), and 2′,5′-oligoadenylate synthase (OAS-1), which inhibit protein synthesis and block viral replication (Dionne et al. 2011; Goody et al. 2007; Chawla-Sarkar et al. 2003). Although IFNs and ISGs cause apoptotic signaling in some cases (Selleri et al. 1997; Castelli et al. 1997), IFN is dispensable for reovirus-induced apoptosis in vitro (Knowlton et al. 2012). In mice, IFNβ limits viral replication and protects against myocardial injury and liver failure following reovirus infection (O'Donnell et al. 2005; Holm et al. 2010). Infection of the heart by T1L is associated with myocarditis, which is characterized by dystrophic calcification and apoptosis of cardiac myocytes. On the other hand, T3D rarely causes cardiac injury (O'Donnell et al. 2005; Hassan et al. 1965; Sherry et al. 1989; Stangl et al. 1987). The difference in heart pathology is attributed to strain-specific differences in the levels of induction of and sensitivity to IFNs, which protect against myocardial injury (O'Donnell et al. 2005; Sherry et al. 1998). Mice that do not express IFNAR succumb to intestinal injury and liver failure in less than a week following inoculation (Dionne et al. 2011). The lack of an apparent role for IFNβ in modulating CNS disease may reflect an inability of the host to control systemic viral replication and the rapid onset of disease in other vital organs.

Mechanism of Reovirus-Induced Apoptosis

Reovirus induces cell death through both the death-receptor-associated (extrinsic) and mitochondrial (intrinsic) apoptosis pathways (Kominsky et al. 2002a) (Fig. 6). Activation of the extrinsic apoptosis pathway occurs via signaling through death-domain (DD)-containing receptors. In cultured neurons, reovirus infection induces the JNK-dependent expression of TNF-related apoptosis-inducing ligand (TRAIL), which signals through the TRAIL receptor (TRAIL-R) via adaptor molecule FADD to activate initiator caspase-8, leading to apoptosis (Beckham et al. 2007; Clarke et al. 2001b). Apoptosis can be inhibited by treatment with a cell-permeable pan-caspase inhibitor or specific inhibitors of caspase-8, TRAIL-R1/TRAIL-R2, TNFR1, and FasR (Richardson-Burns et al. 2002; Danthi et al. 2010a). TRAIL-dependent stimulation of the second phase of NF-kB activation leads to cleavage of a member of the B-cell lymphoma 2 (Bcl-2) family, Bid, to form truncated t-Bid, which leads to activation of caspase-9 (Danthi et al. 2010a; Clarke et al. 2000, 2001b). Thus, Bid

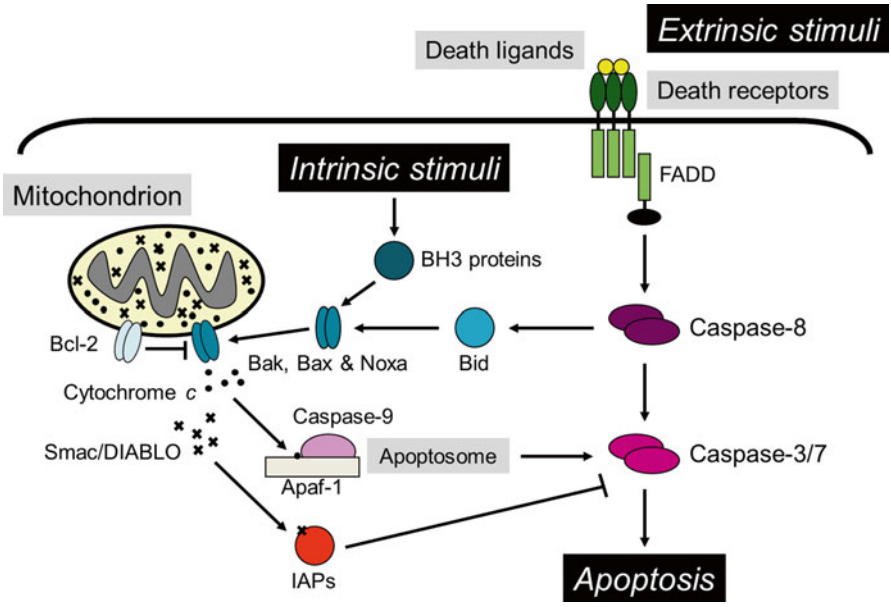

Fig. 6 Reovirus induces cell death via both the death receptor-associated (extrinsic) and mitochondrial (intrinsic) apoptosis pathway. The intrinsic apoptosis pathway is induced following penetration of the endosome and release of μ1 φ fragments into the cytoplasm. BH3-only proteins of the Bcl-2 family facilitate the release of cytochrome c and smac/DIABLO from the mitochondria, which triggers the formation of the apoptosome containing Apaf-1 and activated caspase-9 and inhibits the function of inhibitor of apoptosis proteins (IAPs). Activation of the extrinsic apoptosis pathway occurs via NF-κB-dependent signaling of death-domain (DD) ligands through DD-containing receptors, which leads to the formation of the FADD-containing death-induced signaling complex (DISC) and activation of caspase-8. TRAIL-dependent stimulation of the second phase of NF-κB activation leads to caspase-8 activation and cleavage of Bid to t-Bid, which links the extrinsic and intrinsic apoptosis pathways. Activation of caspase-8 and caspase-9 results in activation of the effector caspase-3 and apoptosis. Figure and legend are modified from Danthi (2011)

links the extrinsic apoptosis pathway with the intrinsic apoptosis pathway, thereby amplifying the apoptotic stimulus. Reovirus-induced apoptosis and neurovirulence are diminished in Bid-deficient cells and mice, suggesting that Bid modulates reovirus-induced encephalitis (Danthi et al. 2010a). Activation of the intrinsic apoptosis pathway leads to mitochondrial depolarization, release of cytochrome *c* and smac/DIABLO from the mitochondria, and activation of initiator caspase, caspase-9 (Kominsky et al. 2002a, b; Li et al. 1997). In cultured cells, anti-apoptotic Bcl-2 family member Bcl-2 inhibits apoptosis, whereas NF-kB- and IRF-3-dependent expression of a BH3-only Bcl-2 family protein, Noxa, enhances apoptosis (Knowlton et al. 2012). Pro-apoptotic Bcl-2 family member Bcl-2-associated x protein (Bax) enhances reovirus replication and apoptosis in the brain by enhancing the release of cytochrome *c* and smac/DIABLO (Berens and Tyler 2011). Upregulation of caspase 8 and Fas as well as release of cytochrome *c* and smac/DIABLO is observed

following infection of the brain, suggesting a role for both the extrinsic and intrinsic apoptosis pathways in reovirus encephalitis (Richardson-Burns et al. 2002; Clarke et al. 2009).

Studies using cultured cells suggest that non-apoptotic cell death pathways also are activated by reovirus under some conditions. Blockade of either NF-κB activation or caspase cleavage activates RIP1 kinase, which leads to a non-apoptotic type of cell death called necroptosis (Berger and Danthi 2013). Autophagy-mediated cell death, a process in which cellular components are degraded by lysosomes, has been implicated in a cell culture model of multiple myeloma (Thirukkumaran et al. 2013). The function of non-apoptotic cell death pathways in reovirus encephalitis has yet to be determined.

Viral Determinants of Apoptosis

Reovirus particles are composed of two concentric shells forming an outer capsid and an inner core. The inner core surrounds the segmented dsRNA genome of approximately 23,500 base pairs (Dryden et al. 1993; Reinisch et al. 2000). The gene segments range from 1196 to 3916 base pairs and are classified into three groups on the basis of size: four small (S), three medium (M), and three large (L) (Shatkin et al. 1968; Ramig et al. 1977). Each segment encodes a single protein, except for S1, which encodes two proteins in overlapping open reading frames, σ1 and σ1s. Virions display icosahedral symmetry and are approximately 85 nm in diameter. The outer capsid is comprised of three proteins, σ1, σ3, and μ1. Attachment protein σ1 extends from the fivefold axis formed by pentamers of core-spike protein λ2 (Dryden et al. 1993). Following receptor binding and integrin-mediated entry of virions into endosomes, the outer-capsid protein σ3 is removed, and μ1 is cleaved into δ and φ. Conformational changes in δ release the myristoylated N-terminal domain (μ1N) and the C-terminal φ fragment, leading to disruption of the endosomal membrane and cytoplasmic release of the transcriptionally active core, along with the μ1 cleavage fragments (Odegard et al. 2004; Nibert and Fields 1992; Chandran et al. 2002, 2003; Nibert et al. 1991) (Fig. 1).

Apoptotic potential is genetically linked to the S1 and M2 gene segments, which encode viral attachment protein σ1 and nonstructural protein σ1s (S1) and outer-capsid protein μ1 (M2). The mechanistic association between S1 and apoptosis has not been fully elucidated. However, binding to sialic acid by σ1 enhances NF-kB-dependent apoptosis induction independent of viral protein synthesis or the production of viral progeny, suggesting that sialic acid engagement activates cellular signaling pathways leading to apoptosis (Connolly et al. 2001). Since viral disassembly also is required for apoptosis induction by reovirus in cultured cells (Connolly and Dermody 2002), subtle differences in σ1 structure may influence interactions of σ1 with other outer-capsid proteins, which in turn could influence capsid disassembly. Susceptibility of outer-capsid protein σ3 to cleavage by cellular proteases influences capsid stability, which in turn dictates the kinetics of viral uncoating. Alteration

of capsid stability strikingly enhances reovirus virulence in vivo (Doyle et al. 2015). Mice inoculated with a virus containing a single amino-acid mutation in σ3 (Y354H) that reduces capsid stability and accelerates viral disassembly succumb to infection at a higher frequency relative to mice inoculated with wild-type virus. Viral loads in key target organs were higher at early times postinfection, and myocarditis was exacerbated and accompanied by an increase in inflammatory cytokines (Doyle et al. 2015). Interestingly, the capsid-stability mutant also was transmitted more efficiently between hosts, providing no evidence for a fitness cost for the virion-destabilizing mutations described in this study.

Ectopic expression of the μ1φ domain leads to NF-κB activation and apoptosis, demonstrating that this portion of μ1 is sufficient for the initiation of apoptotic signaling (Coffey et al. 2006). Mutations in the portion of the μ1φ domain predicted to interact with membranes have yielded viruses with reduced capacity to penetrate endosomal membranes, activate NF-κB, and induce apoptosis (Danthi et al. 2008a). Some mutations uncouple membrane penetration from apoptosis induction, suggesting that φ triggers apoptotic signaling following release of the virus from the endosome (Danthi et al. 2008a). Apoptosis-reducing mutations in φ increase survival rates and reduce neural injury but do not alter viral replication in the CNS. Together, these studies indicate that viral uncoating is a key determinant of reovirus-induced myocarditis and encephalitis.

Relationship Between Viral Replication and Apoptotic Capacity

The capacity of reovirus to replicate in the CNS is linked to apoptotic capacity and neurovirulence. One study found that reovirus strain variant K (VarK), which differs from T3D by a single amino acid substitution in the σ1 head domain, produces lower titers and causes less apoptosis compared with T3D in the cortex while displaying no differences in replication or apoptosis in the hippocampus (Richardson-Burns and Tyler 2004). These differences are partly reversed when neuronal apoptosis by the wild-type strain is inhibited by the pan-caspase inhibitor ZVAD-FMK or when neuronal apoptosis is triggered using an antibody that binds and activates the Fas receptor in brains of mice inoculated with VarK. Two other studies show that mutant viruses with apoptosis-diminishing single amino acid substitutions in φ replicate to lower titers in the CNS compared with their fully apoptotic wild-type counterparts (Danthi et al. 2008a, b; Pruijssers et al. 2013). Conversely, genetic disruption of host determinants of reovirus-induced apoptosis diminished both apoptosis and viral replication in the CNS (Danthi et al. 2010a; O'Donnell et al. 2005; Holm et al. 2010). Increases in apoptotic capacity and viral replication expand CNS tropism in some cases (Pruijssers et al. 2013; Dionne et al. 2011) but not others (Richardson-Burns and Tyler 2004; Danthi et al. 2008a, b, 2010a; O'Donnell et al. 2005; Beckham et al. 2007). It is not clear whether the relationship between viral replication and neuronal apoptosis is causal and how specifically the two properties are related. Perhaps activation of signaling pathways that eventually

lead to apoptosis could promote expression of host factors that promote viral replication. Alternatively, the induction of apoptosis may enhance reovirus release from neurons and facilitate initiation of new rounds of replication in surrounding cells (Pruijssers et al. 2013). Future studies may identify host factors that link the signaling pathways involved in viral replication and apoptosis.

Does Encephalitis Enhance Viral Fitness?

Despite the large body of available data describing mechanisms of reovirus neuronal injury, it is not clear why reovirus would cause encephalitis. From the host perspective, apoptotic cell death is a mechanism to limit viral spread, which would be preferred over necrotic cell death presumably because this death mechanism yields a more subdued inflammatory response. However, apoptosis of non-regenerating cells such as neurons is detrimental to the host, and as a result, infected neonates develop neurological disease with significant morbidity and mortality. Apoptosis induction appears to benefit the virus, as viral replication is reduced in cells lacking essential apoptosis mediators (Danthi et al. 2010a; Pruijssers et al. 2013; Connolly et al. 2000; Berens and Tyler 2011). Moreover, viral mutants that evoke apoptosis less efficiently also replicate less efficiently (Pruijssers et al. 2013; Danthi et al. 2008b). Yet, if fitness is measured by the efficiency with which a virus is transmitted between hosts, killing the host would be beneficial to viral fitness only in cases in which disease and death directly contribute to transmission. Considering the fecal-oral transmission route, inducing a lethal encephalitis is unlikely to enhance reovirus transmission. It has been hypothesized that neuronal apoptosis and neuropathogenesis are an inconsequential side effect of a trait that enhances transmission. Apoptosis is observed in the intestinal epithelium (Fleeton et al. 2004); however, the finding that an apoptosis-proficient virus displays a reduced capacity to transmit to uninfected hosts compared with an apoptosis-deficient virus suggests that apoptotic capacity is detrimental to viral fitness (Pruijssers et al. 2013). Thus, it is possible that apoptotic capacity and neuronal targeting are not evolutionarily desirable traits for reovirus and may be manifestations of infection of a unique host population, the very young.

Age Restriction of Reovirus Encephalitis

Reovirus can replicate in the intestine of mice of all ages, from neonates to adults. However, the efficiency of reovirus replication and dissemination and the capacity to induce disease declines substantially during the first few weeks of life (Tardieu et al. 1983). Mechanisms underlying the age-restriction to reovirus disease are largely unknown, but studies addressing this phenomenon have suggested a contribution for both cell-extrinsic and cell-intrinsic factors.

The age-window for the decline in susceptibility of mice to infection with many viruses coincides with a number of changes occurring in the CNS, including growth of axons and formation of synapses, changes in blood–brain barrier (BBB) permeability, and maturation of immune responses required to control viral infection. The BBB of newborn mice is more permeable than that in adult mice, in which the BBB poses a formidable barrier for virus penetration into the brain parenchyma. The immaturity of adaptive immune responses also could contribute to the increased virulence of reovirus in neonates. Major changes in adaptive immunity occur in the first 2 weeks in mice, which mirrors the interval during which reovirus virulence declines. Although adaptive immune responses are functional in newborn mice, the scope and magnitude of the responses required to limit viral replication and clear infected cells may be insufficient. Newborn mice do not have immunologic memory, and the numbers of circulating immune cells are relatively low. In addition, the T cell response in newborn mice is strongly skewed towards a Th2 phenotype, which is less effective at clearing viral infections (Garcia et al. 2000). Finally, neonatal B cell responses develop more slowly and reach lower peak titers, and the antibodies produced display lower affinity and reduced heterogeneity than those in adults (Aranda et al. 1993).

The role of adaptive immune responses in controlling reovirus infection following oral inoculation has been studied using mice lacking $CD8^+$ T-cell responses, mice without antigen-specific lymphocytes (SCID mice), and mice lacking B-cell responses (MuMT mice) (Barkon et al. 1996). Viral clearance from the intestine is delayed in adult B- or antigen-specific lymphocyte-deficient mice but unaltered in mice lacking $CD8^+$ T-cells (Barkon et al. 1996). Depletion of $CD4^+$ and $CD8^+$ T-cells had little effect on clearance of reovirus from the intestines of newborn mice (Virgin and Tyler 1991). Adult SCID mice infected with reovirus succumbed to liver disease. However, partial protection was achieved by adoptive transfer of PP cells from congenic mice (George et al. 1990), polyclonal reovirus-immune spleen cells, or antireovirus IgG (Barkon et al. 1996). These data suggest that the reovirus-specific adaptive immune response contributes to reduced susceptibility to reovirus infection in adults.

Like adaptive immune responses, innate immune responses also require maturation (Ginaldi et al. 1999). For example, the magnitude of microglial activation in the CNS and the production of pro-inflammatory cytokines decreases with age (Ferrazzano et al. 2013; Inamizu et al. 1985). This observation is in line with the finding that the expression of the inflammatory cytokine, IL-1α following reovirus infection is increased in the neonatal brain compared with the adult brain (Derrien and Fields 1999). The innate immune sensor TLR3 is upregulated in the intestine in association with age (Pott et al. 2012). TLR3 signaling plays an essential role in triggering the production of cytokines involved in modulating the immune response to rotavirus infection. Therefore, an increase in TLR3 expression in adults relative to newborn mice provides an attractive explanation for the increased susceptibility of neonates to rotavirus infection. Similar mechanisms could underlie reovirus age-restriction.

Regardless of cell-extrinsic factors, age-dependent changes in the abundance or activation status of cell-intrinsic factors essential for viral entry, replication, assembly, and release also could contribute to reovirus age-restriction. For example, cell-surface expression of reovirus receptors may decrease with age, thus reducing reovirus binding to target cells. Myelination of axons in the CNS in mice is initiated at postnatal days 7–9 (Tessitore and Brunjes 1988), which coincides with a sharp drop in reovirus disease (Tardieu et al. 1983). Myelination could limit access to reovirus receptors including NgR1, which is ligated by myelin-associated proteins. Specific cellular pathways required for reovirus replication also might undergo some type of age-dependent maturation. The selective replication of reovirus in transformed cells with an activated Ras pathway suggests that activated Ras signaling can enhance reovirus infection (Hashiro et al. 1977; Duncan et al. 1978; Strong et al. 1998; Coffey et al. 1998). Thus, changes in Ras signaling could contribute to reovirus age-restriction.

Perspectives

Although many discoveries have been made in studies of reovirus infection and neuropathogenesis, many questions remain unanswered. For example, it is still unknown how reovirus traverses the BBB and transits from neuron to neuron to produce the highly reproducible antigen signature observed in histological sections of type 3 reovirus-infected brains. In addition, cellular factors that account for the tropism differences displayed by reovirus type 1 and 3 in the nervous system have yet to be identified. Our understanding of the array of molecules mediating reovirus-induced neuronal apoptosis and brain injury is incomplete. In addition to providing a more satisfying understanding of how reovirus causes encephalitis, answers to these questions might allow strategic reengineering of reovirus to improve its potency as an oncolytic therapeutic for CNS neoplasms.

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