4

Neurotropic coronavirus infections

Stanley Perlman and Noah Butler

Introduction/classification

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

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

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

Virus structure

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

Table 4.1. Neurotropic coronaviruses

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

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

Genome organization

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

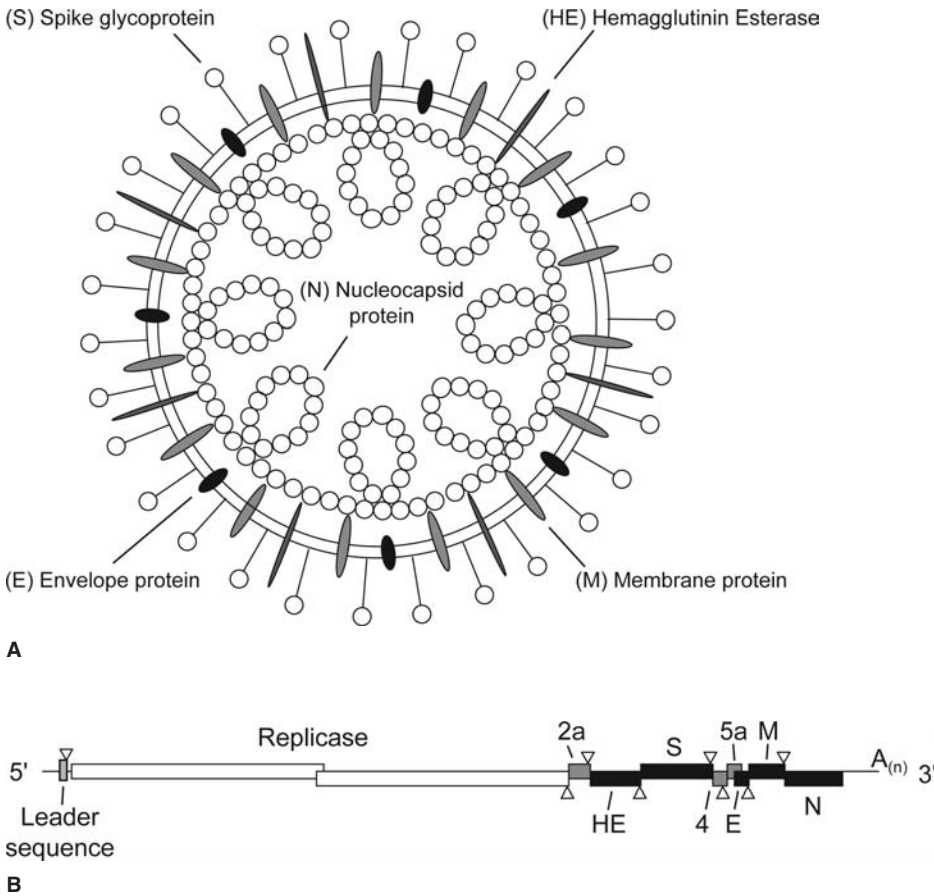


Figure 4.1. (A) Structure of the MHV coronavirus virion depicting structural proteins. (B) Schematic representation of the genomic organization of mouse hepatitis virus. Replicase genes, accessory genes, and structural genes are denoted by open, shaded, and closed rectangles, respectively. Open triangles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

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

Life cycle

Cell entry

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

Table 4.2. Receptors utilized by neurotropic coronaviruses

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

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

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

Genome replication

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

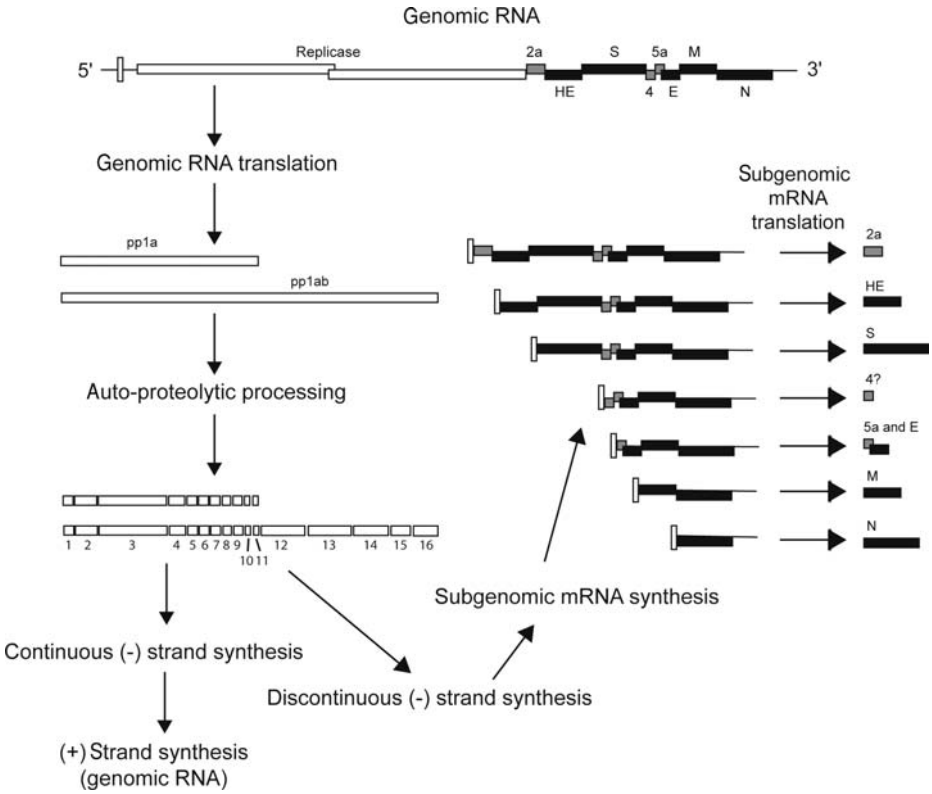


Figure 4.2. Overview of coronavirus replication. Upon uncoating, virus RNA is directly translated via host cell machinery into two large polyproteins, pp1a and pp1ab, the latter a result of a ribosomal frameshift during translation of pp1a. Both polyproteins undergo autoproteolytic processing to generate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

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

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

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

Transcription

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

Virus assembly and egress

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

Coronavirus reverse genetics

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

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

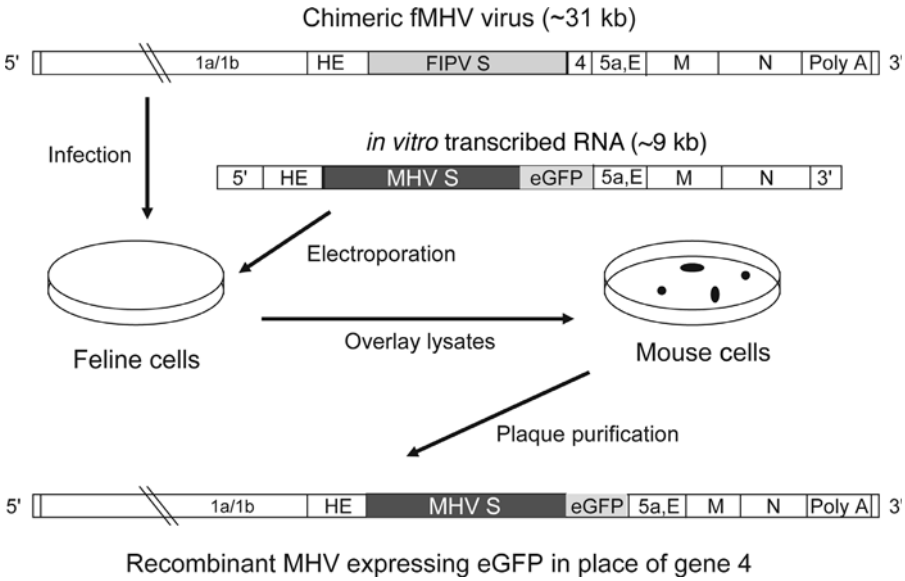


Figure 4.3. Strategy of targeted recombination for generating recombinant MHV variants. Feline tissue culture cells are first infected with a recombinant, chimeric MHV expressing the feline S gene (fMHV). Four hours after inoculation, these cells are electroporated/transfected with a synthetic (*in vitro*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

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

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

Transmission and epidemiology

Intraspecies transmission

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

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

Interspecies transmission

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

Pathogenesis of MHV-induced disease

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

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

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

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

CNS cell tropism and virus spread

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

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

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

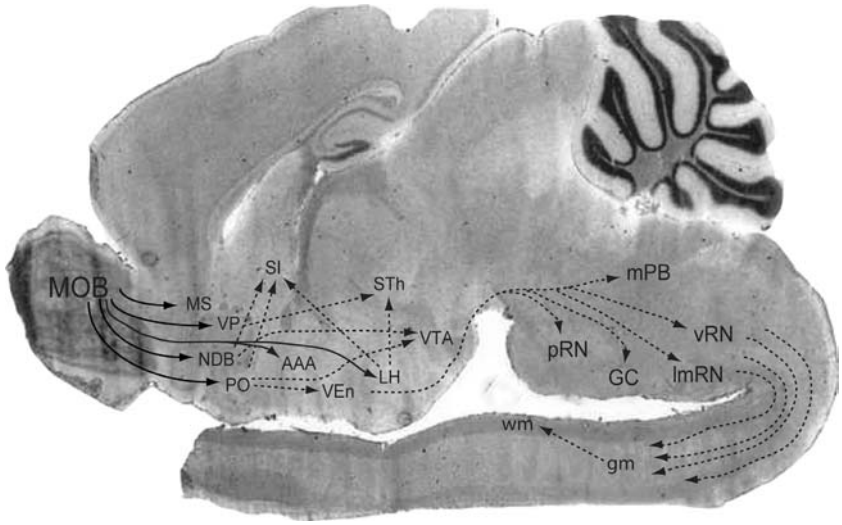


Figure 4.4. Schematic representation of MHV spread in the CNS. Upon intranasal inoculation, MHV initially replicates in the main olfactory bulb (MOB) and nerve. Dissemination to distal parts of the brain and spinal cord occurs via retrograde spread along neuronal tracts that comprise the primary (solid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

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

Acute encephalitis mediated by MHV-JHM

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

Persistent CNS infection by MHV-JHM

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

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

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

Other experimental models of MHV infection

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

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

Role of the MHV S protein in pathogenesis

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

Innate immune response to MHV infection

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

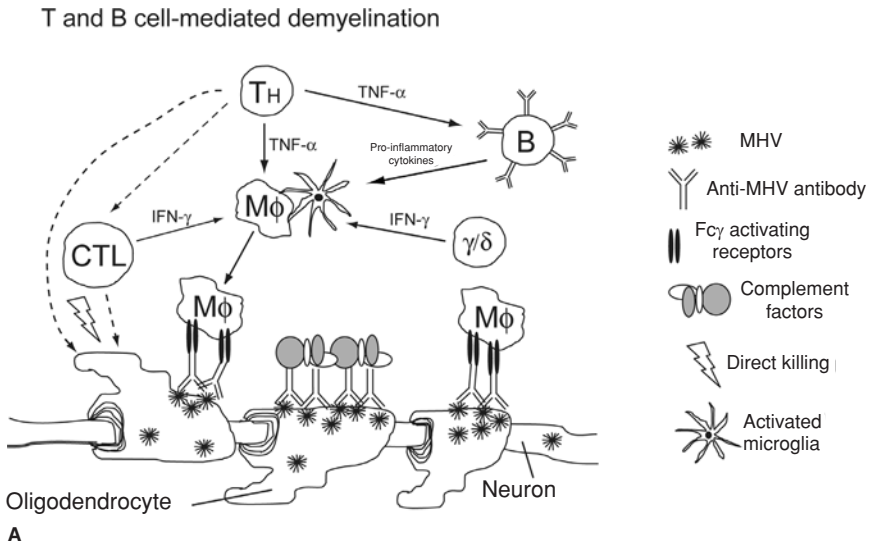
(termed Alb274) resulted in reduced virus titers, enhanced infiltration of NK cells, and protection from acute disease, suggesting that CXCL10 can also recruit natural killer (NK) cells, which may contribute to virus clearance in the absence of T cells [131]. In contrast to a protective role during acute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

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

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

Adaptive immune response to MHV infection

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



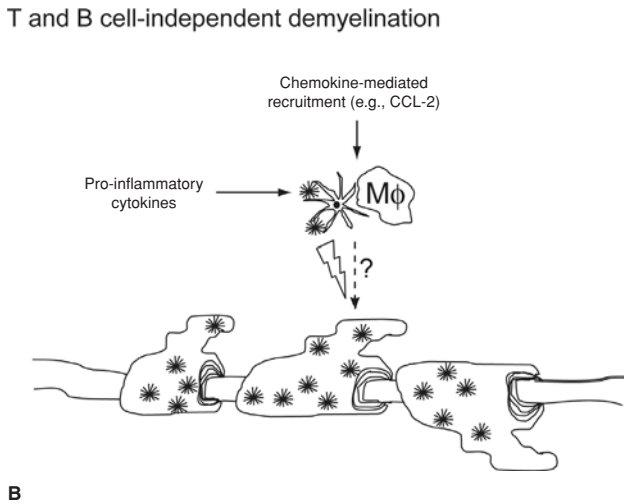


Figure 4.5. Schematic representation of the host-specific factors and cell types that contribute to demyelination in the infected CNS. (A) Intranasal or intracerebral MHV infection initiates an inflammatory cascade that results in the recruitment of CD8 (CTL) and CD4 (T_H) T cells, B cells, γ/δ T cell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

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

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

CD8 T cell responses

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

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

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

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

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

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

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

CD4 T cell responses

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

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

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

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

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

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

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

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

Antibody responses

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

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

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

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

Host-specific factors that influence demyelination

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

Adaptive immune cells

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

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

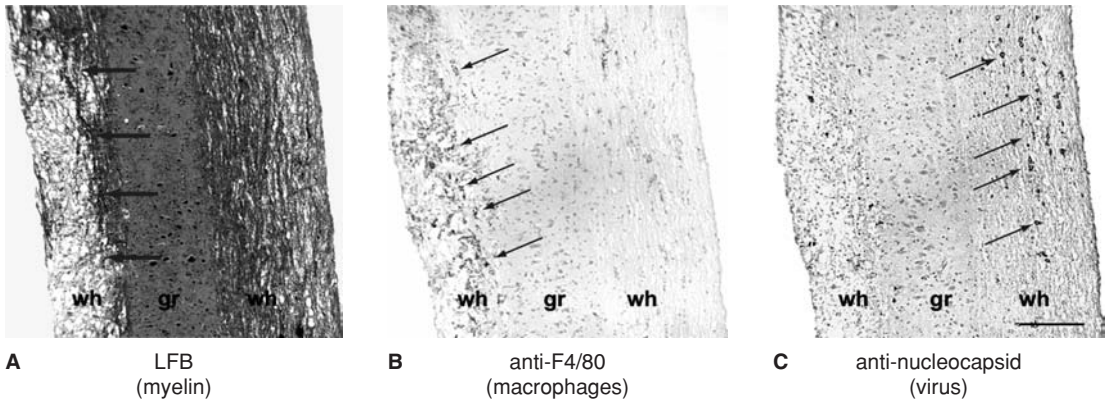


Figure 4.6. Representative serial sections of an MHV-infected spinal cord demonstrating loss of myelin (A), macrophage infiltration (B), and presence of virus antigen (C). Luxol-Fast Blue (LFB) specifically stains myelin that comprises the white matter (wm), and regions of demyelination are denoted by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

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

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

Anti-MHV antibody

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

Innate immune factors

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

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

Murine infection with human coronaviruses

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

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

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

Conclusions and future directions

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

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

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

REFERENCES

[1] Gorbalenya, A.E., Snijder, E.J., and Spaan, W.J., J Virol, 78 (2004) 7863.
[2] Ksiazek, T.G., Erdman, D., Goldsmith, C.S., *et al.*, N Engl J Med, 348 (2003) 1953.
[3] Peiris, J.S., Lai, S.T., Poon, L.L., *et al.*, Lancet, 361 (2003) 1319.

[4] Compton, S.R., Barthold, S.W., and Smith, A.L., Lab Anim Sci, 43 (1993) 15.
[5] Homberger, F.R., Barthold, S.W., and Smith, A.L., Lab Anim Sci, 42 (1992) 347.
[6] Burks, J., DeVald, B., Jankovsky, L., *et al.*, Science, 209 (1980) 933.
[7] Tanaka, R., Iwasaki, Y., and Koprowski, H., J Neurol Sci, 28 (1976) 121.
[8] Murray, R.S., Brown, B., Brian, D., *et al.*, Ann Neurol, 31 (1992) 525.
[9] Stewart, J.N., Mounir, S., and Talbot, P.J., Virology, 191 (1992) 502.
[10] Gu, J., Gong, E., Zhang, B., *et al.*, J Exp Med, 202 (2005) 415.
[11] Xu, J., Zhong, S., Liu, J., *et al.*, Clin Infect Dis, 41 (2005) 1089.
[12] Lee, D.T., Wing, Y.K., Leung, H.C., *et al.*, Clin Infect Dis, 39 (2004) 1247.
[13] Masters, P.S., Adv Virus Res, 66 (2006) 193.
[14] Shi, S.T. and Lai, M.M., Curr Top Microbiol Immunol, 287 (2005) 95.
[15] Godeke, G.J., de Haan, C.A., Rossen, J.W., *et al.*, J Virol, 74 (2000) 1566.
[16] Hurst, K.R., Kuo, L., Koetzner, C.A., *et al.*, J Virol, 79 (2005) 13285.
[17] Baudoux, P., Carrat, C., Besnardeau, L., *et al.*, J Virol, 72 (1998) 8636.
[18] Yu, X., Bi, W., Weiss, S.R., *et al.*, Virology, 202 (1994) 1018.
[19] Kuo, L. and Masters, P.S., J Virol, 77 (2003) 4597.
[20] Vlasak, R., Luytjes, W., Leider, J., *et al.*, J Virol, 62 (1988) 4686.
[21] de Haan, C.A., Masters, P.S., Shen, X., *et al.*, Virology, 296 (2002) 177.
[22] Schwarz, B., Routledge, E., and Siddell, S.G., J Virol, 64 (1990) 4784.
[23] Kazi, L., Lissenberg, A., Watson, R., *et al.*, J Virol, 79 (2005) 15064.
[24] Lai, M.M.C. and Cavanagh, D., Adv Virus Res, 48 (1997) 1.
[25] Ontiveros, E., Kim, T.S., Gallagher, T.M., *et al.*, J Virol, 77 (2003) 10260.
[26] Sperry, S.M., Kazi, L., Graham, R.L., *et al.*, J Virol, 79 (2005) 3391.
[27] Huang, C., Narayanan, K., Ito, N., *et al.*, J Virol, 80 (2006) 210.
[28] Schaecher, S.R., Mackenzie, J.M., and Pekosz, A., J Virol, 81 (2007) 718.
[29] Ito, N., Mossel, E.C., Narayanan, K., *et al.*, J Virol, 79 (2005) 3182.
[30] Williams, R.K., Jiang, G., and Holmes, K.V., Proc Natl Acad Sci USA, 88 (1991) 5533.
[31] Chen, D., Asanaka, M., Yokomori, K., *et al.*, Proc Natl Acad Sci, 92 (1995) 12095.
[32] Hemmila, E., Turbide, C., Olson, M., *et al.*, J Virol, 78 (2004) 10156.
[33] Frana, M.F., Behnke, J.N., Sturman, L.S., *et al.*, J Virol, 56 (1985) 912.
[34] Huang, I.C., Bosch, B.J., Li, F., *et al.*, J Biol Chem, 281 (2006) 3198.
[35] Kubo, H., Yamada, Y.K., and Taguchi, F., J Virol, 68 (1994) 5403.
[36] Suzuki, H. and Taguchi, F., J Virol, 70 (1996) 2632.
[37] Taguchi, F., Kubo, H., Takahashi, H., *et al.*, Virology, 208 (1995) 67.
[38] Kooi, C., Cervin, M., and Anderson, R., Virology, 180 (1991) 108.
[39] Nash, T. and Buchmeier, M.J., Virology, 233 (1997) 1.
[40] Vlasak, R., Luytjes, W., Spaan, W., *et al.*, Proc Natl Acad Sci USA, 85 (1988) 4526.
[41] Li, W., Moore, M.J., Vasilieva, N., *et al.*, Nature, 426 (2003) 450.
[42] Brierley, I., Digard, P., and Inglis, S.C., Cell, 57 (1989) 537.
[43] Ziebuhr, J., Snijder, E.J., and Gorbalenya, A.E., J Gen Virol, 81 (2000) 853.
[44] Snijder, E.J., Bredenbeek, P.J., Dobbe, J.C., *et al.*, J Mol Biol, 331 (2003) 991.
[45] Sawicki, S.G., Sawicki, D.L., and Siddell, S.G., J Virol, 81 (2007) 20.
[46] Vennema, H., Godeke, G-J., Rossen, J.W.A., *et al.*, EMBO J, 15 (1996) 2020.
[47] Molenkamp, R. and Spaan, W.J., Virology, 239 (1997) 78.
[48] Narayanan, K., Chen, C.J., Maeda, J., *et al.*, J Virol, 77 (2003) 2922.
[49] Enjuanes, L., Sola, I., Alonso, S., *et al.*, Curr Top Microbiol Immunol, 287 (2005) 161.
[50] Thiel, V., Herold, J., Schelle, B., *et al.*, J Gen Virol, 82 (2001) 1273.
[51] St-Jean, J.R., Desforges, M., Almazan, F., *et al.*, J Virol, 80 (2006) 3670.
[52] Yount, B., Curtis, K.M., and Baric, R.S., J Virol, 74 (2000) 10600.
[53] Baric, R.S. and Sims, A.C., Curr Top Microbiol Immunol, 287 (2005) 229.

[54] Masters, P.S., Adv Virus Res, 53 (1999) 245.
[55] Kuo, L., Godeke, G.J., Raamsman, M.J., *et al.*, J Virol, 74 (2000) 1393.
[56] Peiris, J.S., Yuen, K.Y., Osterhaus, A.D., *et al.*, N Engl J Med, 349 (2003) 2431.
[57] Peiris, J.S., Guan, Y., and Yuen, K.Y., Nat Med, 10 (2004) S88.
[58] Lipsitch, M., Cohen, T., Cooper, B., *et al.*, Science, 300 (2003) 1966.
[59] Baric, R.S., Yount, B., Hensley, L., *et al.*, J Virol, 71 (1997) 1946.
[60] Baric, R.S., Sullivan, E., Hensley, L., *et al.*, J Virol, 73 (1999) 638.
[61] Schickli, J.H., Zelus, B.D., Wentworth, D.E., *et al.*, J Virol, 71 (1997) 9499.
[62] Schickli, J.H., Thackray, L.B., Sawicki, S.G., *et al.*, J Virol, 78 (2004) 9073.
[63] Thackray, L.B. and Holmes, K.V., Virology, 324 (2004) 510.
[64] Guan, Y., Zheng, B.J., He, Y.Q., *et al.*, Science, 302 (2003) 276.
[65] Lau, S.K., Woo, P.C., Li, K.S., Proc Natl Acad Sci USA, 102 (2005) 14040.
[66] Li, W., Shi, Z., Yu, M., *et al.*, Science, 310 (2005) 676.
[67] Vijgen, L., Keyaerts, E., Moes, E., *et al.*, J Virol, 79 (2005) 1595.
[68] Bailey, O., Pappenheimer, A.M., Cheever, F.S., *et al.*, J Exp Med, 90 (1949) 195
[69] Cheever, F.S., Daniels, J.B., Pappenheimer, A.M., *et al.*, J Exp Med, 90 (1949) 181.
[70] Weiner, L.P., Arch Neurol, 28 (1973) 298.
[71] Weiner, L.P., Johnson, R.T., and Herndon, R.M., N Engl J Med, 288 (1973) 1103.
[72] Manaker, R.A., Piczak, C.V., Miller, A.A., *et al.*, J Natl Cancer Inst, 27 (1961) 29.
[73] Lavi, E., Gilden, D., Highkin, M., *et al.*, Lab Invest, 36 (1986) 130.
[74] Lavi, E., Gilden, D., Wroblewska, Z., *et al.*, Neurology, 34 (1984) 597.
[75] Stohlman, S.A., Bergmann, C.C., and Perlman, S. In R. Ahmed, I. Chen (Eds.), Persistent viral infections, John Wiley & Sons, Ltd., New York, 1998, 537.
[76] Fleming, J.O., Trousdale, M.D., El-Zaatari, F., *et al.*, J Virol, 58 (1986) 869.
[77] Lampert, P.W., Sims, J.K., and Kniazeff, A.J., Acta Neuropathol, 24 (1973) 76.
[78] Wang, F., Stohlman, S.A., and Fleming, J.O., J Neuroimmunol, 30 (1990) 31.
[79] Wu, G.F. and Perlman, S., J Virol, 73 (1999) 8771.
[80] Houtman, J.J. and Fleming, J.O., J Neurovirol, 2 (1996) 101.
[81] Morales, S., Parra, B., Ramakrishna, C., *et al.*, Virology, 286 (2001) 160.
[82] Nakagaki, K., Nakagaki, K., and Taguchi, F., J Virol, 79 (2005) 6102.
[83] Godfraind, C., Havaux, N., Holmes, K.V., *et al.*, J Neurovirol, 3 (1997) 428.
[84] Godfraind, C., Langreth, S.G., Cardellichio, C.B., *et al.*, Lab Invest, 73 (1995) 615.
[85] Gallagher, T., Buchmeier, M., and Perlman, S., Virology, 191 (1992) 517.
[86] Nash, T. and Buchmeier, M.J., Virology, 223 (1996) 68.
[87] Aloisi, F., Ria, F., and Adorini, L., Immunol Today, 21 (2000) 141.
[88] Fabry, Z., Raine, C.S., and Hart, M.N., Immunol Today, 15 (1994) 218.
[89] Hickey, W.F., Glia, 36 (2001) 118.
[90] Wang, F-I., Hinton, D., Gilmore, W., *et al.*, Lab Invest, 66 (1992) 744.
[91] Barnett, E., Cassell, M., Perlman, S., (1993) Neuroscience, 57 (1993) 1007.
[92] Lavi, E., Fishman, P.S., Highkin, M.K., *et al.*, Lab Invest, 58 (1988) 31.
[93] Sun, N. and Perlman, S., J Virol, 69 (1995) 633.
[94] Phillips, J.J., Chua, M.M., Rall, G.F., *et al.*, Virology, 301 (2002) 109.
[95] Adami, C., Pooley, J., Glomb, J., *et al.*, Virology, 209 (1995) 337.
[96] Lavi, E., Gilden, D., Highkin, M., *et al.*, J Virol, 51 (1984) 563.
[97] Rowe, C.L., Baker, S.C., Nathan, M.J., *et al.*, J Virol, 71 (1997) 2959.
[98] Zhou, J., Stohlman, S.A., Hinton, D.R., *et al.*, J Immunol, 170 (2003) 3331
[99] Ransohoff, R.M., Kivisakk, P., and Kidd, G., Nat Rev Immunol, 3 (2003) 569.
[100] Turner, B.C., Hemmila, E.M., Beauchemin, N., *et al.*, J Virol, 78 (2004) 5486.
[101] Zhou, H. and Perlman, S. J Virol, 80 (2006) 2506.
[102] Ramakrishna, C., Bergmann, C.C., Holmes, K.V., *et al.*, J Virol, 78 (2004) 7828.
[103] Bergmann, C.C., Altman, J.D., Hinton, D., *et al.*, J Immunol, 163 (1999) 3379.
[104] Nagashima, K., Wege, H., Meyermann, R., *et al.*, Acta Neuropathol, 44 (1978) 63.
[105] Sorensen, O., Perry, D., and Dales, S., Arch Neurol, 37 (1980) 478.
[106] Watanabe, R., Wege, H., and ter Meulen, V., Lab Invest, 57 (1987) 375.

[107] Barac-Latas, V., Suchanek, G., Breitschopf, H., *et al.*, Glia, 19 (1997) 1.
[108] Murray, R.S., Cai, G-Y., Hoel, K., *et al.*, Virology, 188 (1992) 274.
[109] Watanabe, R., Wege, H., and ter Meulen, V., Nature, 305 (1983) 150.
[110] Schwender, S., Imrich, H., and Dorries, R., Immunology, 74 (1991) 533.
[111] Parker, S.E., Gallagher, T.M., and Buchmeier, M.J., Virology, 173 (1989) 664.
[112] MacNamara, K.C., Chua, M.M., Phillips, J.J., *et al.*, J Virol, 79 (2005) 9108.
[113] Rempel, J.D., Murray, S.J., Meisner, J., *et al.*, Virology, 318 (2004) 381.
[114] Navas, S. and Weiss, S.R., J Virol, 77 (2003) 4972.
[115] Phillips, J.J., Chua, M.M., Lavi, E., *et al.*, J Virol, 73 (1999) 7752.
[116] Bergmann, C.C., Lane, T.E., and Stohlman, S.A., Nat Rev Microbiol, 4 (2006) 121.
[117] Lane, T.E., Asensio, V., Yu, N., *et al.*, J Immunol, 160 (1998) 970.
[118] Trifilo, M.J., Bergmann, C.C., Kuziel, W.A., *et al.*, J Virol, 77 (2003) 4004.
[119] Zhou, J., Stohlman, S.A., Atkinson, R., *et al.*, J Virol, 76 (2002) 7374.
[120] Pewe, L., Haring, J., and Perlman, S., J Virol, 76 (2002) 7329.
[121] Stohlman, S.A., Hinton, D.R., Cua, D., *et al.*, J Virol, 69 (1995) 5898.
[122] Taguchi, F., Siddell, S., Wege, H., *et al.*, J Virol, 54 (1985) 429.
[123] Garlinghouse, L.E., Jr., Smith, A.L., and Holford, T., Arch Virol, 82 (1984) 19.
[124] Pewe, L., Zhou, H., Netland, J., *et al.*, J Virol, 79 (2005) 11335.
[125] Zhou, H. and Perlman, S., J Virol, 81 (2007) 568.
[126] Versteeg, G.A., Bredenbeek, P.J., van den Worm, S.H., *et al.*, Virology, 361 (2007) 18.
[127] Cervantes-Barragan, L., Zust, R., Weber, F., *et al.*, Blood, 109 (2006) 1131.
[128] Iacono, K.T., Kazi, L., and Weiss, S.R., J Virol, 80 (2006) 6834
[129] Liu, M.T., Armstrong, D., Hamilton, T.A., *et al.*, J Immunol, 166 (2001) 1790.
[130] Liu, M.T., Chen, B.P., Oertel, P., *et al.*, J Immunol, 165 (2000) 2327.
[131] Trifilo, M.J., Montalto-Morrison, C., Stiles, L.N., *et al.*, J Virol, 78 (2004) 585.
[132] Xue, S., Sun, N., van Rooijen, N., *et al.*, J Virol, 73 (1999) 6327.
[133] Bukowski, J.F., Woda, B.A., Habu, S., *et al.*, J Immunol, 131 (1983) 1531.
[134] Williamson, J.S. and Stohlman, S.A., J Virol, 64 (1990) 4589.
[135] Biron, C.A. and Brossay, L., Curr Opin Immunol, 13 (2001) 458.
[136] Marten, N.W., Stohlman, S.A., and Bergmann, C.C., J Virol, 74 (2000) 7903.
[137] Williamson, J.S., Sykes, K.C., and Stohlman, S.A., J Neuroimmunol, 32 (1991) 199.
[138] Daniels, K.A., Devora, G., Lai, W.C., *et al.*, J Exp Med, 194 (2001) 29.
[139] Goetzl, E., Banda, M., and Leppert, D., J Immunol, 156 (1996) 1.
[140] Yong, V.W., Power, C., Forsyth, P., *et al.*, Nat Rev Neurosci, 2 (2001) 502.
[141] Zhou, J., Marten, N.W., Bergmann, C.C., *et al.*, J Virol, 79 (2005) 4764.
[142] Haring, J.S., Pewe, L.L., and Perlman, S., J Virol, 75 (2001) 3043.
[143] Matthews, A., Weiss, S.R., Shlomchik, M., J Immunol, 167 (2001) 5254.
[144] Ramakrishna, C., Stohlman, S.A., Atkinson, R.D., *et al.*, J Immunol, 168 (2002) 1204.
[145] Marten, N.W., Stohlman, S.A., Atkinson, R.D., *et al.*, J Immunol, 164 (2000) 4080.
[146] Haring, J.S. and Perlman, S., J Neuroimmunol, 137 (2003) 42.
[147] Haring, J.S., Pewe, L.L., and Perlman, S., J Immunol, 169 (2002) 1550.
[148] Chen, G., Tai, A.K., Lin, M., *et al.*, J Immunol, 175 (2005) 2212.
[149] Dalziel, R.G., Lampert, P.W., Talbot, P.J., *et al.*, J Virol, 59 (1986) 463.
[150] Fleming, J.O., Trousdale, M.D., Bradbury, J., *et al.*, Microb Pathog, 3 (1987) 9.
[151] Lavi, E., Murray, E., Makino, S., *et al.*, Lab Invest, 62 (1990) 570.
[152] Wang, F., Fleming, J.O., and Lai, M.M.C., Virology, 186 (1992) 742.
[153] Lin, M.T., Stohlman, S.A., and Hinton, D.R., J Virol, 71 (1997) 383.
[154] Parra, B., Hinton, D., Marten, N., *et al.*, J Immunol, 162 (1999) 1641.
[155] Parra, B., Lin, M.T., Stohlman, S.A., *et al.*, J Virol, 74 (2000) 2447.
[156] Buchmeier, M.J., Lewicki, H.A., Talbot, P.J., *et al.*, Virology, 132 (1984) 261.
[157] Perlman, S., Schelper, R., Bolger, E., *et al.*, Microb Pathog, 2 (1987) 185.

[158] Pewe, L., Wu, G., Barnett, E.M., *et al.*, Immunity, 5 (1996) 253.
[159] Pewe, L., Xue, S., and Perlman, S., J Virol, 72 (1998) 5912.
[160] Goulder, P.J. and Watkins, D.I., Nat Rev Immunol, 4 (2004) 630.
[161] Dandekar, A.A., Jacobsen, G., Waldschmidt, T.J., *et al.*, J Virol, 77 (2003) 11867.
[162] Kim, T.S. and Perlman, S., J Immunol, 171 (2003) 2006.
[163] Xue, S. and Perlman, S., Virology, 238 (1997) 68.
[164] Pearce, B.D., Hobbs, M.V., McGraw, T.S., *et al.*, J Virol, 68 (1994) 5483.
[165] Sutherland, R.M., Chua, M-M., Lavi, E., *et al.*, J Neurovirol, 3 (1997) 225.
[166] Erlich, S., Matsushima, G., and Stohlman, S., J Neurol Sci, 90 (1989) 203
[167] Stohlman, S.A., Matsushima, G.K., Casteel, N., *et al.*, J Immunol, 136 (1986) 3052.
[168] Stohlman, S.A., Sussman, M.A., Matsushima, G., *et al.*, J Neuroimmunol, 19 (1988) 255.
[169] Yamaguchi, K., Goto, N., Kyuwa, S., *et al.*, J Neuroimmunol, 32 (1991) 1.
[170] Körner, H., Schliephake, A., Winter, J., *et al.*, J Immunol, 147 (1991) 2317.
[171] Stohlman, S.A., Bergmann, C.C., Lin, M.T., *et al.*, J Immunol, 160 (1998) 2896.
[172] Heemskerk, M., Schoemaker, H., Spaan, W., *et al.*, Immunology, 84 (1995) 521.
[173] Wijburg, O.L.C., Heemskerk, M.H.M., Sanders, A., *et al.*, Immunology, 87 (1996) 34.
[174] Lane, T.E., Liu, M.T., Chen, B.P., *et al.*, J Virol, 74 (2000) 1415.
[175] Anghelina, D., Pewe, L., and Perlman, S., Am J Pathol, 169 (2006) 209.
[176] Lin, M.T., Hinton, D.R., Marten, N.W., *et al.*, J Immunol, 162 (1999) 7358.
[177] Burrer, R., Buchmeier, M.J., Wolfe, T., *et al.*, Am J Pathol, 170 (2007) 557.
[178] Dandekar, A., Wu, G., Pewe, L.L., *et al.*, J Virol, 75 (2001) 6115.
[179] Trapp, B., Peterson, J., Ransohoff, R., *et al.*, N Engl J Med, 338 (1998) 278.
[180] Houtman, J.J. and Fleming, J.O., J Neurovirol, 2 (1996) 361.
[181] Wu, G.F., Dandekar, A.A., Pewe, L., *et al.*, J Immunol, 165 (2000) 2278.
[182] Wu, G.F., Pewe, L., and Perlman, S., J Virol, 74 (2000) 7683.
[183] Pewe, L.L. and Perlman, S., J Immunol, 168 (2002) 1547.
[184] Bergmann, C.C., Parra, B., Hinton, D.R., *et al.*, J Virol, 78 (2004) 1739.
[185] Huseby, E.S., Liggitt, D., Brabb, T., *et al.*, J Exp Med, 194 (2001) 669.
[186] Tran, E.H., Prince, E.N., and Owens, T., J Immunol, 164 (2000) 2759.
[187] Dandekar, A.A. and Perlman, S., Am J Pathol, 161 (2002) 1255.
[188] Kim, T.S. and Perlman, S., Am J Pathol, 166 (2005) 801.
[189] Kim, T.S. and Perlman, S., J Virol, 79 (2005) 7113.
[190] McIntosh, K., Becker, W.B., and Chanock, R.M., Proc Natl Acad Sci USA, 58 (1967) 2268.
[191] Butler, N., Pewe, L., Trandem, K., *et al.*, Virology, 347 (2006) 410.
[192] Jacomy, H. and Talbot, P.J., Virology, 315 (2003) 20.
[193] Jacomy, H. and Talbot, P.J., Adv Exp Med Biol, 581 (2006) 473.
[194] Subbarao, K. and Roberts, A., Trends Microbiol, 14 (2006) 299.
[195] Nagata, N., Iwata, N., Hasegawa, H., *et al.*, J Virol, 81 (2007) 1848.
[196] Roberts, A., Deming, D., Paddock, C.D., *et al.*, PLoS Pathog, 3 (2007) e5.
[197] McCray, P.B., Jr., Pewe, L., Wohlford-Lenane, C., *et al.*, J Virol, 81 (2006) 813.
[198] Tseng, C.T., Huang, C., Newman, P., *et al.*, J Virol, 81 (2006) 1162.
[199] Bergmann, C.C., Yao, Q., Lin, M., *et al.*, J Gen Virol, 77 (1996) 315.
[200] Castro, R.F. and Perlman, S., J Virol, 69 (1995) 8127.
[201] Bergmann, C., McMillan, M., Stohlman, S.A., J Virol, 67 (1993) 7041.
[202] Heemskerk, M., Schoemaker, H., De Jong, I.,*et al.*, Immunology, 85 (1995) 517.
[203] Van der Veen, R.C., Virology, 225 (1996) 339.