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CHAPTER

44

Experimental Models of Virus-Induced Demyelination

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MEDICAL RELEVANCE OF ANIMAL MODELS OF DEMYELINATING DISEASE

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the human central nervous system (CNS) and is the most common cause of acquired nontraumatic neurologic disability in young adults. The pathologic hallmark of the disease is damage to oligodendrocytes and CNS myelin, which results in demyelinated white matter lesions, followed by axonal loss and glial scarring (Lucchinetti *et al*., 1997; Noseworthy *et al*., 2000; Trapp *et al*., 1998). Areas of active disease display a prominent inflammatory response with tissue infiltration by mononuclear cells, primarily T cells and macrophages. Despite decades of research, the causes of myelin damage and neurologic dysfunction in MS remain largely unknown. Chapters 29 through 33 in Section IV of these volumes provide an overview of the classification, pathology, genetics, and potential pathogenic mechanisms of multiple sclerosis.

There is no cure for MS, and attempts to develop effective therapies have met with only limited success (Noseworthy, 1998). Immunosuppression with corticosteroids is commonly used as a short-term therapy to control MS relapses. Long-term therapy generally consists of treatment with immunomodulatory drugs such as glatiramer acetate (Copolymer-1/Copaxone), interferon-β-1a (Avonex), or interferon-β-1b (Betaseron). Each of these drugs has been shown to reduce relapses of the disease, but whether they alter the long-term clinical endpoint is still being debated.

Our continued lack of understanding about the causes of MS and the mechanisms of disease progression, and the lack of truly effective therapies for the disease, underscore the need for good animal models of MS on which to conduct research. In this chapter we review two of the most widely studied animal models of virus-induced demyelinating disease: Theiler's murine encephalomyelitis virus and murine hepatitis virus. Both viruses produce acute inflammatory encephalitis that is followed by chronic CNS demyelinating disease. The clinical and pathologic correlates of virus-induced demyelination are largely immune mediated. Several pathologic mechanisms have been proposed to explain the development of myelin damage and neurologic deficits, and each of the proposed mechanisms may play a role in disease progression depending on the genetic constitution of the infected animal. The induction of demyelinating disease by virus may be directly relevant to human MS. Several viruses are known to cause demyelination in humans, and viral infection is an epidemiologic factor that is consistently associated with clinical exacerbation of MS (Sarchielli *et al*., 1993; Sibley *et al*., 1985). It has been suggested that viral infection may be a cause of MS, although no specific virus has been identified as a causative agent.

The clinical and pathologic presentation of virus-induced demyelination, the immune-mediated nature of the disease mechanisms, and the variability of the disease presentation depending on genetic background are all very similar to what is observed in MS. The many similarities between human MS and the demyelinating diseases that are induced by infection with TMEV and MHV make these animal models very attractive for the study of multiple sclerosis.

THEILER'S MURINE ENCEPHALOMYELITIS VIRUS (TMEV)

Theiler's murine encephalomyelitis Virus (TMEV) belongs to the cardiovirus genus of the picornavirus family (Nitayaphan *et al.*, 1986; Pevear *et al.*, 1987). The original isolation and characterization of the virus was reported by Theiler in 1937 when the effects of TMEV infection of the CNS were observed in a mouse that spontaneously developed flaccid paralysis. The virus was subsequently transmitted to other mice by intracerebral inoculation with a suspension made from the brain and spinal cord of the infected mouse.

TMEV is a ubiquitous enteric pathogen that usually causes asymptomatic intestinal infections in mice. Occasionally, the virus will spread beyond the intestinal tract and enter the CNS, resulting in both acute and chronic CNS infections. Chronic CNS infection results in extensive demyelination with accumulating neurologic deficits and has provided a valuable experimental model for human demyelinating disease.

Virus Biology and Life Cycle

TMEV Subgroups

Since its initial discovery, isolates of TMEV have been recovered in several laboratories. The different TMEV strains have been divided into two subgroups depending on the disease that they induce after infection of the CNS (Lorch *et al.*, 1981). The GDVII and FA strains are highly virulent with an LD_{50} as low as 1 to 10 plaque forming units (PFU). CNS infection causes acute encephalitis that is characterized by the destruction of a large number of CNS neurons, especially in the cortex, hippocampus, thalamus, brain stem, and in the anterior horns of the spinal cord. The encephalitis is usually fatal, and the highly virulent strains do not result in persistent infection of the CNS.

A second TMEV subgroup is far less virulent ($LD_{50} > 10^6$ PFU) and results in a distinctly different disease presentation. This subgroup, which includes the TO4, DA, BeAn 3886, Yale, and WW strains, induces a biphasic CNS disease (Daniels *et al.*, 1952; Lipton, 1978; Wroblewska *et al.*, 1977). Following intracerebral infection, these strains replicate in neurons in the brain resulting in encephalitis similar to that observed with the GDVII strain but nonlethal. The virus is cleared from the brain by the host immune response but persists in the spinal cord, eventually resulting in the development of chronic demyelinating disease (Lehrich *et al.*, 1976; Lipton, 1975; Njenga *et al.*, 1997; Rodriguez *et al.*, 1987). The disease is characterized by viral persistence in oligodendrocytes (Rodriguez *et al.*, 1983) and macrophages (Lipton *et al.*, 1995), with chronic demyelination and progressive loss of motor function (McGavern *et al.*, 1999). The pathology is largely immune mediated with animals demonstrating a range of disease phenotypes depending on their genetic background. In the SJL strain, demyelination is evident within 30 days after infection. By 90 days, infected animals begin to develop spasticity and gait abnormalities, and weakness of the lower extremities, with paralysis eventually occurring by 6 to 9 months (Lipton and Dal Canto, 1976a).

Capsid Structure

The X-ray crystallographic structures of DA, BeAn and GDVII viruses have been determined to about 3 angstrom resolution (Grant *et al.*, 1992; Luo *et al.*, 1992, 1996; Toth *et al.*, 1993). A schematic representation of the TMEV capsid, based on the crystallograghic data, is presented in Figure 44.1. Each virus consists of a protein shell that is composed of 60

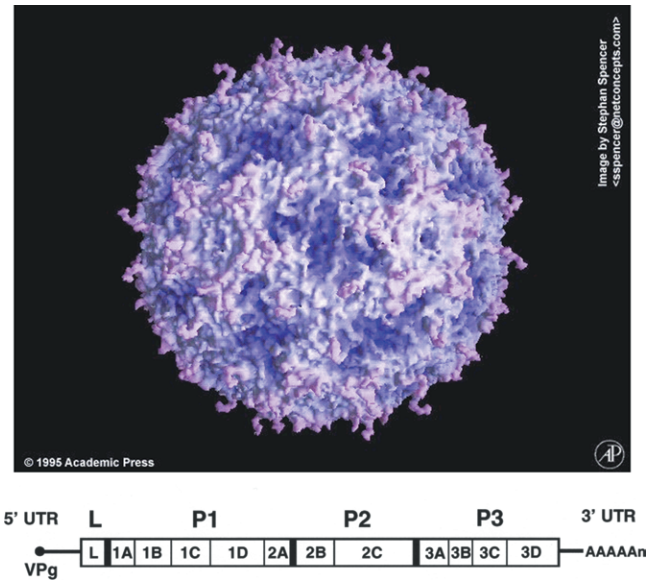

FIGURE 44.1
TMEV capsid and genome structure. The upper panel shows a schematic representation of the TMEV capsid, based on the X-ray crystallograghic data. Lower panel shows a map of the TMEV DA strain genome. The 5′ and 3′ untranslated (UTR) regions are indicated. The 5′ end of the RNA is bound by the viral VPg protein (ball) and the 3′ end is polyadenylated (AAAAAn). The open bar indicates the open reading frame from nucleotides 1066 to 7968. The heavy lines dividing the bar indicate the positions of the initial proteolytic cleavages after the leader peptide (L) and between the P1, P2, and P3 precursor peptides. The lighter lines dividing the bar indicate the arrangement of the final gene products. The identities of the final gene products are discussed in the text. The TMEV capsid structure is reproduced with permission from Webster and Granoff (1995).

protomers, arranged as 12 pentamers with icosahedral symmetry. Each protomer contains one of each of the four capsid peptides (VP1, VP2, VP3, VP4). The VP1, VP2, and VP3 capsid proteins are each composed of an eight-stranded antiparallel β-barrel. The loops that connect the strands of the β-barrel form the surface structure of the viral capsid and may play important roles as sites of binding for neutralizing antibody and in conformational determinants of virus persistence. A second surface feature, the ''pit'' formed at the contact region between VP1 and VP3, is the likely binding site for the cellular receptor (Zhou, *et al*., 2000; Jnaoui, *et al*., 2002).

Viral Genome

The TMEV genome consists of a positive sense, single stranded RNA molecule. Genomes for both the GDVII and TO subgroups have been sequenced and found to be very similar, with about 90% identity at the nucleotide level and 96% at the amino acid level (Pevear *et al*., 1988). The genome of the DA strain is 8093 nucleotides long (Ohara *et al*., 1988) and contains a single 6903 nucleotide open reading frame that starts at nucleotide 1066 and encodes a 2301 amino acid polyprotein. The genome terminates with a poly(A) tail. The 12 mature TMEV gene products are generated from the polyprotein by post-translational proteolytic cleavage. The gene products include a leader peptide, four capsid polypeptides (VP1-4), two viral proteases, a polymerase/helicase, the RNA-dependent RNA polymerase that is involved in genome replication, and a small basic protein which becomes covalently linked to the 5′ end of viral RNAs. Two other gene products have unknown functions.

Virus Life Cycle

The TMEV infection cycle is typical of the picornavirus family (Rueckert, 1996). Attachment of the virion to specific cell surface receptors serves to position the virus close to the cell membrane. Attachment also induces a conformational change in the virion that results in the loss of the VP4 capsid protein and the translocation of the RNA genome across the cell membrane and into the cytoplasm. Co-opting the translational machinery of the cell,

the RNA genome directs the synthesis of a single large polyprotein. While nascent on the ribosome, the polyprotein is cleaved by virus encoded protease activity, into three large precursor proteins: P1, P2, and P3. The precursor proteins are further cleaved to produce the viral structural proteins and proteins necessary for completion of the infection cycle (Fig. 44.1).

Precursor P3 is processed to produce a viral proteinase (designated 3C), a protein involved in initiating replication of the viral genome (3B or VPg) and the viral RNA-dependent RNA polymerase (3D). The viral polymerase copies the incoming viral genome to produce a negative-strand RNA that then serves as the template for replication of the positive-strand viral genome. The newly formed positive-strand RNA molecules may be copied to form more negative-strand templates or be packaged into virions as virus assembly proceeds.

The P1 precursor protein is cleaved to produce the viral capsid proteins VP0 (1A+B), VP1 (1D), and VP3 (1C). As the concentration of capsid proteins increases in the cell, they begin to aggregate into protomers composed of one copy of each of the capsid proteins. These protomers then assemble into pentamers, and 12 pentamers assemble with a positive-stranded, VPg bound viral RNA, to produce a noninfectious provirion. The final step in maturation to infectious virus requires a maturation cleavage in which the VP0 protein is cleaved to give the final VP2 (1B) and VP4 (1A) capsid proteins that are found in the mature virus. In neonates and in some cells in culture, mature virus may accumulate to very high levels in infected cells and can often be observed as paracrystalline arrays in electron micrograghs (Fig. 44.2). Virus is generally released from the cell by infection-mediated cytolysis. The time that is required to complete the infection cycle is generally 7 to 12 hours.

TMEV Receptor

In culture, TMEV is able to infect a wide variety of cell types from several different species. Although a unique protein receptor for the virus has not been identified, several lines of evidence suggest that cell surface carbohydrate residues may play a central role in the binding and entry of virus. Removal of sialic acid from the cell surface by treatment with sialidase significantly reduces the infectivity of BeAn virus in BHK cells (Fotiadis *et al.*, 1991). Similar decreases are observed when binding to sialic acid is blocked with wheat germ agglutinin, which binds to sialic acid on the cell surface (Fotiadis *et al.*, 1991).

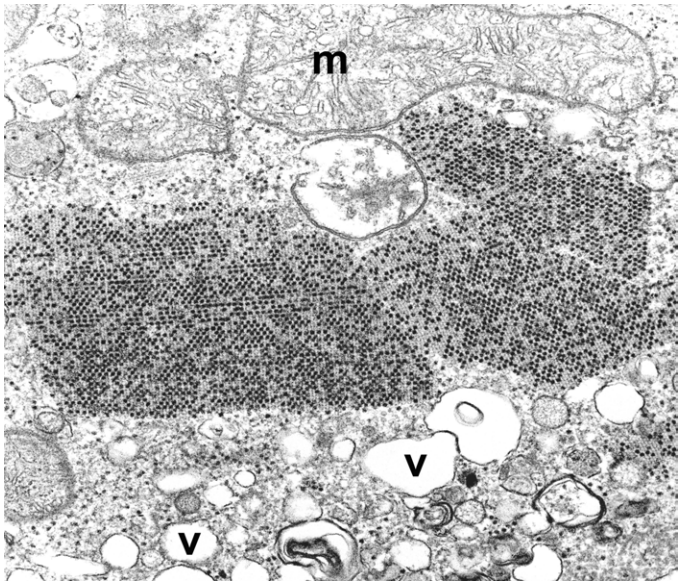

FIGURE 44.2
Crystalline virus in neonatal brain. A paracrystalline viral array in the cytoplasm of a spinal cord neuron from a neonatal SJL mouse infected with the DA strain of TMEV. An intact mitochondrion is visible (m), but the numerous cytoplasmic vacuoles (v) indicate the cytopathic effects of viral infection.

Sialyllactose comprises the terminal three sugar residues of cell surface oligosaccarides, and inclusion of sialyllactose in cell culture medium inhibits viral binding and infectivity by blocking receptor binding sites on the virus (Zhou *et al*., 1997). Together these data demonstrate an important role for sialic acid in the recognition of the cell surface by the TO subgroup TMEV strains. Sialyloligosaccaride addition is a common post-translation modification of cell surface proteins, which is consistent with the observation that TMEV can infect a variety of cell types. Which specific sialic acid containing proteins might be required for virus binding and entry has not yet been established.

The neurovirulent strains of TMEV (GDVII and FA) do not require sialic acid for cell surface binding (Fotiadis *et al*., 1991) but instead use cell surface heparin sulfate proteoglycan. Pretreatment of virus with heparin sulfate, treatment of host cells with heparin sulfate cleaving enzymes, or treatment with heparin sulfate specific antibodies all inhibit GDVII binding and infection (Reddi and Lipton, 2002). Proteoglycan deficient CHO cells are resistant to infection, further supporting a role for proteoglycans in GDVII binding. The interaction of proteins with heparin sulfate is usually mediated by heparin-binding domains (HBD) and a heparin-binding domain consensus sequence has been described (Cardin and Weintraub, 1989). A putative HBD is present in the amino acid sequence of the VP1 capsid protein of both the GDVII and TO subgroups. This sequence may play a role in the binding of GDVII to heparin sulfate but if so, conformational differences must exist between VP1 peptides of the TMEV subgroups because the TO subgroup viruses do not bind heparin sulfate. Proteoglycans are known to serve as co-receptors or attachment factors for a variety of different viruses. For several of these viruses, heparin sulfate acts primarily as an attachment factor and is not fully sufficient to mediate virus entry. The extent to which additional protein entry receptors are needed for GDVII infection is not yet clear (Reddi and Lipton, 2002).

Disease Pathology

The encephalitic phase of TMEV-induced disease develops rapidly after intracerebral infection. Virus infects and replicates in neurons of the brain and spinal cord, with titers of infectious virus reaching their peak by 5 to 7 days. Immunohistochemical staining demonstrates the highest viral antigen loads in the thalamus, hypothalamus, midbrain, brain stem, and spinal cord gray matter. Neuronal infection and lysis is particularly pronounced with the GDVII subgroup viruses, and infection usually results in death soon after inoculation (Patick *et al*., 1990).

Acute disease following infection with the TO subgroup viruses is also characterized by intense mononuclear cell infiltration in the brain and spinal cord. The combined effects of direct viral lysis of neurons and damage from infiltrating cells may result in transient flaccid paralysis within 2 weeks after infection, but in many cases the early phase of the disease is largely asymptomatic. The acute phase of the disease is followed by clearance of virus from the gray matter by the host immune response (Njenga *et al*., 1997). Resistant strains of mice achieve virtually complete viral clearance. In susceptible strains, virus is cleared from the gray matter but persists in the white matter of the spinal cord for the lifetime of the animal.

During persistent infection, the amount of infectious virus, and the number of cells with detectable virus antigen in the spinal cord is usually quite low. This is in contrast to the high level of viral genome that is present throughout the disease (Trottier *et al*., 2001). Infected cells most often appear in the lateral or anterior columns of the thoracic spinal cord and the presence of infected cells is often accompanied by an inflammatory parenchymal infiltrate and perivascular cuffing. The diffuse inflammatory infiltrate consists of large numbers of $CD4^+$ and $CD8^+$ T lymphocytes, activated macrophages and microglia, B cells, and reactive astrocytes (Fig. 44.3; Lindsley *et al*., 1989). The presence of inflammatory cells correlates closely with the development of myelin damage, and electron microscopy reveals infiltrating mononuclear cells stripping myelin from axons. Macrophages containing engulfed myelin debris are numerous in demyelinating lesions. The specific roles that these different cell types play in the development of demyelination and neurologic deficits will be discussed in subsequent sections.

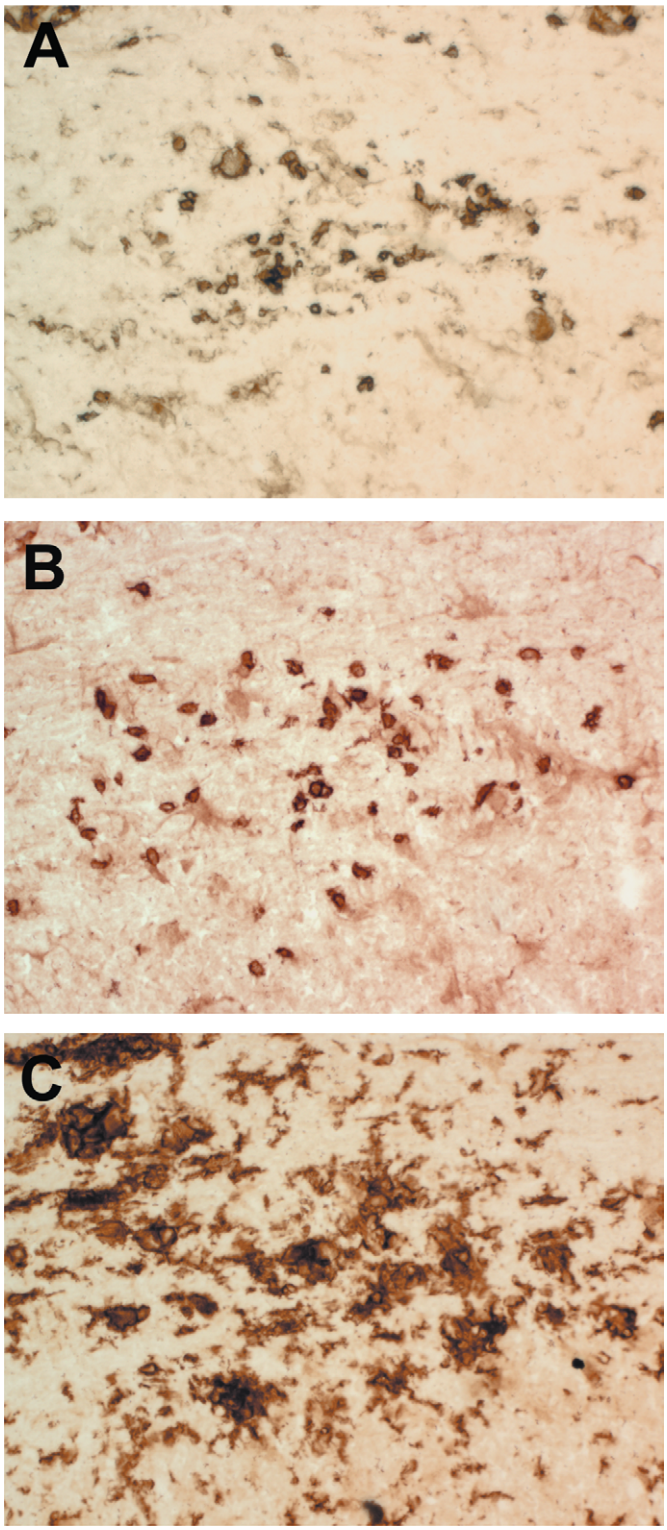

FIGURE 44.3
Immunochemical characterization of a chronic demyelinated lesion. Adjacent sections from the spinal cord of an SJL mouse, 8 months post-infection, were stained for $CD4^+$ T cells with the Ly-2 antibody (A), for $CD8^+$ T cells with the GK1.5 antibody (B) and for macrophages with the M1/70 antibody against Mac-1 (C).

Demyelination is apparent by as early as 22 days after infection and in many mouse strains, as demyelination progresses, neurologic deficits begin to appear. Demyelination pathology is illustrated in Figure 44.4. The development of neurologic deficits in

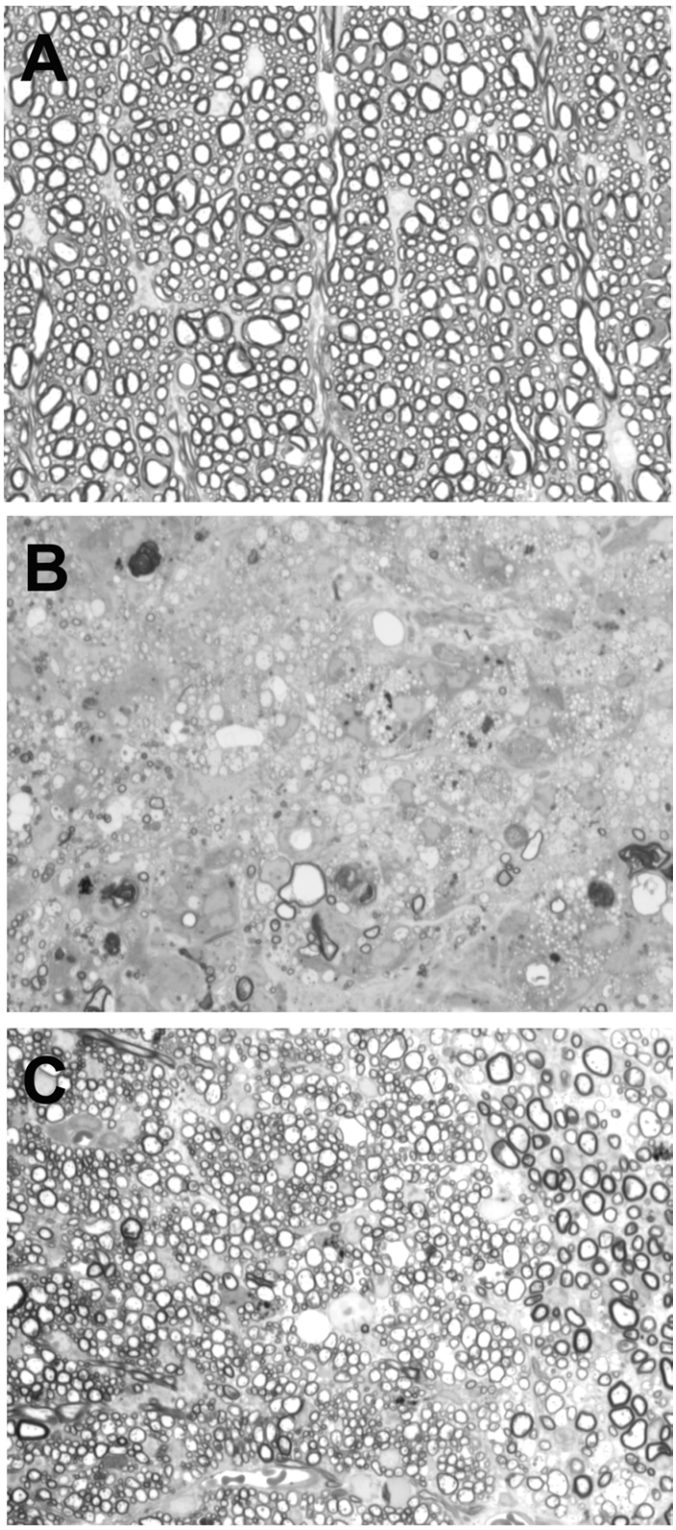

FIGURE 44.4
Demyelinated and remyelination in the TMEV-infected spinal cord. (A) Normal spinal cord white matter stained for myelin sheaths with para-phenylene-diamine. (B) A demyelinated lesion from an FVB mouse, 3 months post-infection with the DA strain of TMEV. Almost complete myelin destruction is apparent. (C) Extensive remyelination is apparent by 8 months post-infection. Schwann cell remyelination is evident as heavily stained myelin figures in the upper right corner of the panel, while the lightly staining myelin in the center of the panel is oligodendrocyte remyelination.

infected mice involves not just the disruption of neuronal function but neuronal and axonal loss as well (McGavern *et al*., 2000). Quantitative assessment of CNS injury during TMEV-induced disease shows that significant atrophy of the lateral and anterior white matter regions of the spinal cord can be detected by 45 days post-infection. Atrophy is most pronounced in the posterior cervical and anterior thoracic regions of the spinal cord. While levels of demyelination reach a peak by 100 days post-infection, spinal cord atrophy continues to increase between 100 and 220 days. A 25% reduction was observed in the area of the anterior and lateral white matter at the C8-T11 level of the spinal cord by 195 to 229 days after infection. In this study, atrophy was specifically assessed in areas of normal appearing white matter and not in lesion areas, to avoid complications due to area changes resulting from inflammation and edema. This marked spinal cord atrophy is the result of a 30% loss of medium to large axonal fibers by 195 to 229 days post-infection. The progressive loss of axons correlates well with electrophysiologic changes in the spinal cord and with changes in motor ability. These observations demonstrate that the neurologic deficits that develop during chronic TMEV-induced demyelinating disease are primarily due to axonal damage and loss rather than to demyelination alone.

Viral Persistence

Intracerebral inoculation with the highly virulent GDVII subgroup viruses results in fatal encephalitis, while infection with the less virulent TO viruses results in milder encephalitis, followed by clearance of virus from the brain and virus persistence in the spinal cord. There is still some debate concerning whether the difference in disease that is induced by these viruses is due simply to differences in virulence. Would the GDVII strains persist and cause demyelination if animals survived the initial encephalitis or are determinants for persistence and demyelination a specific genetic property of the TO strains? Recombinant chimeric viruses, resulting from the exchange of sequences between the GDVII and BeAn (or DA) virus genomes, have been useful in beginning to map the determinants of virus neurovirulence and persistence.

Initial studies localized the majority of neurovirulence to a segment of the GDVII genome from the middle of 1B to the middle of 2C, although additional upstream sequences had a more minor effect (Fig. 44.1; Fu *et al*., 1990a). Infection with recombinant DA virus that contains these GDVII regions results in death at a much reduced LD_{50} compared to wildtype DA virus. Subsequent studies narrowed the critical segment to a region of P1 from 1B to 1D, the capsid coding region (Zhang *et al*., 1993). Several studies have since confirmed that major determinants of viral persistence lie in the region of the viral genome that encodes the capsid proteins and have identified regions in two of these proteins, VP1 and VP2, that appear to be necessary for persistence (Adami *et al*., 1998; Lin *et al*., 1998). Molecular modeling suggests that these sequences may be positioned on the surface of the virus in a manner consistent with a role in the binding of the virus to its cellular receptor (Zhou *et al*., 1997). Differences in receptor binding between the TMEV subgroups may therefore play an important role in determining the balance between neurovirulence and the ability to produce persistent infection.

Several mutated strains of the GDVII virus have been characterized in which neurovirulence is greatly attenuated, and while these strains are viable, replicate in the brain, and may even induce encephalitis, they do not persist or induce chronic demyelinating disease (Lipton *et al*., 1991, 1998). These experiments suggest that ability of a TMEV strain to produce persistent infection and demyelination is not solely a function of neurovirulence but may be a specific genetic property of the strain. Other investigators have reported that insertion into the GDVII backbone, of DA strain segments from across the length of the DA genome, attenuates virulence and results in virus persistence and demyelination (Fu *et al*., 1990b; Rodriguez and Roos, 1992; see also Jakob and Roos, 1996). Further studies will be needed to determine whether there are specific genetic determinants in the TO subtype that control persistence and to map these determinants to specific viral proteins.

An additional interesting difference between the GDVII and TO subgroups involves the expression of the leader peptide region (L) of the TMEV genome at the 5′ end of the open

reading frame (see Fig. 44.1). The TO strains use an alternative initiation codon to produce L*, a protein whose coding sequence is out of frame with the polyprotein reading frame. The GDVII strains do not encode the L* protein. Mutations in L* dramatically decrease virus-induced demyelination indicating that it plays a role in the development of disease (Chen *et al*., 1995). The L* protein is essential for replication of the DA strain in a macrophage-like cell line in culture, while the GDVII strain does not replicate in these cells (Takata *et al*., 1998). L* appears to function by preventing apoptosis of the DA-infected cells (Ghadge, *et al*., 1998). The production of the L* protein therefore plays an essential role in the ability of TO subtype viruses to persist in specific cell types in the CNS. Analysis of the cytotoxic T lymphocyte (CTL) response in the CNS of mice infected with L* mutant virus, suggests that the inhibition of apoptosis by L* may prevent development of virus-specific cytotoxicity, permitting persistent virus infection and demyelinating disease (Lin *et al*., 1999).

TMEV persistence in susceptible mice is associated with continuous virus replication and infectious virus and viral RNA can be isolated from the CNS for many months after initial infection. There is no single obvious cellular site of virus persistence in the spinal cord. Both viral antigen and RNA have been reported in oligodendrocytes, macrophages, and astrocytes (Aubert *et al*., 1987; Brahic *et al*., 1981; Lipton *et al*., 1995; Rodriguez *et al*., 1983). The typical observation that only very small numbers of infectious virus can be recovered from chronically infected spinal cord initially led to the notion that the virus persists at low levels in the tissue. However, recent quantitative studies on the level of viral RNA in the CNS have demonstrated the presence of large amounts of full-length viral RNA, about 10^9 copies/spinal cord, throughout the course of the chronic disease (Trottier *et al*., 2001). In contrast, infectious virus reaches a peak of $>10^6$ PFU/spinal cord by 15 days after intracerebral inoculation but then falls significantly to 10^2 to 10^4 PFU/spinal cord after 28 days. This difference between viral genome load and infectious virus may reflect the rapid neutralization of virus by virus-specific antibodies. These data indicate that active viral replication continues throughout persistent viral infection.

The identification of a primary cellular site and specific mechanism of TMEV persistence has been difficult. Based on immunostaining experiments, the predominant virus antigen burden in infected spinal cord has been reported to reside in either oligodendrocytes or macrophages (Lipton *et al*., 1995; Rodriguez *et al*., 1983). More recently, fractionation of macrophages and oligodendrocytes from the chronically infected CNS revealed large amounts of viral genome in both cell types (Trottier *et al*., 2001). Restriction of virus replication has been suggested as a mechanism for virus persistence and such restriction has been reported in macrophages isolated from TMEV infected CNS (Clatch *et al*., 1990; Levy *et al*., 1992). However, the ratio of plus- to minus-strand TMEV RNA appears to be similar in macrophages and oligodendrocytes from infected spinal cord, suggesting that neither cell type is generally restricted for virus replication (Trottier *et al*., 2001).

As a potential site for TMEV persistence, macrophages have been the most widely studied. As mentioned earlier, restriction of virus replication has been reported in macrophages isolated from the TMEV infected CNS, and such restriction has been suggested as a possible mechanism for persistence. Depletion of blood borne macrophages early during the course of disease (7 to 21 days post-infection) decreases the amount of viral RNA in the CNS, supporting a role for macrophages in viral persistence (Rossi *et al*., 1997). Studies in macrophage cell lines have demonstrated a block at the point of virus assembly rather than replication and it has been demonstrated that the differentiation state of the macrophage may be critical for the ability of the cell to support persistence (Jelachich *et al*., 1995; Shaw-Jackson and Michiels, 1997). Similar experiments, to look at potential viral persistence in the precursors of other glial types, have not yet been reported.

Other investigators have suggested a role in virus persistence for other CNS cells, such as astrocytes and vascular endothelial cells (Sapatino *et al*., 1995; Zheng *et al*., 2001). Whether there is a primary cellular site and a unique mechanism for TMEV persistence in the chronically infected CNS, is still an unresolved matter.

Host Determinants of Resistance and Susceptibility

Inbred strains of mice can be either resistant or susceptible to persistent TMEV infection and demyelinating disease. Upon intracerebral infection with virus, resistant strains mount an anti-viral immune response that controls virus replication and eventually clears virus from the animal. Upon clearance, infectious virus, virus antigen, and viral RNA can no longer be detected. Susceptible strains also mount a strong immune response against virus but virus is not cleared. A persistent viral infection is established along with the development of demyelinating disease, as described earlier. Since virus clearance is mediated by the immune response, mutations that disrupt major functional elements of the immune system generally result in some level of susceptibility, even in an animal with an otherwise resistant genetic background. The effects of mutations of this type will be discussed in subsequent sections. Here we review naturally occurring genetic loci that act as modifiers of the response to TMEV infection and that have been identified using standard techniques of genetic analysis in mice.

Early studies with different strains of mice demonstrated a wide range of susceptibility to TMEV-induced disease (Lipton and Del Canto, 1979). F1 and F2 backcrossing experiments between resistant and susceptible strains revealed that several loci were important for virus clearance and that one of these loci was linked to the H-2 major histocompatibility complex on chromosome 17 (Lipton and Melvold, 1984). Further genetic analysis of this region showed that mice with certain H-2 haplotypes ($H\text{-}2^{f,\ p,\ q,\ r,\ s,\ v}$) are susceptible to persistent virus infection, while others ($H\text{-}2^{d,b,k}$) conferred resistance (Rodriguez and David, 1985). Resistance was a dominant effect (Patick *et al*., 1990) and mapped to the D region of the H-2 complex, suggesting that a class-I mediated element of the immune response was essential for virus resistance (Clatch *et al*., 1985; Rodriguez *et al*., 1986b). Direct demonstration that an H-2 linked class-I gene was responsible for virus resistance was provided when the $H\text{-}2D^{d}$ class-I gene was introduced into a susceptible mouse strain and these transgenic animals were shown to be resistant (Azoulay *et al*., 1994). A key role for class-I restricted $CD8^{+}$ T cells as effectors of resistance has since been confirmed with targeted mutations in CD8 and β2-microglobulin (which lacks MHC class-I function). Both of these mutations result in TMEV susceptibility in animals with otherwise resistant genetic backgrounds (Fiette *et al*., 1993; Murray *et al*., 1998; Pullen *et al*., 1993; Rodriguez *et al*., 1993). The $H\text{-}2D^{b}$ gene exerts its effect on the stimulation of virus-specific CTL through the presentation of an immunodominant peptide from the VP2 capsid protein: $VP2_{121–130}$ (Dethlefs *et al*., 1997; Johnson *et al*., 1999). There are other class-I molecules whose peptide binding sites are identical to those of the D locus and therefore have the potential to present TMEV peptide. These molecules, however, have not been shown to play a role in establishing resistance, possibly due to differential patterns of expression or to structural differences outside of the peptide binding domain that are important for antigen presentation (Altintas *et al*., 1993; Lin *et al*., 1997a).

Non-MHC loci also have an influence on virus resistance/susceptibility. The T-cell receptor genes lie on chromosome 6 (the *Tcrb* locus) in the mouse, and genetic mapping studies have indicated that a gene affecting resistance maps close to the *Tcrb* locus. Several commonly used strains of susceptible mice have large deletions of the Vβ T-cell receptor genes, further suggesting a link between T-cell function and virus resistance (Rodriguez *et al*., 1992). Genetic analysis of congenic mice with specific Vβ deletions indicates that in susceptible genetic backgrounds, Vβ deletions enhance susceptibility but that have little effect on resistance. These data generally support a role for T-cell receptor genes in determining virus resistance (Rodriguez *et al*., 1994).

Mapping experiments using the susceptible SJL strain and the resistant B10.S strain, two strains that both have the same $H\text{-}2^{S}$ haplotype, have identified loci on chromosomes 10 and 18 (Bihl *et al*., 1999; Bureau *et al*., 1993). There are two loci close to the gene for interferon-γ (Ifn-γ) on chromosome 10. While the possibility of a resistance locus at the site of the Ifn-γ gene was of obvious interest, fine mapping experiments have excluded Ifn-γ as a candidate (Bihl *et al*., 1999). A second locus is near the gene for myelin basic protein (MBP) on chromosome 18. The exact identity of the gene involved has not yet been

established but the possibility that TMEV resistance might be influenced by one of the major structural genes for myelin is intriguing. Studies on TMEV infection of MBP deficient *shiverer* mice demonstrate enhanced susceptibility in the absence of MBP, supporting the idea that MBP might play a role in determining resistance (Bihl *et al*., 1997).

Loci that affect TMEV resistance and persistence have also been identified on chromosome 3 near the carbonic anhydrase-2 (*Car-2*) locus (Melvold *et al*., 1990), on chromosome 11 (Aubagnac *et al*., 1999), and on chromosome 14 (Bureau *et al*., 1998). The identity of the genes that are involved at these loci has not yet been established. Gender has also been reported to influence the susceptibility of certain mouse strains to TMEV-induced demyelinating disease (Kappel *et al*., 1990).

Mechanisms of Demyelination, Axonal Damage and the Development of Neurologic Deficits

When considering TMEV-induced disease as a model for human demyelinating disease, questions regarding the mechanisms of demyelination and axonal damage are of central importance. Several different mechanisms have been proposed to explain the pathology and neurologic deficits that usually accompany persistent TMEV infection, but there is still disagreement in the field about the relative importance of the various potential mechanisms for the development of demyelination and axonal damage.

Whatever the exact mechanisms of disease resistance and susceptibility, it seems certain that a balance between persistent virus infection and immune cell activation determines whether demyelinating disease will develop in immunocompetent mice. Mice with severe combined immunodeficiency (SCID) normally die from acute encephalitis when infected with TMEV. Adoptive transfer of normal spleen cells can result in survival and the development of demyelinating disease, but the results of adoptive transfer depend critically on the number of cells transferred (Rodriguez *et al*., 1996). Transfer of too few cells fails to ameliorate the SCID phenotype and most mice die. Transfer of too many cells results in complete virus clearance and the mice survive but do not develop disease. Only when an intermediate number of cells is transferred do the surviving mice develop persistent virus infection and demyelination. These results demonstrate the balance between immune activation and virus persistence that determines the quality and extent of demyelinating disease.

Mechanisms of Demyelination

Proposed mechanisms of TMEV-induced demyelination include (1) demyelination resulting from direct virus lysis of infected oligodendrocytes or as a ''dying back'' response of oligodendrocytes following infection; (2) TMEV-specific, immune-mediated destruction of infected oligodendrocytes; (3) ''bystander'' damage to glia by toxic mediators from activated macrophages; (4) ''epitope spreading,'' an autoimmune attack against myelin antigens resulting from the *de novo* processing and immune recognition of myelin antigens that follows the initial events of virus-induced myelin damage; and (5) ''molecular mimicry,'' an autoimmune attack against myelin antigens based on the activity of virus-activated T cells that are cross-reactive with myelin antigens. While several distinct mechanisms of demyelination have been proposed and supported by experimental evidence, this does not preclude the likelihood that different combinations of these mechanisms may be active at various times during the course of TMEV-induced disease and in animals with varying genetic composition.

Direct virus effects on oligodendrocytes While it is generally agreed that immune activation plays a major role in demyelination, several lines of evidence also support a role for direct effects of viral infection on oligodendrocytes. In cultures of mixed neonatal brain cells, TMEV infection of oligodendrocytes is primarily lytic (Graves *et al*., 1986; Rodriguez *et al*., 1988). Two days after infection, many cultured oligodendrocytes display cytopathic ultrastructural changes characteristic of picornavirus infection, such as numer-

ous cytoplasmic vacuoles and disruption of the endoplasmic reticulum, with preservation of mitochondria (see Fig. 44.2). The number of oligodendrocytes in these cultures begins to diminish within 48 hours after infection and by 7 days has decreased to 50% of the value in uninfected control cultures (Rodriguez *et al*., 1988). Since TMEV infects oligodendrocytes *in vivo* (Graves *et al*., 1986; Ohara *et al*., 1990; Rodriguez *et al*., 1988) and kills these cells *in vitro*, demyelination as a direct cellular consequence of the infection of oligodendrocytes seems plausible.

A direct demonstration that infection can produce demyelination without the need for an accompanying activation of the immune response comes from studies of demyelination in nude mice which lack circulating T cells and cell mediated immunity (Roos and Wollmann, 1984; Rosenthal *et al*., 1986). In these studies, infection of BALB/c *nu/nu* mice with the DA strain of TMEV resulted in the development of significant neurologic deficits by 2 to 3 weeks and death within 4 to 8 weeks. Demyelinated foci were observed in the spinal cord by 14 days post-infection even though inflammatory infiltrates were minimal. Electron microscopy revealed numerous infected and degenerating glial cells, some that were identified as oligodendrocytes. Damaged myelin was observed around intact axons as well as completely demyelinated axons. In general, the demyelinated lesions in these mice were small and inflammation is minimal compared to what might be expected in most susceptible strains of mice, suggesting that other mechanisms of demyelination may normally play a more prominent role. However, these observations do indicate that TMEV-induced glial damage can occur in the absence of a T-cell mediated immune response.

In immunocompetent mice, the cytoplasm of most oligodendrocytes from infected animals appears normal on electron microscopic examination (Dal Canto and Lipton, 1975; Rodriguez *et al*., 1983), even though viral RNA and antigen have been detected in oligodendrocytes throughout the course of persistent infection (Brahic *et al*., 1981; Rodriguez *et al*., 1983; Trottier *et al*., 2001). Rodriguez (Rodriguez *et al*., 1983; Rodriguez, 1985) reported ultrastructural abnormalities of the innermost extent of the myelin sheath and has proposed a ''dying back'' mechanism for demyelination. In persistently infected SJL mice, the inner tongue of the myelin sheath, which is the most distal extent of the myelin lamellae, often exhibits swelling and abnormalities in the normal cytoachitecture. Abnormalities included vacuolization, degeneration of mitochondria, increased numbers of microfilaments, and the appearance of electron-dense bodies and debris. These indications of damage to the innermost layer of the myelin sheath were observed in the absence of any signs of damage to the external surface of the sheath, indicating that damage to the oligodendrocyte may occur before immune-mediated damage to the myelin surface. Immunoperoxidase studies revealed that viral antigen was present on both the inner and outer extents of the myelin lamellae, even though the ultrastructural abnormalities were restricted to the inner loops. The observed degeneration appears to begin in the inner loop of the oligodendrocyte lamellae and then proceeds proximally toward the cytoplasm with the ultimate destruction of the myelin sheath.

The ''dying back'' concept was initially proposed as a mechanism underlying peripheral neuropathies in which the cell body is unable to support the structure and metabolism of a distant cellular process (Spencer and Schaumburg, 1976). It seems particularly relevant that Ludwin and Johnson (1981) initially reported the ''dying back'' of oligodendrocytes in a very different model for CNS demyelination in which demyelination is induced by ingestion of the toxic, copper-chelating agent, cuprizone. Dying back oligodendrogliopathy may therefore represent a general mechanism for demyelination in the CNS.

TMEV-specific immune damage Dying back oligodendrogliopathy could be the direct result of viral infection on the cellular physiology of oligodendrocytes. However, it has been demonstrated that the demyelination observed following persistent TMEV infection in susceptible mouse strains is, at least in part, immune mediated (Lipton and Dal Canto, 1976b). Dying back might also be the result of damage due to TMEV-specific immune reactions directed against infected oligodendrocytes.

In cultures of mixed neonatal brain cells, immunoperoxidase staining visualized with electron microscopy demonstrated large amounts of viral antigen on the oligodendrocyte cell membrane (Rodriguez *et al*., 1988). Similarly, staining *in vivo* can detect virus antigen in persistently infected oligodendrocytes by 28 days post-infection (Rodriguez *et al*., 1983). Antigen is present in the cell cytoplasm as well as in the myelin lamellae. Infected animals typically produce high titers of virus-specific antibody and the recognition of virus antigen on the cell surface by these immunoglobulins may result in oligodendrocyte injury through several antibody dependent mechanisms including complement activation, antibody-dependent cell-mediated cytotoxicity (ADCC), or receptor mediated phagocytosis by macrophages.

Alternatively, viral antigen might also be presented on the cell surface in the context of the MHC class I or class II proteins. MHC class I, in particular, is known to be up-regulated in oligodendrocytes following TMEV infection of the CNS (Altintas *et al*., 1993; Lindsley *et al*., 1992). In susceptible mouse strains, class-I MHC is up-regulated in the CNS by one day after infection and remains high in the spinal cord white matter throughout chronic infection and demyelination. A novel antigen such as viral peptide, presented on the cell surface by MHC class-I, might generate a class-I restricted cytotoxic T lymphocyte (CTL) response, which might cause primary demyelination as the result of chronic non-lethal injury to TMEV-infected oligodendrocytes. However, mice with a disrupted β_2-microglobulin (β_2-m) gene do not express significant levels of MHC class-I, do not have functional $CD8^+$ T cells, and therefore cannot mount a CTL response. When β_2-m knockout mice from a resistant haplotype are infected with TMEV, resistance is abrogated and the virus establishes persistent CNS infection. However, significant demyelination develops in these mice (Fiette *et al*., 1993; Pullen *et al*., 1993; Rodriguez *et al*., 1993), demonstrating that $CD8^+$ class I-restricted cytotoxic T cells are probably not the primary mediators of CNS demyelination. In general agreement with these observations, β_2-m knockout mice from a susceptible haplotype display somewhat enhanced levels of demyelination (Begolka *et al*., 2001). Similar results have been obtained with mice carrying a genetic deletion of the gene encoding CD8, which display little alteration in the disease phenotype in either resistant or susceptible strains (Murray *et al*., 1998).

Bystander damage In contrast to observations with MHC class-I and CD8, treatment of susceptible mice with neutralizing antibodies against MHC class-II (anti-Ia) and anti-CD4 has been shown to reduce demyelination (Gerety *et al*., 1994; Rodriguez *et al*., 1986a; Welsh *et al*., 1987). These observations suggest that class-II restricted $CD4^+$ T lymphocytes might play a role in disease pathogenesis. Clatch and coworkers (1986) attempted to correlate the development of clinical disease with several pathophysiologic parameters including virus titer, titer of anti-TMEV specific antibody, delayed-type hypersensitivity (DTH), and T-cell proliferative responses. The extent of TMEV-induced demyelinating disease correlated most closely with the presence of high levels of TMEV-specific DTH.

The development of TMEV specific delayed-type hypersensitivity would require an initial presentation of virus antigen to T_H cells by MHC class-II, resulting in activation and clonal expansion. Generally, the activated T cells are of the $CD4^+$ T_H1 subtype and are often referred to as T_{DTH} cells. Upon secondary contact with antigen, T_{DTH} cells secrete cytokines that recruit and activate macrophages and other nonspecific inflammatory cells. Development of the DTH response may take 48 to 72 hours and by the time the reaction is fully developed, the vast majority of the activated cells at the site of inflammation are nonspecific immune cells and macrophages which act as the primary effector cells of the DTH response. These cells release a variety of cytotoxic mediators which results in nonspecific "bystander" damage to cells in the area of the response. In the CNS of an animal with persistent TMEV infection, any cell that presents virus antigen with MHC class-II might therefore serve as a nidus for the development of a DTH response.

The concept of DTH as a mechanism of demyelination is supported by adoptive transfer experiments using the sTV1 T-cell line or using TMEV sensitized lymph node T cells. sTV1 is a DTH-mediating $CD4^+$ T_H1 line that is specific for the immunodominant

$VP2_{74–86}$ peptide of TMEV. Transfer of 5×10^6 peptide-stimulated sTV1 cells into SJL mice that had been infected with a suboptimal dose of virus, significantly potentiated clinical disease in the recipient mice. Animals that received cells but did not receive virus to provide secondary cellular stimulation did not develop disease. An experimental scheme using lymph node T cells from mice primed with UV-inactivated virus instead of sTV1 cells resulted in similar potentiation of disease but only when virus was present for secondary stimulation (Gerety *et al*., 1994). Further supporting the notion of DTH mediated demyelination, induction of peripheral immune tolerance to TMEV significantly reduces the development of DTH, general inflammation, virus-specific immune responses, and demyelination, following infection with the virus (Karpus *et al*., 1995).

Epitope spreading While the precise mechanisms may not yet be clear, it is well accepted that TMEV persistence in the CNS stimulates a virus-specific immune response that initiates damage to CNS cells and myelin. Recent studies have also detected myelin-reactive T cells in chronically infected mice. The appearance of T cells that are reactive to myelin autoepitopes is presumably due to the development of an immune response to myelin antigens that are released during the initial stages of TMEV-induced demyelination. It has been proposed that these autoreactive T cells are pathogenic and that they may be a major source of pathology during the chronic stages of demyelinating disease. The generation of a myelin-specific pathologic immune response, following an initial event of virus-induced demyelination, is called ''Epitope spreading'' (Lehmann *et al*., 1993; McRae *et al*., 1995).

Earlier attempts to detect myelin reactive T cells from TMEV infected mice had been unsuccessful (Miller *et al*., 1990). However, Miller and coworkers (1997) have now reported that 3 to 4 weeks after initiation of demyelinating disease, T-cell responses to a series of myelin autoepitopes begin to appear in an ordered progression. T-cell proliferation and DTH responses to myelin peptides were first demonstrated at about 50 days post-infection. By 164 days post-infection, reactivity was shown for multiple peptide epitopes from proteolipid protein (PLP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and mouse spinal cord homogenate. T-cell hybridoma clones specific for the immunodominant peptides of TMEV, and lymph node T cells from TMEV primed mice, did not show reactivity to myelin peptides. Conversely, lymph node T cells from myelin primed mice did not show reactivity to TMEV peptides. These results suggest that the reactivity to myelin epitopes was specific and not due to cross-reactivity with TMEV. Additionally, sequence comparison of the reactive myelin peptides to TMEV showed no strong homologies that might indicate a likely source of cross-reactivity.

An epitope spreading mechanism for TMEV-induced demyelinating disease would be very similar to the pathologic mechanism of EAE. Adoptive transfer of myelin reactive immune cells from animals with EAE effectively transfers the disease to naïve animals. However, attempts to transfer TMEV-induced demyelinating disease by the transfer of T cells from infected animals have been unsuccessful (Barbano *et al*., 1984). Initial attempts to suppress TMEV-induced disease by inducing neuroantigen-specific tolerance to a heterogeneous mixture of CNS antigens were unsuccessful (Miller *et al*., 1990). More recently, induction of myelin-specific tolerance with a fusion protein containing MBP and PLP sequences has been reported to reduce demyelination and CNS inflammation in mice with TMEV-induced demyelinating disease (Neville *et al*., 2002).

Studies of TMEV-induced disease in CD4 knockout mice are in potential disagreement with the idea of CD4-dependent mechanisms of demyelination such as bystander damage (DTH) or epitope spreading. Murray and coworkers (1998) examined the effect of CD4 deletion in both resistant (C57BL/6) and susceptible (SJL and PLJ) genetic backgrounds. In a resistant background, the absence of CD4 resulted in the establishment of persistent viral infection in the CNS and with the development of demyelination by 90 days post-infection. In both susceptible strains, demyelination was dramatically increased.

It is not immediately obvious how best to address and reconcile the various observations described here. It might be noted, however, that CD4 plays an important role in immune control of viral infection and that TMEV titers in CD4 knockout mice were generally

elevated during the disease. The development of TMEV-induced pathology likely reflects a delicate balance between viral titer and activity and a dynamic immune response to the virus. As experimental protocols alter various aspects of this balance, such as virus titer and the quality and quantity of the participating immune cells, the mechanisms of pathology may also change in ways that make the interpretation of the data less straightforward than expected. In addition to the complexity of the system, different strains of TMEV are currently being used by the most active research groups. The BeAn 8386 and Daniel's (DA) strains are both widely used and although both establish robust, persistent infections of the spinal cord, the BeAn strain is generally less virulent that the DA strain. The precise significance of the use of these two strains to the interpretation and comparison of data is not yet clear.

Molecular mimicry Like the epitope spreading model, molecular mimicry involves pathogenesis due to myelin reactive autoimmunity. However, in molecular mimicry, the autoimmune response results from structural homologies between TMEV and myelin proteins. Immune recognition of the virus therefore also gives rise to an anti-myelin response, and this autoimmunity is pathogenic.

While molecular mimicry is probably an important mechanism for other CNS diseases (Rouse and Deshpande, 2002; Yuki, 2001), several observations suggest that it may not play a prominent role in TMEV-induced disease. TMEV infection is followed by a rapid and robust immune response that includes the development of both TMEV-specific antibodies and T cells by 7 days post-infection. Demyelinating disease is first observed by about 21 days post-infection and does not reach a peak until 90 to 100 days. The temporal difference between the development of the TMEV-specific immune response and the appearance of demyelinating disease argues against molecular mimicry as an important component of the demyelination mechanism. Furthermore, the initial immune response to TMEV does not cross-react with several myelin antigens including MBP, PLP, and MOG (Miller *et al*., 1997). No strong homologies have been reported between TMEV protein sequences and those of the major myelin proteins.

Mechanisms of Axonal Damage and the Development of Neurologic Deficits

In the past, demyelination alone has often been considered to be sufficient for the development of axonal damage and neurologic deficits. However, both the timing and mechanisms of demyelination and axonal damage can be distinct and different in the TMEV model. Quantitative assessment of demyelination and neurologic ability in two susceptible mouse strains shows that small demyelinated lesions, accompanied by statistically significant decreases in motor ability, can occur as early as 24 days post-infection (McGavern *et al*., 1999). However, axonal damage and neurologic deficits do not necessarily follow demyelination. Several reports have documented that deficiency of MHC class-I (β_2-microglobulin mutants) or MHC class-II (Ab^o mutants) function, will abrogate resistance to TMEV-induced disease in otherwise resistant strains of mice (Fiette *et al*., 1993; Njenga *et al*., 1996; Pullen *et al*., 1993; Rodriguez *et al*., 1993). Resistant mice that are homozygous for either of these mutations develop extensive demyelination. However, class-II deficient mice develop severe neurologic deficits as indicated by significant reductions in spontaneous activity, while class-I deficient mice show no clinical signs. Electrophysiologic measurement of the conduction velocity of motor-evoked potentials shows that decreased conduction velocities accompany deficits in class-II deficient mice with chronic demyelination. Conduction velocities in class-I deficient mice are no different that those in uninfected controls (Rivera-Quinones *et al*., 1998). These findings demonstrate that demyelination and neurologic deficits are genetically and functionally separable, and they implicate a role for MHC class-I in the development of neurologic deficits following demyelination.

A role for MHC class-I in the development of neurologic deficits implicates $CD8^+$ T cells as the pathologic effector cell for the induction of neurologic disease. Granule exocytosis (perforin release) and Fas ligand expression are two of the common cytopathic effector functions of CD8 cells, and mice deficient for perforin, Fas, and Fas ligand were tested to determine whether these molecules play a role in the development of

demyelination and clinical disease (Murray *et al.*, 1998). *Lpr* (Fas mutation) and *Gld* (Fas ligand) mutant mice, on a TMEV-resistant genetic background, maintained resistance to viral persistence and demyelinating disease. Perforin-deficient mice developed persistent viral infection and chronic demyelination of spinal cord white matter. Despite demyelination and virus persistence, these mice showed only minimal neurologic deficits. These studies indicate that perforin release is the pathologic effector for the induction of neurologic disease by $CD8^+$ T cells.

The experiments described earlier for the determination of the roles played by class-I and class-II related mechanisms for the development of axonal damage were all performed in mice with the C57BL/6 or C57BL/6×129 genetic background. C57BL/6 mice mount a vigorous CTL response to TMEV. This response is predominantly directed against a single immunodominant TMEV peptide that represents amino acids 121 to 130 of the VP2 capsid protein, which is presented in the context of the H-$2D^b$ MHC class-I molecule (Borson *et al.*, 1997). At 7 days post-infection, staining of $CD8^+$ brain infiltrating lymphocytes with D^b:$VP2_{121–130}$ tetramers shows that 50 to 63% of the infiltrating $CD8^+$ cells are virus specific (Johnson *et al.*, 1999). Mice with a targeted disruption of the Ifn-γ receptor gene respond to virus infection with rapidly progressing disease and severe clinical deficits. These mice also develop a class-I restricted anti-viral response that is dominanted by the $VP2_{121–130}$ epitope. In these mice, intravenous injection of the VP2 peptide one day before virus infection completely eliminates the immunodominant T-cell response (Johnson *et al.*, 2001). Elimination of the response does not cause a significant increase in viral titer. There is no change in the overall pattern of infection: by 45 days virus is primarily localized to the white matter of the spinal cord, there is no significant increase in inflammation, and demyelination is similar in extent to control animals. Elimination of the response did, however, significantly preserve motor function in infected animals. At 45 days after infection, VP2 peptide treated animals performed significantly better than control animals in tests of motor ability, although not as well as uninfected animals. These results suggest that the $CD8^+$ T cells that mediate neurologic dysfunction are most likely the TMEV-specific cells that infiltrate the CNS following infection.

In contrast to the work done in mice with the TMEV-resistant C57BL/6 genetic background, susceptible SJL mice that lack $CD8^+$ effector function (β_2-microglobulin knockouts) are not rescued from TMEV-induced neurologic deficits (Begolka *et al.*, 2001). Instead, these mice show an earlier onset of neurologic disease, with enhanced CNS demyelination and macrophage infiltration at 50 days post-infection. These results indicate that in these mice, $CD8^+$ cells are not required for the initiation or progression of demyelinating disease and may illustrate the strong influence that genetic background can have on the development of this disease.

Remyelination Following TMEV-Induced Demyelination

Myelin repair, or remyelination, is a normal physiologic response to myelin damage. However, the ability of mice to repair the damage caused by TMEV-induced demyelinating disease is strongly dependent on the genetic constitution of the animal. β_2-microglobulin deficient mice lack MHC class-I function. On a C57BL/6×129 genetic background, these mice develop extensive demyelination following TMEV infection (Rivera-Quinones *et al.*, 1998). Despite the presence of persistent viral infection, these animals show extensive remyelination by 6 months post-infection (Miller *et al.*, 1995). This spontaneous remyelination of CNS axons involves both oligodendrocytes and Schwann cells. In contrast, MHC class-II deficient mice on the same genetic background also develop demyelinating disease but subsequent remyelination is largely absent (Njenga *et al.*, 1999). Class-II deficient mice fail to produce TMEV-specific IgG and eventually developed high viral titers. However, the animals do not die during the acute phase of the disease but survive well into the chronic demyelinating phase, even though most die by 120 days post-infection. Failure to observe remyelination may simply reflect insufficient time for myelin repair to occur before death. Significant axonal damage is also apparent in these mice and this damage may also preclude remyelination.

Like MHC class-II deficient mice, the SJL strain is susceptible to TMEV infection, develops extensive demyelinating disease, and does not repair myelin damage. In contrast to class-II deficient mice, SJL mice control viral infection effectively and can survive for over a year with persistent infection and chronic demyelinating disease. The disease in SJL mice progresses from demyelination, through mild neurologic deficits, to profound deficits and paralysis. Two general hypotheses have been proposed to explain the absence of remyelination in these mice (Miller *et al*., 1996). First, an inhibitory local environment may be present, which prevents spontaneous repair. Local inflammation, reactive astrocytes, microglia, or damaged axons might play a role. Second, an absence of growth factors or oligodendrocyte precursor cells may prevent efficient remyelination. Which of these mechanisms is the most important remains unclear. The absence of spontaneous remyelination in SJL mice, however, has provided an excellent experimental system for the study of ways to enhance the remyelination process, and several potential therapeutic approaches have been studied and developed using this system (Asakura *et al*., 1998; Drescher *et al*., 1998; Njenga *et al*., 2000; Ure and Rodriguez, 2002; Warrington *et al*., 2000).

Whatever the mechanisms of remyelination, it seems clear that remyelination is associated with improved neurologic function. Like the SJL strain, PL/J mice develop chronic demyelinating disease but do not remyelinate. PL/J mice that are deficient for either $CD8^+$ or $CD4^+$ T cells show a significant increase in the severity of disease pathogenesis (Murray *et al*., 1998). The disease is particularly severe in PL/J CD4 mutant mice. While early mortality is common in these animals, many survive up to 6 months post-infection, and many of the mice that survive to the chronic phase of the disease have nearly complete spontaneous remyelination. Importantly, all of the surviving mice show partial recovery of motor function that coincides with myelin repair, and the extent of recovery correlates strongly with the percentage of lesioned white matter area that has remyelinated (Murray *et al*., 2001). These observations clearly demonstrate that functional recovery is possible despite previous demyelination and ongoing persistent virus infection, and that remyelination correlates with this recovery.

MOUSE HEPATITIS VIRUS (MHV)

Murine hepatitis virus (MHV) is a member of the *Coronaviridae*, a family of large, enveloped, plus-strand RNA viruses. MHV is a common enteric pathogen of mice and rats that occasionally disseminates to the CNS. Like TMEV, the original isolation and characterization of one of the most commonly studied strains, MHV-JHM, occurred with the spontaneous appearance of two paralyzed mice, and the subsequent passage of the virus in mouse brain were it continued to produce paralytic disease (Cheever *et al*., 1948; Pappenheimer, 1958). Later passages of the virus resulted primarily in encephalitic disease with accompanying demyelination. The demyelinating phase of MHV infection has been used as a model for human demyelinating disease

Virus Biology and Life Cycle

The MHV genome is a 31 kb message-strand RNA that is capped and polyadenylated. The genomic RNA is complexed with the viral nucleocapsid phosphoprotein (N) to form a long, helical nucleocapsid. The nucleocapsid lies within a lipoprotein envelop that consists largely of intracellular host cell membrane and three viral capsid proteins: membrane protein (M), spike protein (S), and hemagglutinin-esterase protein (HE).

MHV virions bind to the plasma membrane of the host cell by the interaction of the spike proteins with the host receptor. Biliary glycoprotein 1a (Bgp1a), a member of the immunoglobulin superfamily, is a known MHV receptor (Williams *et al*., 1991). The main determinant of cellular tropism is the ability of the spike protein to react with a suitable receptor on the cell surface and infected cell populations in the animal closely match those

with demonstrated expression of the Bgp1a receptor. However, very little Bgp1a is expressed on CNS glia and neurons, and the mechanism of virus entry into these cells is still uncertain. The MHV virion also contains the HE protein. Binding of HE to 9-O-acetylated neuraminic acid residues on the plasma membrane may serve as an important pre-receptor function that facilitates viral entry.

After receptor binding, the viral envelope fuses with the plasma membrane in an S-protein mediated event. Once the RNA genome has entered the cytoplasm of the cell, it is translated into a polyprotein that is co- and post-translationally processed into multiple proteins. These proteins, which include the capsid proteins (N, M, S, and HE), the virus-specific RNA-dependent RNA polymerase, and virus encoded proteases, play various roles in the replication and development of infectious virus. The RNA-dependent RNA polymerase uses the positive-strand genomic RNA as a template to produce full-length negative-strand RNA, which in turn is used to make more positive-strand genomic RNA. Overlapping sets of 3′ co-terminal subgenomic positive-strand RNAs are also produced and translated into viral gene products. The newly synthesized genomic RNA assembles with nucleocapsid (N) protein to form the helical nucleocapsid. The S, HE, and M glycoproteins are all translated on membrane bound polysomes and are co-translationally inserted into intracellular membranes. Virions begin to form when nucleocapsids bind to intracellular membranes that contain the viral M protein and virus then buds from these specialized membranes. S and HE proteins are incorporated into the virions during budding while host proteins are excluded. After budding, mature virions accumulate in large, smooth-walled vesicles that release virus when they fuse with the plasma membrane.

Disease Pathology

The outcome of MHV infection depends on several variables such as virus strain, virus dose and route of infection, age, and genetic composition of the host. Two different MHV strains, MHV-A59 and MHV-JHM, have been used in most studies of MHV-induced demyelination. As the name suggests, hepatitis is a common outcome of MHV infection but while infection with the MHV-A59 strain results in prominent hepatitis, MHV-JHM infected animals show little or no evidence of hepatitis. However, intracerebral or intranasal infection with either strain results in acute, sometimes fatal, encephalitis, followed by the development of chronic demyelination in animals that survive. To decrease mortality and thereby facilitate the study of MHV pathogenesis, several MHV-JHM variants have been developed that have limited neurotropism related lethality but that continue to produce an early encephalitis followed by acute primary demyelination (Erlich *et al.*, 1987; Fleming *et al.*, 1986; Haspel *et al.*, 1978).

MHV infects a variety of CNS cell types including astrocytes, oligodendrocytes, microglia and neurons. As virus replicates to high titers in the brain and spinal cord, it is controlled by the development of an anti-viral immune response that is largely restricted to the CNS. Control of infection correlates well with the appearance of an inflammatory infiltrate by 4 to 7 days post-infection. This acute inflammatory response is characterized by the influx of many types of immune effector cells including $CD4^+$ and $CD8^+$ T cells, B cells, macrophages, and natural killer cells (Williamson *et al.*, 1991). Virus neutralizing antibodies appear in the serum by 7 to 9 days post-infection but probably do not play a critical role in initial virus clearance. Infectious virus is cleared from the CNS by 10 to 14 days but complete clearance is not achieved and virus persists in the CNS. Infectious virus is rarely recovered from spinal cord tissue after the acute phase of infection. Virus antigen can be detected for several months after initial clearance and appears to persist in astrocytes and oligodendrocytes (Sun *et al.*, 1995). Viral RNA can be detected in the CNS for at least a year after infection (Lavi *et al.*, 1984).

Demyelination with axonal sparing can be detected as early as 5 days post-infection. MHV-induced demyelination is illustrated in Figure 44.5. Relatively few inflammatory cells but large numbers of debris containing macrophages characterize demyelinated lesions, and their appearance correlates well with the development of lesions (Lane *et al.*, 2000). Electron microscopy demonstrates macrophage processes extending between the

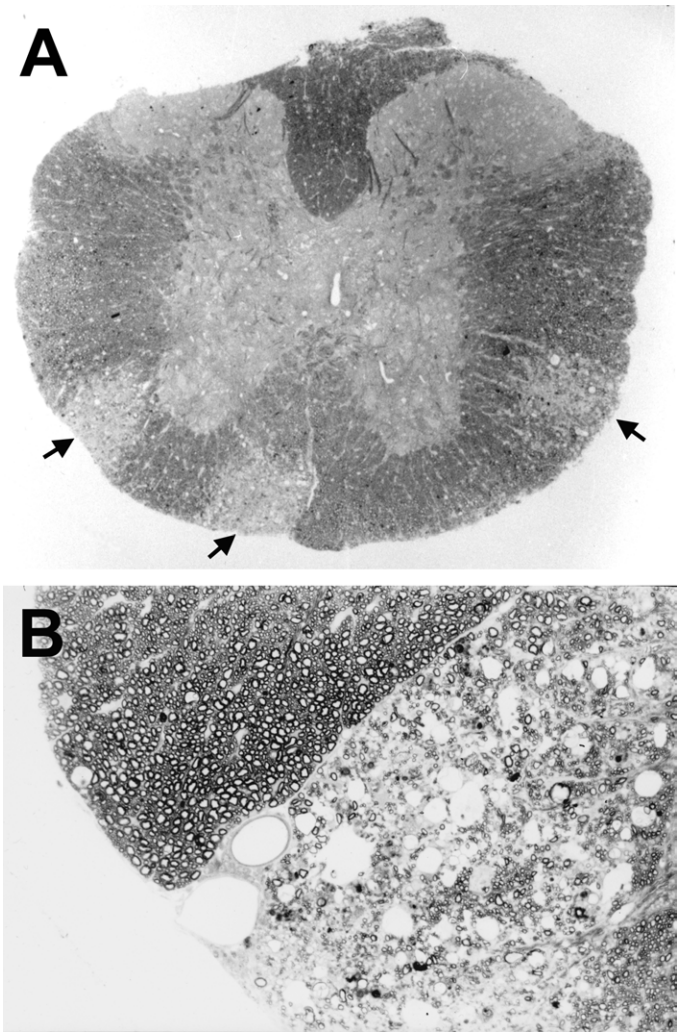

FIGURE 44.5
Pathology of the MHV-infected spinal cord. (A) A section of spinal cord tissue from an SJL mouse infected with MHV-A59 and then stained for myelin with para-phenylene-diamine shows multiple white matter lesions (arrows). (B) Normal white matter lies adjacent to a demyelinated lesion in the ventral spinal cord tracts. Myelin destruction in the lesioned area is apparent.

layers of myelin sheaths, strongly suggesting an active role for macrophages in the demyelination process (Powell and Lampert, 1975). Demyelination continues to develop until about 21 days after infection and then begins to slow. Spontaneous remyelination is common following the initial phase encephalitis and demyelination. After the initial phase of encephalitis, demyelination, and viral clearance, virus persistence results in a second phase that probably continues for the life of the animal and that is associated with the recurrent episodes of demyelination.

Virus Resistance

Infected adult mice generate a potent immune response that clears the virus in 10 to 14 days. $CD4^+$ and $CD8^+$ T cells are the most critical elements of this response. Viral infection results in the rapid recruitment of large numbers of $CD8^+$ T cells to the CNS parenchyma, while $CD4^+$ cells are primarily localized to the perivasculature and meninges.

$CD8^+$ T cells are the primary immune effectors of virus clearance. MHC class-I tetramer staining shows that over 50% of the $CD8^+$ T cells in the CNS after infection are virus specific (Bergmann *et al.*, 1999). As virus is cleared, the total number of $CD8^+$ cells in the

CNS decreases but the percentage of cells that are virus-specific remains high. Initially the infiltrating $CD8^+$ cells display virus-specific cytolytic activity. As clearance progresses and persistence is established, these cells lose their cytolytic function while retaining their ability to secrete Ifn-γ, indicating differential regulation of $CD8^+$ effector functions as the course of infection progresses. Consistent with a role for $CD8^+$ cells in virus clearance, CD8 deficient mice show increases in viral titer, increased mortality, and an inability to efficiently clear virus after infection (Lane *et al*., 2000; Williamson *et al*., 1990).

$CD8^+$ cells probably use two different effector mechanisms to clear virus from different subsets of infected cells. Perforin-mediated cytolysis may play an essential role in the control of virus infection of astrocytes and microglia. Perforin deficient mice are able to clear MHV from the CNS but the clearance is delayed by several days (Lin *et al*., 1997b), indicating that perforin-dependent CTL plays a role in viral clearance from the CNS. Histologic analysis has shown that virus-specific CTL are more efficient at the clearance of virus from astrocytes and macrophages than from oligodendrocytes (Stohlman *et al*., 1995). Ifn-γ is the antiviral mechanism primarily responsible for controlling the infection of oligodendrocytes (Parra *et al*., 1999). In Ifn-γ deficient mice, MHV infection results in increased neurologic disability and mortality that is associated with increased viral titers and delayed viral clearance. The anti-viral antibody response and the CTL response are not affected. Increases of viral antigen in Ifn-γ deficient mice are located almost exclusively in oligodendrocytes and are accompanied by increased numbers of $CD8^+$ cells in CNS white matter. $CD8^+$ cells are therefore major participants in MHV clearance and employ at least two different effectors mechanisms to clear virus from different types of infected cells. Some studies have also suggested that the Fas-Fas ligand system may play a role (Parra *et al*., 2000).

Although the ability to control MHV replication is predominantly controlled by $CD8^+$ cells, this response is highly dependent on the $CD4^+$ T-cell response (Stohlman *et al*., 1998). Adoptive transfer of activated CTL into recipients that have been depleted of $CD4^+$ cells shows that although the $CD8^+$ cells can effectively traffic to sites of CNS infection in the absence of $CD4^+$ cells, once they arrive they rapidly undergo apoptosis. These observations indicate that the survival of the $CD8^+$ cells that are primarily responsible for virus clearance may depend critically on support from $CD4^+$ cells. Consistent with these observations, studies on CD4 deficient mice show increases in viral titer, increased mortality, and an inability to efficiently clear virus, a pathologic phenotype very similar to that observed for CD8 deficient mice (Lane *et al*., 2000).

CD4 deficient mice also exhibit a significant reduction in the number of activated macrophages and microglia in the CNS of infected animals (Lane *et al*., 2000). This may result from a decrease in the expression of the chemokine RANTES that is secreted by $CD4^+$ cells and acts to recruit infiltrating mononuclear cells to the CNS. In strong support of this idea, *in vivo* depletion of RANTES with RANTES-specific antiserum mimics the phenotype seen in CD4 deficient mice.

Despite the increases in viral titer, increased mortality, and inability to clear virus, CD4 mutant mice that survive infection show a general decrease in inflammation and demyelination. It has been proposed that these decreases may result directly from a reduction in the infiltration of macrophages that are involved in myelin destruction. However, depletion of blood-borne macrophages with toxic liposomes prior to infection has little effect on demyelination (Xue *et al*., 1999). Large numbers of activated macrophages/microglia are still present in the CNS after liposome treatment suggesting that the activity of resident microglia may play a central role in demyelination. Macrophage depletion prior to infection does result in increased mortality, suggesting a role for macrophages in the control of viral titers prior to the development of the T-cell responses (Wijburg *et al*., 1997; Xue *et al*., 1999).

The role of antibody in viral clearance and the establishment of persistence has been studied in mice that lack a normal humoral immune response (Lin *et al*., 1999). Mice homozygous for a disruption in the immunoglobulin mu gene lack B cells. In these mice, virus is cleared normally during acute infection but then virus titers increase dramatically after about 11 days post-infection, when the titer of infectious virus in wild-type animals

has fallen to undetectable levels. Passive transfer of MHV immune serum following initial virus clearance prevents this secondary rise in virus titers. These data demonstrate that initial clearance of virus is largely antibody independent but that antibody may play a key role in controlling the reemergence of virus in the CNS during persistent infection.

Mechanisms of Demyelination and Axonal Damage

The mechanisms of demyelination during MHV infection are not yet entirely clear. *A priori,* the potential mechanisms involved in MHV-induced demyelination might be similar to those involved in TMEV-induced demyelinating disease—that is, direct virus lysis of infected oligodendrocytes, virus-specific immune-mediated destruction of oligodendrocytes and myelin, macrophage-mediated ''bystander'' damage to oligodendrocytes and myelin, and myelin damage by ''epitope spreading'' or ''molecular mimicry.''

Several studies have shown that virus actively replicates in oligodendrocytes and that morphologic and biochemical changes accompany oligodendrocyte infection *in vivo* (Barac-Latas *et al*., 1997; Powell *et al*., 1975). Down-regulation of oligodendrocyte gene expression along with oligodendrocyte destruction by necrosis and apoptosis have been reported for both the early and late stages of demyelinating disease (Barac-Latas *et al*., 1997). These types of observations have supported the suggestion that direct viral cytolysis might play a role in the development of demyelination. However, it seems clear that an immune component is essential for the full development of demyelinating disease. JHM-MHV infection of severe combined immunodeficiency (SCID) mice, which lack T lymphocytes, does not result in either demyelination or paralysis (Houtman and Fleming, 1996). Similarly, mice deficient in recombinase-activating gene (RAG) activity lack both B- and T-cell function and do not develop demyelination after MHV infection (Wu and Perlman, 1999). Infected RAG mice, whose immune system has been reconstituted by the adoptive transfer of spleen cells from genetically identical immunocompetent mice that have been immunized for MHV, developed demyelination within 7 to 9 days after transfer. Demyelination rarely occurs if donor mice have not been preimmunized against MHV, demonstrating that MHV-specific T cells are critical for the development of demyelination.

The demyelination that follows reconstitution of immune function is characterized by the extensive recruitment of activated macrophages and microglia to sites of demyelinating lesions, consistent with a potential role for these cells in the development of myelin damage. These observations are also consistent with the idea that RANTES, secreted by $CD4^+$ cells, plays a key role in the recruitment of macrophages to lesions and that activated macrophages and microglia may be primary mediators of myelin destruction (Lane *et al*., 2000). A mechanism of $CD4^+$-dependent, nonspecific damage mediated by infiltrating macrophages bears strong resemblance to the ''bystander'' damage induced by the development of a DTH response in the TMEV model.

Although these data suggest a significant role for the immune system in the development of demyelination, a recent report on the infection of RAG mice with MHV-A59 demonstrated typical demyelinated lesions (Matthews *et al*., 2002). T cells may therefore play a central role in the development of demyelination but other mechanisms may also be important, and the relative contributions of specific mechanisms may depend on variables such as virus strain and the host genetic background.

The question of axonal damage after MHV infection is separate from the question of demyelination. MHV infection is generally characterized by demyelination with axonal sparing, but axonal injury at sites of demyelination has been described (Stohlman and Weiner, 1981). In contrast to the TMEV model, where neurologic deficits tend to occur well after primary demyelination is established, most studies have shown that MHV induced paralysis and demyelination occur almost simultaneously, indicating that axonal damage occurs early in disease. In the experiments described earlier, animals that lack T cells, such as RAG and SCID mice, do not develop demyelination, but reconstitution of these animals by the adoptive transfer of spleen cells results in demyelination by 7 days post-transfer. Axonal damage appears with exactly the same pattern following MHV

infection in these mice (Dandekar *et al*., 2001). Using the appearance of nonphosphorylated neurofilament as a marker for axonal damage, the onset of axonal damage was shown to be coincident with the appearance of demyelination. Damaged axons were detected not only at sites of active demyelination but also in areas with heavy macrophage infiltration but no obvious demyelination. Axonal damage therefore occurs very early in the disease process and nonspecific inflammation may play a key role.

In the TMEV model, the secondary development of a pathogenic immune response directed against myelin antigens or ''epitope spreading'' has been proposed as an important mechanism for the exacerbation and maintenance of demyelination. JHM-MHV infection of either Brown Norway or Lewis rats results in a subacute demyelinating disease. Infection of Lewis rats also results in lymphocytes that proliferate in response to myelin basic protein and that produce mild encephalitis upon adoptive transfer into naïve recipients (Watanabe *et al*., 1983). Lymphocytes from infected Brown Norway rats did not develop MBP sensitivity, demonstrating a strong host strain dependence for this response (Watanabe *et al*., 1987). The production of pathogenic anti-myelin antibodies demonstrates potential for an epitope spreading mechanism of MHV-induced disease, but the extent to which this mechanism contributes to the development of the chronic demyelinating disease is an unresolved issue.

Antibodies, either autoreactive or anti-viral, might directly mediate demyelination by mechanisms such as antibody-dependent or complement-mediated cytotoxicity. In general, little correlation has been found between anti-MHV antibody titers and the severity of demyelination. The role of B cells and antibody has been studied directly in mice with mutations in the immunoglobulin mu gene; these mice lack B cells and make no antibody. In B-cell deficient mice, demyelination following MHV infection is significantly more severe than in controls at 30 to 60 days post-infection (Matthews *et al*., 2002). This increase may be due to the inability of the animals to control virus after the acute phase of the disease. Animals that can produce antibody but lack antibody receptors (Fc) or components of the complement pathway are able to clear virus but still develop demyelination (Matthews *et al*., 2002). These data indicate that antibody and antibody-dependent effector mechanisms are probably not central to the demyelination process.

Demyelination after MHV infection is clearly a complex event and probably results from some combination of direct viral destruction and immune-mediated damage. There does not, however, appear to be any single lymphocyte or monocyte function that is primarily responsible for the immune component of the damage. Both MHV-specific and nonspecific immune responses are probably involved.

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