The Journal of Neuroscience, December 1995, 15(12): 8345–8352

Spontaneous CNS Remyelination in β_2 Microglobulin-Deficient Mice Following Virus-Induced Demyelination

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Animal models with selective genetic immunodeficiencies are useful tools to identify pathogenic mechanisms of disease. Resistant (C57BL/6F 129/J) (H-2^b) mice are rendered susceptible to Theiler's murine encephalomyelitis virus-induced demyelination by genetic disruption of the β_2 microglobulin gene [β_2m(−/−)]. The absence of β_2m prevents the expression of major histocompatibility complex class I molecules and normal levels of functional CD8$^+$ T cells. We tested whether genetic depletion of β_2m would permit CNS remyelination after chronic demyelination induced by the Daniel's strain of Theiler's virus. In contrast to the minimal spontaneous remyelination observed in SJL/J mice after infection with the Daniel's strain of Theiler's virus, chronically infected β_2m(−/−) mice showed extensive and progressive spontaneous CNS remyelination at 6, 12, and 18 months after infection. Spontaneous remyelination by both oligodendrocytes and Schwann cells occurred despite the presence of persistent virus antigen and RNA, but was associated with diminished virus-specific humoral and delayed-type hypersensitivity responses. These experiments support the hypothesis that the immune response inhibits myelin regeneration after virus-induced CNS demyelination.

[Key words: myelin, oligodendrocyte, Schwann cell, MHC class I, CD8$^+$ T cell, delayed-type hypersensitivity, Theiler's virus]

Experimental models of CNS demyelination provide valuable information concerning the morphological, cellular, and molecular mechanisms surrounding myelin repair. The spontaneous remyelination in rodents after toxin-induced CNS demyelination (Ludwin, 1988) indicates that myelin repair is a normal physiological response to myelin damage. In contrast, the relative lack of spontaneous remyelination in chronic multiple sclerosis (MS) lesions suggests that either the presence of inhibitory factors or the absence of endogenous stimulatory factors prevents the full expression of myelin repair in MS patients. Infection of susceptible strains of mice with the Daniel's (DA) strain of Theiler's murine encephalomyelitis virus (TMEV) produces chronic, progressive, inflammatory CNS demyelination that is similar clinically and pathologically to the chronic progressive form of MS (Rodriguez et al., 1987). Spontaneous remyelination in susceptible SJL/J mice infected with the DA strain of TMEV is minimal and limited to the periphery of chronic lesions (Dal Canto and Lipton, 1975; Rodriguez and Lennon, 1990; Rodriguez and Lindsley, 1992; Miller et al., 1994), in part due to an inhibitory T cell-mediated immune response preventing spontaneous myelin repair (Rodriguez and Lindsley, 1992). This suggests that mechanisms preventing complete remyelination in MS patients may also be operative in mice chronically infected with TMEV.

Genetic animal models with selective immunodeficiencies have been used to define the pathogenic mechanisms of CNS demyelination during TMEV infection. Chronic TMEV-induced demyelination is controlled in part by host genes encoded in the major histocompatibility complex (MHC) class I locus (Clatch et al., 1985; Rodriguez et al., 1986b). Mice with the H-2^b haplotype are resistant to chronic infection with TMEV, and clear the virus early during disease without developing CNS demyelination. However, H-2^b mice that have had the β_2 microglobulin gene disrupted by targeted mutation [β_2m(−/−)], and therefore do not have significant levels of MHC class I expression or functional CD8$^+$ T cells (Koller et al., 1990), are no longer resistant and develop CNS demyelination after chronic infection with TMEV (Fiette et al., 1993; Pullen et al., 1993; Rodriguez et al., 1993). Although the chronic demyelinated CNS lesion induced by the DA strain of TMEV in susceptible SJL/J mice shows minimal spontaneous remyelination (Dal Canto and Lipton, 1975; Rodriguez and Lennon, 1990; Rodriguez and Lindsley, 1992; Miller et al., 1994), some CNS lesions in β_2m(−/−) mice showed evidence of remyelination after 90 d (Rodriguez et al., 1993). In this report we extend this observation and demonstrate by quantitative morphometry that β_2m(−/−) (H-2^b) mice showed progressive spontaneous CNS remyelination 6, 12, and 18 months after TMEV infection despite the presence of persistent virus antigen and RNA.

Materials and Methods

Virus. The DA strain of TMEV was used for all experiments. The tissue culture origin and production of this virus have been previously described (Rodriguez et al., 1983a). Virus antigens for enzyme-linked immunosorbant assays (ELISA) and delayed-type hypersensitivity (DTH) assays were purified from infected BHK-21 cells by ultracentrifugation on cesium chloride gradients as described (Rodriguez et al., 1983a).

Mice. Male and female (C57BL/6F × 129/J) (H-2^b) β_2m(−/−) mice were originally obtained from R. Jaenisch (Whitehead Institute, Cambridge, MA) and subsequently bred at the Mayo Clinic. The mice used for the present experiments were an incipient recombinant inbred strain

Received July 31, 1995; accepted Aug. 30, 1995.

This work was supported by National Institutes of Health Grant NS24180, National Multiple Sclerosis Society Grant RG-2203-A-5, and the generous contributions of K. Peterson and E. Applebaum. We thank M. Pierce, K. Pavelko, and S. Reneker for their technical assistance.

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Table 1. Spontaneous CNS remyelination in β_2m (−/−) H-2^b mice chronically infected with TMEV

Months after infection	N	Area of lesion (mm^2)	Area of remyelination (mm^2)	Area remyelination/ area lesion (%)
6	7	1.16 ± 0.35	0.42 ± 0.14	39.5 ± 7.5
12	8	0.40 ± 0.14	0.28 ± 0.10	66.1 ± 7.7
18	7	0.94 ± 0.29	0.79 ± 0.24	81.9 ± 6.2

β_2m(−/−) mice were infected with TMEV and CNS remyelination was measured by a quantitative morphological assessment on 10–12 spinal cord sections from each mouse at 6, 12, or 18 months after infection. The area of lesion represented demyelination in 13%, 3%, and 11% of the total spinal cord white matter area in β_2m(−/−) mice 6, 12, and 18 months after infection, respectively. The data are a composite of three experiments and represent the mean ± SEM, where *N* indicates the number of mice.

derived from the original (C57BL/6F × 129/J) β_2m(−/−) mice. Four week old mice were inoculated intracerebrally with 2 × 10^5 plaque-forming units (PFU) of TMEV in a 10 μl volume. Handling of all animals conformed to the National Institutes of Health and Mayo Clinic institutional guidelines.

Preparation of tissue for light and electron microscopy. Chronically infected β_2m(−/−) mice were anesthetized with 0.2 mg intraperitoneal pentobarbital, exanguinated by cardiac puncture, and killed by intracardiac perfusion with Trump's fixative (100 mM phosphate buffer, pH 7.2, with 4% formaldehyde and 1.5% gluteraldehyde). The entire spinal cord was remove from the spinal canal and sectioned into 1 mm transverse blocks. Every third block was postfixed in 1% osmium tetraoxide and embedded in Araldite (Polysciences, Warrington, PA). One micrometer Araldite sections were cut and stained with *p*-phenylenediamine. To avoid bias, stained slides were coded before quantitation of remyelination. Selected blocks were trimmed and processed for electron microscopy. Thin sections for electron microscopy were counterstained with uranyl acetate and lead citrate.

Assessment of CNS remyelination. Remyelination on Araldite-embedded spinal cord sections was quantitated using a Zeiss interactive digital analysis system and camera lucida attached to a Zeiss photomicroscope (Carl Zeiss Inc., Thornwood, NY) as previously described (Rodriguez and Lennon, 1990). Abnormally thin myelin sheaths relative to axonal diameter was used as the criterion for remyelination by oligodendrocytes, whereas Schwann cell remyelination was identified by abnormally thick myelin sheaths and a one Schwann cell per axon relationship (Ludwin, 1988). Ten to 12 spinal cord sections from each mouse were examined, which corresponded to 8–10 mm^2 of white matter examined per mouse.

DTH responses to TMEV. TMEV-specific DTH responses were elicited in the ear by intradermal injection of 2.5 μg purified TMEV antigens. Ear thickness was measured before injection and at 24 and 48 hr after injection with a dial gauge micrometer.

Antibody responses to TMEV. TMEV-specific immunoglobulin G (IgG) in the serum was quantitated by an ELISA using purified TMEV antigens as previously described (Rodriguez et al., 1986a) with two modifications. We used 0.5 μg of purified TMEV antigens per well in 96 well polystyrene microtiter plates (Corning, Corning, NY). To enhance detection sensitivity, biotinylated goat anti-mouse IgG and streptavidin conjugated alkaline phosphatase (Jackson Immunoresearch, West Grove, PA) was used.

Viral plaque assay. Infectious TMEV titers were determined in clarified CNS homogenates by plaque assay as previously described (Rodriguez et al., 1983b). Brains and spinal cords from chronically infected β_2m(−/−) mice were homogenized in a 10-fold volume of media, sonicated, and clarified by centrifugation. Plaque assays were performed in duplicate on coded samples. The detection limit of the assay was 170 PFU/gm CNS tissue.

Immunohistochemical staining of TMEV antigens. Spinal cords from chronically infected β_2m(−/−) mice were quick frozen in isopentane chilled in liquid nitrogen before liquid nitrogen storage. Longitudinal cryostat sections 10 μm thick were fixed with acetone and immunostained with polyclonal rabbit anti-TMEV sera (Rodriguez et al., 1983a) using the immunoperoxidase technique previously described (Miller et al., 1994). Slides were counterstained with Mayer's hematoxylin.

In situ *hybridization for TMEV RNA.* Spinal cord cryostat sections from chronically infected β_2m(−/−) mice were fixed for 20 min in ice-cold 0.1 M phosphate buffer containing 0.5% paraformaldehyde, 0.5% gluteraldehyde, 0.002% calcium chloride, 1.6% glucose, and 1% dimethyl sulfoxide. Fixed sections were quenched with ice-cold 0.15 M ethanolamine (pH 7.5) for 20 min, washed in phosphate-buffered saline (PBS; 0.1 M NaCl, 50 mM phosphate, pH 7.4), and treated with 1 μg proteinase K in PBS for 30 min at 37°C, and 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min at room temperature. After acetylation, slides were dehydrated in ethanol and prehybridized with 0.5 mg/ml sonicated salmon sperm DNA, 0.5 mg/ml yeast total RNA, and 50 μg/ml yeast tRNA in 50% deinonized formamide, 0.6 M NaCl, 20% 10 × Denhardt's solution, and 1 mM EDTA for 4 hr at room temperature. Slides were hybridized with ^{35}S-labeled 253 (nucleotides 3053–3305) or 363 (nucleotides 3306–3668) base pair cDNA probes corresponding to the VP1 region of the DA strain of TMEV (Ohara et al., 1988). cDNA probes were obtained by double digesting VP1 with KpnI and SalI restriction enzymes and were radiolabeled with α-^{35}S-dATP to between 0.5 and 0.8 × 10^8 cpm/μg DNA by nick translation. Hybridization was carried out overnight at 37°C in 50% formamide, 20% 10 × Denhardt's solution, 1 mM EDTA, 10 mM dithiothreitol, 0.1% SDS, and 20% dextran sulfate containing 0.1 mg/ml sonicated salmon sperm DNA, 0.5 mg/ml total yeast RNA, and 50 μg/ml yeast tRNA. After hybridization, slides were washed with 2 × standard sodium citrate (SSC) containing 1% sodium thiosulfate, 0.05% sodium pyrophosphate, and 0.1% β_2-mercaptoethanol for 2 hr at 55°C and 1 hr at room temperature. Slides were further washed in 1 × SSC containing 1% sodium thiosulfate, 0.05% sodium pyrophosphate, and 0.1% β_2-mercaptoethanol for 1 hr at 55°C, 30 min at room temperature, 1 hr at 37°C, rinsed sequentially in 2 × SSC containing 50% formamide for 15 min and 1 × SSC for 10 min, and dehydrated in a graded series of ethanol containing 0.3 M ammonium acetate. Slides were air dried, immersed in NTB-2 emulsion (Eastman Kodak Company, New Haven, CT), and exposed for 1–3 d at 4°C before developing and fixing. Slides were counterstained with Mayer's hematoxylin.

Statistics. We used an unpaired Student's *t* test assuming unequal variances to evaluate differences in DTH responses. Statistical results were considered significant when $p < 0.05$.

Results

Quantitation of CNS demyelination and remyelination in β_2m(−/−) mice chronically infected with TMEV

Previous studies of TMEV infection in H-2^b β_2m(−/−) mice have primarily addressed the role of CD8$^+$ T cells in the pathogenesis of virus-induced demyelination (Fiette et al., 1993; Pullen et al., 1993; Rodriguez et al., 1993). To assess the effect of the absence of MHC class I and functional CD8$^+$ T cells on remyelination, we quantitated by detailed morphometric analyses CNS demyelination and spontaneous remyelination in the spinal cords of β_2m(−/−) mice 6, 12, and 18 months after infection with TMEV. In contrast to the minimal spontaneous remyelination seen in chronically infected SJL/J mice (Dal Canto and Lipton, 1975; Rodriguez and Lennon, 1990; Rodriguez and Lindsley, 1992; Miller et al., 1994), spontaneous remyelination in β_2m(−/−) mice was extensive and progressive (Table 1). The

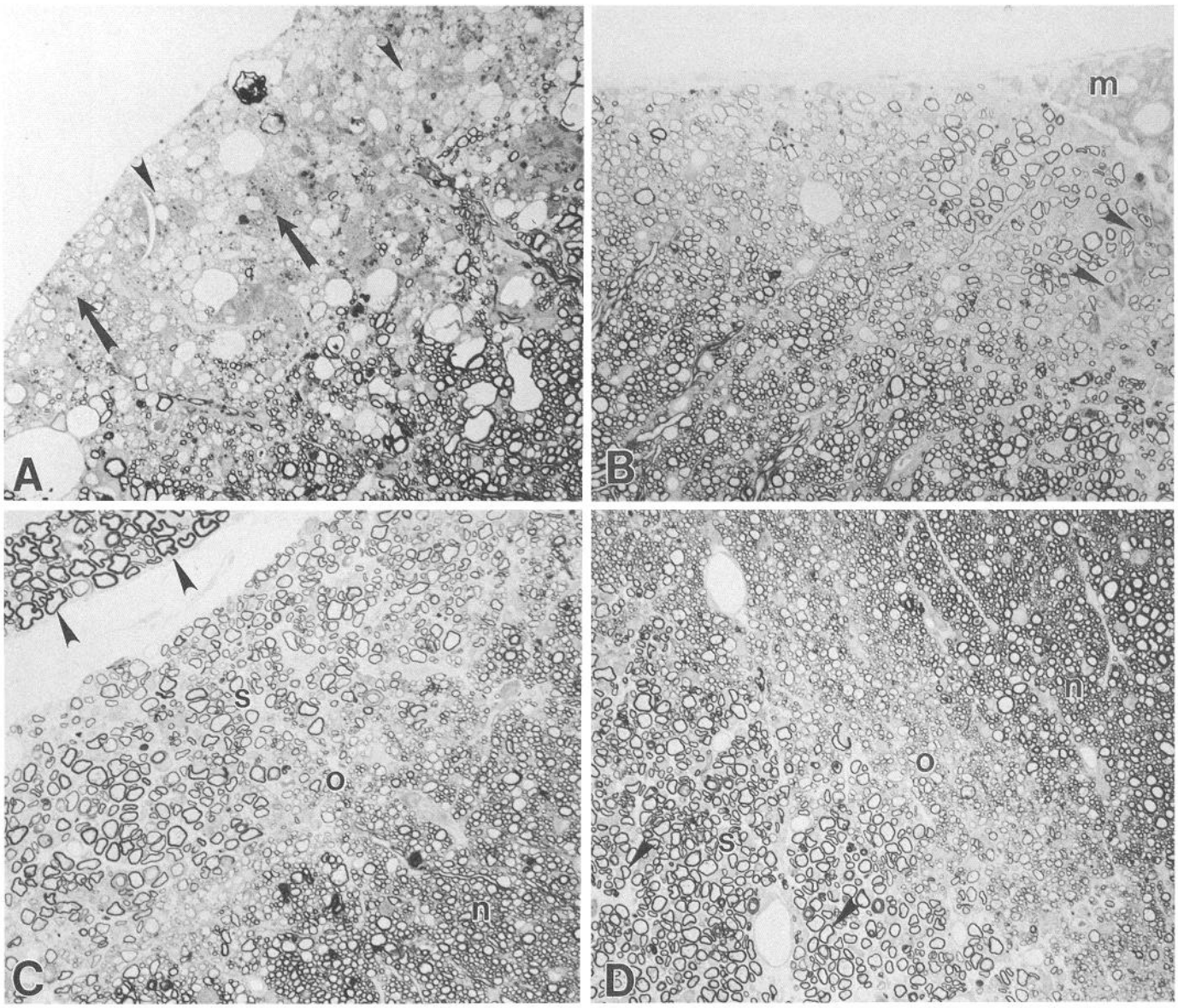
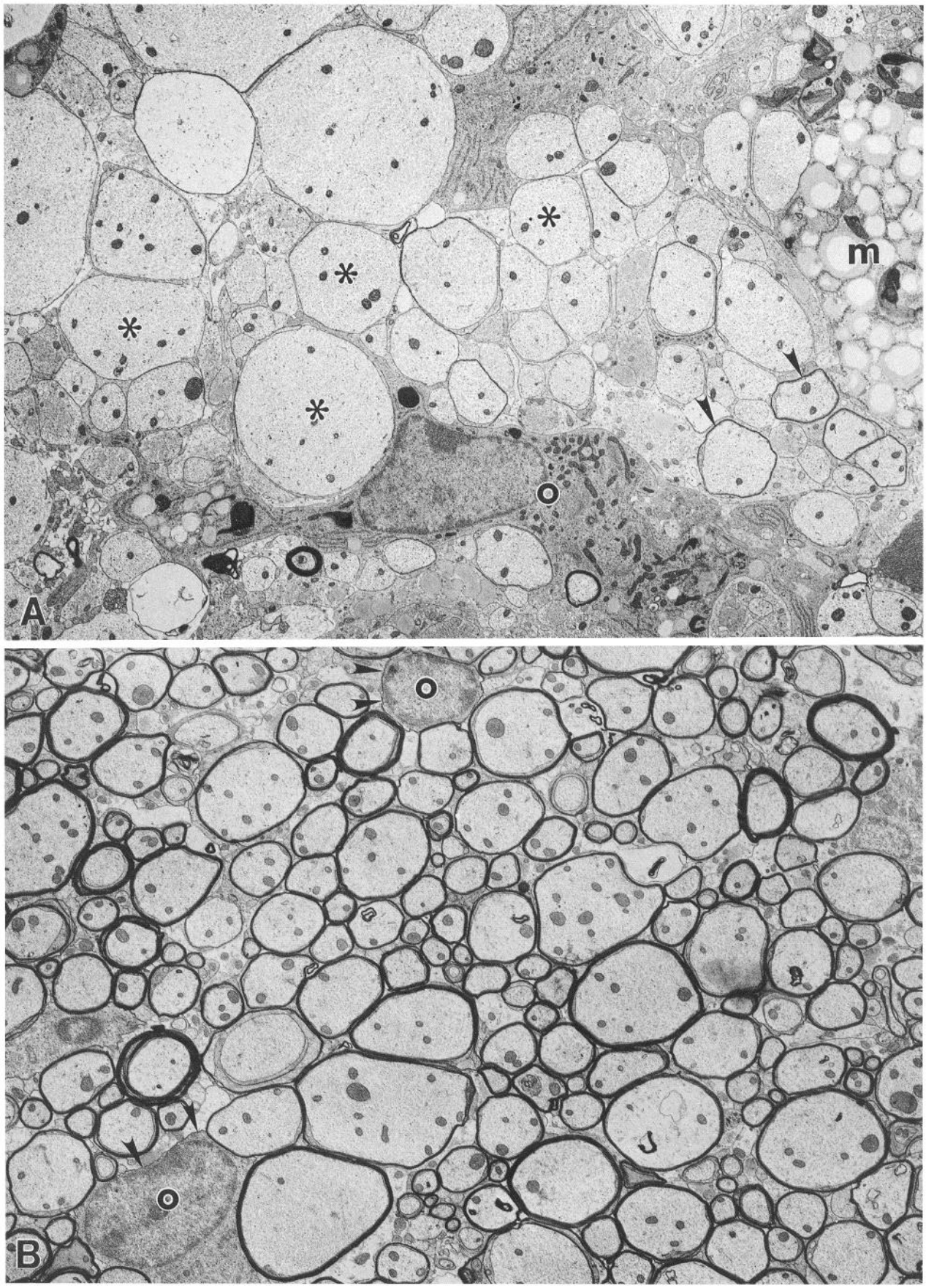


Figure 1. Light micrographs of demyelination and remyelination in β_2m(−/−) mice chronically infected with TMEV. *A*, Demyelinated lesion in the spinal cord of a β_2m(−/−) mice 6 months after infection with TMEV. The lesion is well demarcated and contains primarily naked axons (*arrowheads*) with minimal inflammation. The *arrows* indicate macrophages with ingested myelin debris. *B*, Spinal cord section of a β_2m(−/−) mice 6 months after infection with TMEV showing extensive remyelination directly adjacent to a meningeal perivascular infiltrate (*m*). There are also several inflammatory cells migrating into the parenchyma (*arrowheads*). *C*, Remyelinated lesion in the spinal cord of a β_2m(−/−) mice 12 months after infection with TMEV. A zone of thinly myelinated axons suggestive of oligodendrocyte remyelination (*o*) is between an area of normal myelin (*n*) and an area of thickly myelinated axons suggestive of Schwann cell remyelination (*s*). For comparison, the structure in the upper left is a spinal nerve which contains axons myelinated by Schwann cells (*arrowheads*). *D*, Remyelinated lesion in the spinal cord of a β_2m(−/−) mice 18 months after infection with TMEV showing the characteristic morphology of oligodendrocyte remyelination (*o*) between normal myelin (*n*) and Schwann cell remyelination (*s*). Within this latter area, there are several identifiable Schwann cells with a one to one relationship with axons (*arrowheads*). Alternatively, remyelinated lesions in chronically infected β_2m(−/−) mice may represent different stages of myelin repair by oligodendrocytes, with the thickly myelinated axons representing almost complete restoration of normal CNS myelin thickness. However, although there are no published data that directly address the issue of myelin sheath thickness after virus-induced demyelination, a quantitative morphological study in a toxic model of CNS demyelination indicates that axons remyelinated by oligodendrocytes never reacquire normal myelin sheath thickness (Ludwin and Maitland, 1984). This suggests that Schwann cells are primarily responsible for new myelin synthesis in the areas with thicker myelin sheaths. Magnification, 450×.

total area of demyelination was similar in β_2m(−/−) mice 6 and 18 months after infection, whereas for unknown reasons β_2m(−/−) mice 12 months after infection had less CNS demyelination. Although the extent of CNS disease varied, there was a strong correlation between the area of CNS demyelination and remyelination in individual mice ($R = 0.79$, $p < 0.00002$). Therefore, the most accurate representation of myelin repair was the percentage of lesion area showing remyelination When CNS remyelination was expressed as the percentage of lesion area showing remyelination, CNS remyelination increased with the duration of infection from 40% at 6 months to >80% at 18 months (Table 1). For comparison, SJL/J mice show spontaneous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remyelination in 3–8% of the cumulative demyelinated lesion area 6–8 months after infection with TMEV, and treatment with either immunosuppression or immunotherapy increases remyelination up to 20–30% (Rodriguez and Lennon, 1990; Rodriguez and Lindsley, 1992; Miller et al., 1994). We concluded from these data that H-2^b β_2m(−/−) mice show extensive spontaneous remyelination during chronic infection with the DA strain of TMEV.

Morphology of CNS demyelination and remyelination

CNS demyelination and remyelination were readily identified morphologically in chronically infected β_2m(−/−) mice by both light (Fig. 1) and electron (Fig. 2) microscopy. Consistent with previous observations (Rodriguez et al., 1993), demyelinated lesions in the spinal cords of β_2m(−/−) mice were well circumscribed with minimal parenchymal lymphocyte inflammation (Fig. 1*A*). Demyelinated lesions contained some macrophages with ingested myelin debris, but consisted primarily of naked axons (Fig. 2*A*).

Extensive remyelination was observed in spinal cord lesions of β_2m(−/−) mice 6 (Fig. 1*B*), 12 (Fig. 1*C*), and 18 (Fig. 1*D*) months after infection. Abnormally thin myelin sheaths, a characteristic of remyelination by oligodendrocytes (Ludwin, 1988), were evident by electron microscopy (Fig. 2*B*). Several remyelinated lesions in β_2m(−/−) mice 12 and 18 months after infection had a characteristic appearance (Fig. 1, *C* and *D*, respectively). A zone of thinly myelinated axons surrounded an area of axons with thick myelin sheaths suggestive of Schwann cell remyelination. However, not all of these remyelinated axons were associated with a readily identifiable Schwann cell by light microscopy (Fig. 1*D*). Remyelinated lesions with a similar morphology have been observed in C3H/He (Dal Canto and Lipton, 1979) and strain A (D. J. Miller and M. Rodriguez, unpublished observations) mice infected with the DA strain of TMEV, in CBA mice infected with the BeAn strain of TMEV (Blakemore et al., 1988), and in outbred Swiss mice (CD-1) infected with the attenuated WW strain of TMEV (Dal Canto and Lipton, 1980; Dal Canto and Barbano, 1984). These data indicated that both oligodendrocytes and Schwann cells contributed to the spontaneous remyelination in β_2m(−/−) mice chronically infected with the DA strain of TMEV.

TMEV-specific DTH responses in chronically infected β_2m(−/−) mice

Several studies have indicated a strong correlation between the DTH response to virus antigens and demyelination during chronic infection with TMEV (Clatch et al., 1985, 1986). To investigate the potential relationship between virus-specific DTH responses and remyelination, we measured DTH responses to purified TMEV antigens in β_2m(−/−) mice 6 and 18 months after infection (Table 2). At 6 months after infection, β_2m(−/−) mice showed a strong DTH response at both 24 and 48 hr after intradermal injection of TMEV antigens. In contrast, β_2m(−/−) mice 18 months after infection showed only minimal DTH responses that were not statistically different from uninfected β_2m(−/−) mice (Table 2). These data indicated an inverse relationship between spontaneous remyelination and virus-specific DTH responses in β_2m(−/−) mice chronically infected with TMEV.

Table 2. DTH responses to TMEV antigens in chronically infected β_2m(−/−) H-2^b mice

Months after infection	N	Δ Ear swelling at 24 hr (10^{-2} mm)	p	Δ Ear swelling at 48 hr (10^{-2} mm)	p
None	9	4.6 ± 0.2	—	2.8 ± 0.5	—
6	8	10.0 ± 0.4	$<10^{-7}$	8.6 ± 0.5	$<10^{-6}$
18	11	6.1 ± 0.7	>0.06	3.8 ± 0.6	>0.19

β_2m(−/−) mice were infected with TMEV and DTH responses were assessed at 6 and 18 months after infection. For uninfected controls, 6 week old β_2m(−/−) mice were used. Mice were injected intradermally in the ear with 2.5 μg purified TMEV antigens. Ear swelling is expressed as the change in thickness over the preinjection measurement. The data are a composite of two experiments and represent the mean ± SEM, where *N* indicates the number of mice. Statistical comparisons were made with the data from uninfected β_2m(−/−) mice.

TMEV-specific antibody responses in chronically infected β_2m(−/−) mice

We measured by ELISA the humoral immune response directed against TMEV antigens in β_2m (−/−) mice (Fig. 3). TMEV-specific IgG was detected in the serum of β_2m(−/−) mice at all time points after infection, from 7 d to 18 months after intracerebral inoculation with TMEV. The TMEV-specific antibody responses in chronically infected β_2m(−/−) peaked at 6 months after infection, but subsequently diminished and reached a plateau by 12–18 months after infection (Fig. 3, inset).

Persistent virus antigen and RNA in the spinal cords of chronically infected β_2m(−/−) mice

We examined whether the CNS of β_2m(−/−) mice contained infectious virus 6 and 18 months after infection with TMEV using a viral plaque assay. We detected infectious virus in one of four β_2m(−/−) mice 6 months after infection, but none of four β_2m(−/−) mice 18 months after infection. These data are consistent with the observation that virus titers in β_2m(−/−) mice diminish with increasing duration of infection (Rodriguez et al., 1993).

Although the presence or absence of infectious virus may be a reliable measure of susceptibility or resistance to TMEV-induced demyelination in some cases (Rodriguez et al., 1986b), local virus antigen and RNA production in the spinal cord correlate better with pathology (Chamorro et al., 1986; Patick et al., 1990, 1991; Rodriguez et al., 1992). Therefore, we examined the expression of TMEV antigens and RNA in the spinal cords of chronically infected β_2m(−/−) mice by immunohistochem-

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Figure 2. Electron micrographs of demyelination and remyelination in β_2m(−/−) mice chronically infected with TMEV. *A*, Spinal cord section from a β_2m(−/−) mice 6 months after infection with TMEV showing multiple demyelinated ("naked") axons (*asterisks*) and a macrophage process (*m*) containing vacuoles with degraded myelin debris. There are also several axons with very thin myelin sheaths (*arrowheads*) suggestive of early remyelination. These axons are adjacent to a cell with a morphology suggestive of an oligodendrocyte (*o*), with dense chromatin, minimal cytoplasm, and the absence of filaments. *B*, Spinal cord section from a β_2m(−/−) mice 6 months after infection with TMEV showing numerous axons with abnormally thin myelin sheaths characteristic of oligodendrocyte remyelination. Directly adjacent to several remyelinated axons are two oligodendrocytes (*o*) with a thin layer of myelin around their cytoplasm (*arrowheads*), indicative of active myelin regeneration. Magnification, 5400×.

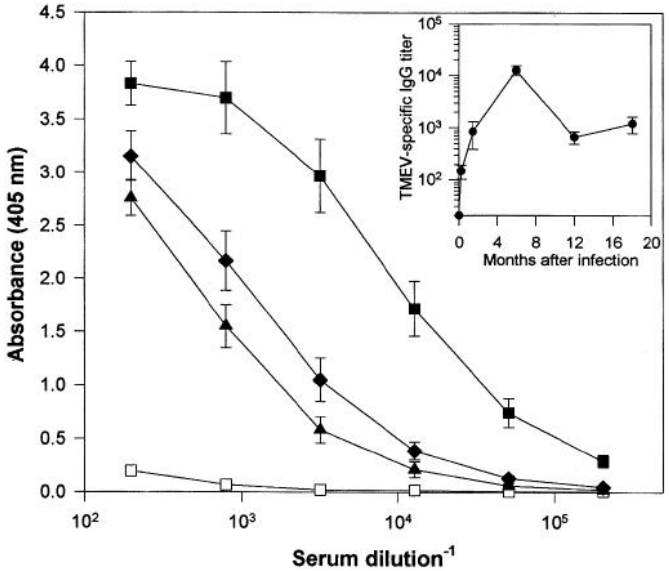

Figure 3. Virus-specific IgG responses in β_2m(−/−) mice infected with TMEV. Serum from uninfected β_2m(−/−) mice (□) and β_2m(−/−) mice 6 (■), 12 (▲), and 18 (♦) months after infection with TMEV were assayed for virus-specific IgG by ELISA using purified TMEV antigens. Data represent the mean ± SEM of five to nine mice per group. *Inset*, Time course of the TMEV-specific IgG response in β_2m(−/−) mice infected with TMEV. The IgG titer is the serum dilution giving a half-maximal absorbance at 405 nm in a TMEV antigen-specific ELISA. Data represent the mean ± SEM of five to nine mice at each time point after infection.

istry and *in situ* hybridization, respectively (Fig. 4). We detected TMEV antigen-positive cells in spinal cord white matter lesions in three of four β_2m(−/−) mice 6 months after infection and three of four β_2m(−/−) mice 18 months after infection (Fig. 4*A*). Similarly, TMEV RNA-positive cells were present in the spinal cords of β_2m(−/−) mice 6 months after infection (Fig. 4*B*). These data indicated that spontaneous remyelination in β_2m(−/−) mice occurred despite the presence of persistent virus antigen and RNA, consistent with previous observations (Patick et al., 1991).

Discussion

Experimental manipulation of established animal models of CNS demyelination has provided potential explanations for the relative absence of spontaneous remyelination in chronic MS lesions. For example, nearly complete remyelination is seen in rodents after acute ingestion of the toxin Cuprizone (Blakemore, 1973; Ludwin, 1988). However, chronic ingestion of Cuprizone prevents spontaneous remyelination due to the depletion of oligodendrocytes needed for myelin synthesis (Ludwin, 1980), which may mimic the situation in chronic MS lesions. The presence of an inhibitory immune response can also prevent spontaneous remyelination. In guinea pigs with chronic experimental autoimmune encephalomyelitis, injection of myelin basic protein and galactocerebroside in incomplete adjuvant promotes oligodendrocyte proliferation and remyelination through postulated suppression of a deleterious immune response (Traugott et al., 1982; Raine et al., 1988). We have used the TMEV model of CNS demyelination to identify the immunological mechanisms preventing spontaneous remyelination in chronically infected SJL/J mice. Immunosuppression using cyclophosphamide or monoclonal antibodies against CD4 or CD8 enhances remyelination (Rodriguez and Lindsley, 1992). In addition, treatment with a monoclonal natural autoantibody with potential immunomodulatory activity promotes CNS remyelination in chronically infected SJL/J mice (Miller et al., 1994; Miller and Rodriguez, 1995). In this report we demonstrate that β_2m(−/−) mice chronically infected with the DA strain of TMEV showed extensive spontaneous remyelination associated with diminished virus-specific immune responses. These observations indicate that chronic infection of β_2m(−/−) mice with the DA strain of

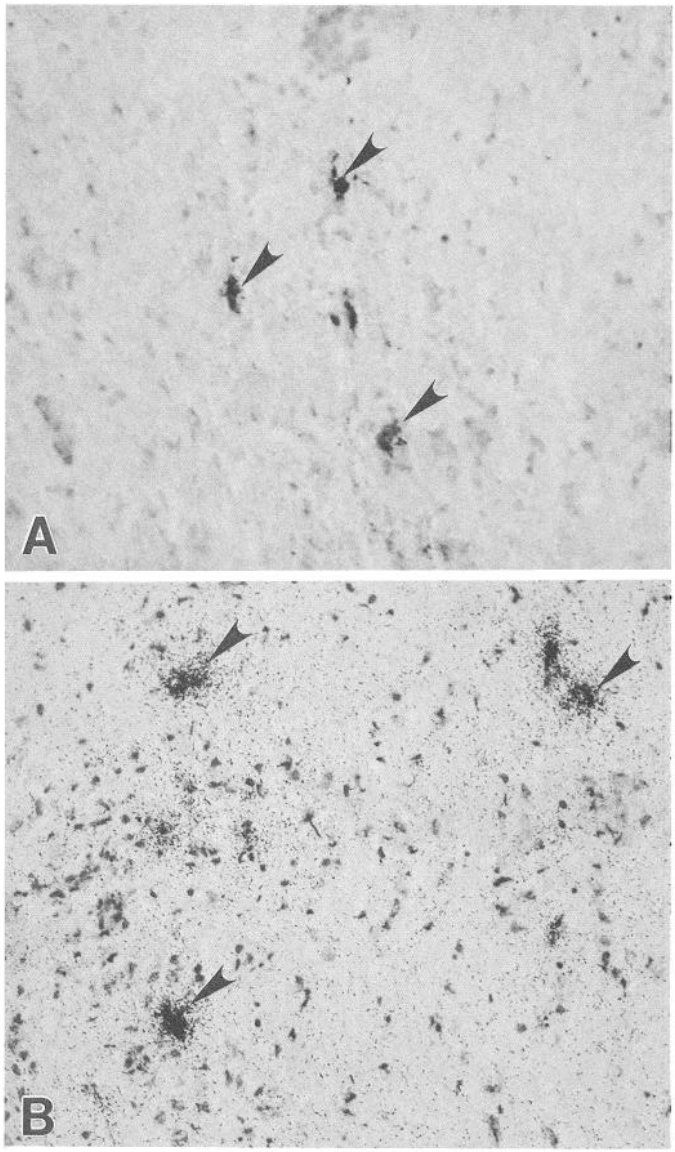

Figure 4. TMEV antigen- and RNA-positive cells in the spinal cord of chronically infected β_2m(−/−) mice. *A*, Immunoperoxidase staining for TMEV antigens in a cryostat section from a β_2m(−/−) mouse 6 months after infection showing virus antigen-positive cells (*arrowheads*) in a spinal cord white matter lesion. The dark reaction product indicates immunoreactivity with polyclonal rabbit anti-TMEV sera. *B*, *In situ* hybridization for TMEV RNA in a cryostat section from a β_2m(−/−) mouse 6 months after infection showing virus RNA-positive cells (*arrowheads*) in a spinal cord white matter lesion. The dark silver grains indicate hybridization with an ^{35}S-labeled cDNA probe corresponding to the VP1 region of TMEV. *In situ* hybridization on spinal cord sections from uninfected β_2m(−/−) mice showed no hybridization with the VP1 cDNA probes. Sections were counterstained with Mayer's hematoxylin. Magnification, 280×.

TMEV provides the opportunity to further investigate the inhibitory factors preventing spontaneous remyelination that may be relevant to MS.

Spontaneous CNS remyelination in β_2m(−/−) mice associated with diminished virus-specific immune responses is consistent with the observation that treatment with monoclonal antibodies to CD4 or CD8 promotes CNS remyelination in SJL/J mice chronically infected with TMEV (Rodriguez and Lindsley, 1992). It is difficult to directly compare the β_2m(−/−) mice used in the present experiments, which have the normally resistant H-2^b haplotype, with SJL/J mice, which have the susceptible H-2^s haplotype. At 6 months after infection, SJL/J mice show demyelination in 15–20% of their spinal cord white matter, with minimal spontaneous remyelination. In β_2m(−/−) (H-2^b) mice 6 months after infection there was demyelination in approximately 13% of the spinal cord white matter, with significant spontaneous remyelination. This suggests that the initial severity of demyelination may influence the subsequent degree of myelin repair. However, we observed a strong correlation between the area of demyelination and remyelination in chronically infected β_2m(−/−) mice, indicating that initial disease severity did not adversely affect subsequent myelin repair.

The association between genetic deletion of β_2m and extensive spontaneous remyelination after chronic infection with TMEV suggests that $CD8^+$ T cells inhibit myelin repair, consistent with the implication of cytotoxic $CD8^+$ T cell responses in the pathogenesis of TMEV-induced demyelination (Rodriguez and Sriram, 1988; Lindsley et al., 1991). Although β_2m(−/−) mice were originally thought to have no functional $CD8^+$ T cells (Koller, 1990), several reports now indicate that functional $CD8^+$ cytotoxic T cells can be induced in β_2m(−/−) mice (Apasov and Sitkovsky, 1994; Glas et al., 1994; Udaka et al., 1994). These $CD8^+$ T cells require *in vivo* priming, and have been shown to react primarily with syngeneic or allogeneic MHC molecules. Chronic virus infection may have provided the necessary stimulatory signals to expand $CD8^+$ T cells in β_2m(−/−) mice. However, treatment with monoclonal antibodies to CD8 did not alter disease in β_2m(−/−) mice infected with lymphocytic choriomeningitis virus (Quinn et al., 1993) or Sendai virus (Hou et al., 1992). No $CD8^+$ T cells were present in the CNS of β_2m(−/−) mice 45 d after infection with TMEV (Rodriguez et al., 1993), nor did we find $CD8^+$ T cells in spinal cord cryostat sections of β_2m(−/−) mice 6 or 18 months after infection (data not shown). In addition, we have not been able to demonstrate virus-specific $CD8^+$ cytotoxic T cells in CNS infiltrates from chronically infected β_2m(−/−) mice (X. Lin, personal communication). These observations indicate that chronic virus infection does not stimulate the production of $CD8^+$ T cells in β_2m(−/−) mice and argues against the presence of functional $CD8^+$ T cells in β_2m(−/−) mice chronically infected with TMEV. Although the data presented in this report suggest an inhibitory role for $CD8^+$ T cells, it was not possible to directly compare remyelination in H-2^b β_2m(−/−) mice with the proper control animals, as H-2^b mice without the β_2m deletion do not develop demyelination (Fiette et al., 1993; Pullen et al., 1993; Rodriguez et al., 1993). We are currently investigating spontaneous CNS remyelination in chronically infected β_2m(−/−) mice that express a susceptible MHC haplotype.

The extensive spontaneous remyelination in chronically infected β_2m(−/−) mice was associated with diminished TMEV-specific antibody and DTH responses in the absence of complete virus clearance. This suggests that immune responses directed by $CD4^+$ T cells may also inhibit myelin repair in mice chronically infected with TMEV. Previous studies have demonstrated that the humoral immune response plays a beneficial rather than pathogenic role for both demyelination (Rodriguez et al., 1988, 1990, 1994; Rossi et al., 1991) and remyelination (Rodriguez and Lennon, 1990; Miller et al., 1994). In contrast, the virus-specific DTH response has been implicated in the pathogenesis of TMEV-induced demyelination (Clatch et al., 1985, 1986). Susceptible SJL/J mice, which show minimal spontaneous remyelination, have a prominent TMEV-specific DTH response which remains elevated for up to 6 months after infection (Clatch et al., 1986). Although we also observed a strong TMEV-specific DTH response in β_2m(−/−) mice 6 months after infection at a time of substantial spontaneous remyelination, genetic differences between mouse strains and different assay techniques make a direct comparison between our data and previously published results difficult to interpret. However, when we assessed DTH responses at 6 and 18 months after infection, myelin repair in β_2m(−/−) mice was inversely related to the TMEV-specific DTH response. This suggests that in the absence of $CD8^+$ T cells, the $CD4^+$ T cell-mediated DTH immune response directed against virus also inhibits remyelination following virus-induced demyelination.

The outcome of a chronic demyelinating disease depends on the balance between myelin destruction and myelin repair. The demonstration of recurrent demyelination in previously remyelinated lesions in both MS patients (Prineas et al., 1984) and in mice infected with TMEV (Dal Canto and Lipton, 1979) also indicate that these processes are not mutually exclusive. The observation that remyelination in β_2m(−/−) mice begins early during infection (Rodriguez et al., 1993) and increases during chronic infection suggests that in chronically infected β_2m(−/−) mice the balance shifts from predominantly myelin destruction to predominantly myelin repair. One explanation for this phenomena is that demyelinating activity decreases with a subsequent increase in endogenous myelin repair activity. This explanation is based on the hypothesis that the disease process responsible for demyelination (i.e., the TMEV-specific DTH response) also indirectly inhibits remyelination. Consequently, current experimental therapies for MS designed to prevent the demyelinating process through inhibition of presumably pathogenic $CD4^+$ T cells (Steinman, 1991) may also secondarily promote myelin repair. An alternative explanation is that the unique inhibitory factors normally preventing remyelination during chronic TMEV infection are absent or depressed in β_2m(−/−) mice, thereby allowing myelin repair to progress. The absence of functional $CD8^+$ T cells in β_2m(−/−) mice make these immune effectors likely inhibitory candidates, and suggest that manipulation of the number of $CD8^+$ T cells or their function might enhance remyelination in MS patients.

References

Apasov SG, Sitkovsky MV (1994) Development and antigen specificity of $CD8^+$ cytotoxic T lymphocytes in β_2-microglobulin-negative, MHC class I-deficient mice in response to immunization with tumor cells. J Immunol 152:2087–2097.

Blakemore WF (1973) Remyelination of the superior cerebellar peduncle in the mouse following demyelination induced by feeding Cuprizone. J Neurol Sci 20:73–83.

Blakemore WF, Welsh CJR, Tonks P, Nash AA (1988) Observations on demyelinating lesions induced by Theiler's virus in CBA mice. Acta Neuropathol (Berl) 76:581–589.

Chamorro M, Aubert C, Brahic M (1986) Demyelinating lesions due

to Theiler's virus are associated with ongoing central nervous system infection. J Virol 57:992–997.

Clatch RJ, Melvold RW, Miller SD, Lipton HL (1985) Theiler's murine encephalomyelitis virus (TMEV) induced demyelinating disease in mice is influenced by the H-2D region: correlation with TMEV-specific delayed-type hypersensitivity. J Immunol 135:1408–1414.

Clatch RJ, Lipton HL, Miller SD (1986) Characterization of Theiler's murine encephalomyelitis virus (TMEV)-specific delayed-type hypersensitivity responses in TMEV-induced demyelinating disease: correlation with clinical signs. J Immunol 136:920–927.

Dal Canto MC, Barbano RL (1984) Remyelination during remission in Theiler's virus infection. Am J Pathol 116:30–45.

Dal Canto MC, Lipton HL (1975) Primary demyelination in Theiler's virus infection: an ultrastructural study. Lab Invest 33:626–637.

Dal Canto MC, Lipton HL (1979) Recurrent demyelination in chronic central nervous system infection produced by Theiler's murine encephalomyelitis virus. J Neurol Sci 42:391–405.

Dal Canto MC, Lipton HL (1980) Schwann cell remyelination and recurrent demyelination in the central nervous system of mice infected with attenuated Theiler's virus. Am J Pathol 98:101–122.

Fiette L, Aubert C, Brahic M, Rossi CP (1993) Theiler's virus infection of β_2-microglobulin-deficient mice. J Virol 67:589–592.

Glas R, Ohlen C, Hoglund P, Karre K (1994) The $CD8^+$ T cell repertoire in β_2-microglobulin-deficient mice is biased toward reactivity against self-major histocompatibility class I. J Exp Med 179:661–672.

Hou S, Doherty PC, Zijlstra M, Jaenisch R, Katz JM (1992) Delayed clearance of Sendai virus in mice lacking class I MHC-restricted $CD8^+$ T cells. J Immunol 149:1319–1325.

Koller BH, Marrack P, Kappler JW, Smithies O (1990) Normal development of mice deficient in β_2m, MHC class I proteins, and $CD8^+$ T cells. Science 248:1227–1230.

Lindsley MD, Thiemann R, Rodriguez M (1991) Cytotoxic T cells isolated from the central nervous system of mice infected with Theiler's virus. J Virol 65:6612–6620.

Ludwin SK (1980) Chronic demyelination inhibits remyelination in the central nervous system: an analysis of contributing factors. Lab Invest 43:382–387.

Ludwin SK (1988) Remyelination in the central nervous system and the peripheral nervous system. Adv Neurol 47:215–254.

Ludwin SK, Maitland M (1984) Long-term remyelination fails to reconstitute normal thickness of central nervous system myelin sheaths. J Neurol Sci 64:193–198.

Miller DJ, Rodriguez M (1995) A monoclonal antibody which promotes central nervous system remyelination in a model of multiple sclerosis is a natural autoantibody encoded by germline immunoglobulin genes. J Immunol 154:2460–2469.

Miller DJ, Sanborn KS, Katzmann JA, Rodriguez M (1994) Monoclonal autoantibodies promote central nervous system repair in an animal model of multiple sclerosis. J Neurosci 14:6230–6238.

Ohara Y, Stein S, Fu J, Stillmann L, Klaman R, Roos RP (1988) Molecular cloning and sequence determination of DA strain of Theiler's murine encephalomyelitis viruses. Virology 164:245–255.

Patick AK, Pease LR, David CS, Rodriguez M (1990) Major histocompatibility complex-conferred resistance to Theiler's virus-induced demyelinating disease is inherited as a dominant trait in B10 congenic mice. J Virol 64:5570–5576.

Patick AK, Thiemann RL, O'Brien PC, Rodriguez M (1991) Persistence of Theiler's virus infection following promotion of central nervous system remyelination. J Neuropathol Exp Neurol 50:523–537.

Prineas JW, Kwon EE, Cho ES, Sharer LR (1984) Continual breakdown and regeneration of myelin in progressive multiple sclerosis plaques. Ann NY Acad Sci 436:11–32.

Pullen LC, Miller SD, Dal Canto MC, Kim BS (1993) Class I-deficient resistant mice intracerebrally inoculated with Theiler's virus show an increased T cell response to viral antigens and susceptibility to demyelination. Eur J Immunol 23:2287–2293.

Quinn DG, Zajac AJ, Frelinger JA, Muller D (1993) Transfer of lymphocytic choriomeningitis disease in β_2-microglobulin-deficient mice by $CD4^+$ T cells. Int Immunol 5:1193–1198.

Raine CS, Moore GRW, Hintzen R, Traugott U (1988) Induction of oligodendrocyte proliferation and remyelination after chronic demyelination. Relevance to multiple sclerosis. Lab Invest 59:467–476.

Rodriguez M, Lennon VA (1990) Immunoglobulins promote remyelination in the central nervous system. Ann Neurol 27:12–17.

Rodriguez M, Lindsley MD (1992) Immunosuppression promotes CNS remyelination in chronic virus-induced demyelinating disease. Neurology 42:348–357.

Rodriguez M, Sriram S (1988) Successful therapy of Theiler's virus-induced demyelination (DA strain) with monoclonal anti-Lyt-2 antibody. J Immunol 140:2950–2955.

Rodriguez M, Leibowitz JL, Lampert PW (1983a) Persistent infection of oligodendrocytes in Theiler's virus-induced encephalitis. Ann Neurol 13:426–433.

Rodriguez M, Leibowitz JL, Powell C, Lampert PW (1983b) Neonatal infection of oligodendrocytes in Theiler's virus-induced encephalomyelitis. Lab Invest 49:672–679.

Rodriguez M, Lafuse WP, Leibowitz J, David CS (1986a) Partial suppression of Theiler's virus-induced demyelination *in vivo* by administration of monoclonal antibodies to immune response gene products (Ia antigens). Neurology 36:964–970.

Rodriguez M, Leibowitz J, David CS (1986b) Susceptibility to Theiler's virus-induced demyelination: mapping of the gene within the H-2D region. J Exp Med 163:620–631.

Rodriguez M, Oleszak E, Leibowitz J (1987) Theiler's murine encephalomyelitis virus: a model of demyelination and viral persistence. CRC Crit Rev Immunol 7:325–365.

Rodriguez M, Lucchinetti CF, Clark RJ, Yaksh TL, Markowitz H, Lennon VA (1988) Immunoglobulins and complement in demyelination induced in mice by Theiler's virus. J Immunol 140:800–806.

Rodriguez M, Kenny JJ, Theimann RL, Woloschak GE (1990) Theiler's virus-induced demyelination in mice immunosuppressed with anti-IgM and in mice expressing the *xid* gene. Microbiol Pathol 8:23–35.

Rodriguez M, Patick AK, Pease LR, David CS (1992) Role of T cell receptor Vβ genes in Theiler's virus-induced demyelination in mice. J Immunol 148:921–927.

Rodriguez M, Dunkel AJ, Thiemann RL, Leibowitz J, Zijlstra M, Jaenisch R (1993) Abrogation of resistance to Theiler's virus-induced demyelination in $H\text{-}2^b$ mice deficient in β_2-microglobulin. J Immunol 151:266–276.

Rodriguez M, Pavelko KD, McKinney CW, Leibowitz JL (1994) Recombinant human IL-6 suppresses demyelination in a viral model of multiple sclerosis. J Immunol 153:3811–3821.

Rossi CP, Cash E, Aubert C, Coutinho A (1991) Role of the humoral immune response in resistance to Theiler's virus infection. J Virol 65:3895–3899.

Steinman L (1991) The development of rational strategies for selective immunotherapy against autoimmune demyelinating diseases. Adv Immunol 49:357–379.

Traugott U, Stone SH, Raine CS (1982) Chronic relapsing experimental autoimmune encephalomyelitis. Treatment with combinations of myelin components promotes clinical and structural recovery. J Neurol Sci 56:65–73.

Udaka K, Marusic-Galesic S, Walden P (1994) $CD4^+$ and $CD8^+$ alpha beta, and gamma delta T cells are cytotoxic effector cells of β_2-microglobulin-deficient mice against cells having normal MHC class I expression. J Immunol 153:2842–2850.