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Theiler's Virus Persistence and Demyelination in Major Histocompatibility Complex Class II-Deficient Mice

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Mice with targeted disruption of the A_β gene of major histocompatibility complex class II molecules (Ab^o) were used to investigate the role of class II gene products in resistance or susceptibility to virus-induced chronic demyelination in the central nervous system (CNS). Class II-deficient mice from the resistant $H\text{-}2^b$ [$H\text{-}2^b(Ab^o)$] and nonmutant $H\text{-}2^b$ backgrounds were infected with Theiler's murine encephalomyelitis virus intracerebrally and examined for CNS virus persistence, demyelination, and neurologic clinical signs. Virus titers measured by plaque assays showed that 8 of 10 normally resistant nonmutant $H\text{-}2^b$ mice had cleared the virus within 21 days, whereas the other 2 mice had low titers. In contrast, all class II-deficient Ab^o mice had high virus titers for up to 90 days after infection (4.30 $\log_{10}$ to 6.18 $\log_{10}$ PFU per g of CNS tissue). Virus antigens and RNA were localized to the brains (cortex, hippocampus, thalamus, and brain stem) and spinal cords of Ab^o mice. Colocalization identified persistent Theiler's murine encephalomyelitis virus in oligodendrocytes and astrocytes but not in macrophages. There was demyelination in 11 of 23 and 6 of 9 Ab^o mice 45 and 90 days after virus infection, respectively, whereas no demyelination was observed in infected nonmutant $H\text{-}2^b$ mice. Demyelinating lesions in Ab^o mice showed virus-specific $CD8^+$ T cells and macrophages but no $CD4^+$ T cells. Spasticity and paralysis were observed in chronically infected Ab^o mice but not in the nonmutant $H\text{-}2^b$ mice. These findings demonstrate that class II gene products are required for virus clearance from the CNS but not for demyelination and neurologic disease.

Mutant mice generated by targeted gene disruption have markedly enhanced the understanding of disease pathogenesis (7, 16). A single gene is inactivated by homologous recombination, resulting in nonexpression of the products. Major histocompatibility complex (MHC) class I and II genes control the development and function of numerous cells of the immune system. Therefore, selective disruption of these genes can provide an understanding of the role of T cells in resistance to infection and pathogenesis of diseases suspected to be immune mediated. In Theiler's murine encephalomyelitis virus (TMEV)-induced central nervous system (CNS) demyelination, the immune system is thought to play a deleterious role in susceptible mice (22, 41, 43). Demyelination and neurologic disease in this animal model of multiple sclerosis are the result, at least in part, of class I- and class II-mediated immune responses directed against virus or virus-induced peptides (22, 41–43, 46). Immunosuppression with cyclophosphamide (22), cyclosporin (41), or anti-lymphocyte (43) or anti-T-cell monoclonal antibodies (MAbs) directed against CD4 (46) or CD8 (42) decreases the number and extent of demyelinating lesions in susceptible SJL/J ($H\text{-}2^s$) mice infected with TMEV. Some studies have also reported amelioration of chronic paralysis and demyelination following treatment with MAb to class II I-A antigens (12, 33), although others have not observed improvement in class II MAb-treated animals (36).

Mice with the $H\text{-}2^b$ haplotype are resistant to TMEV-induced disease and clear the virus from the CNS by day 21 postinfection (p.i.). However, disruption in $H\text{-}2^b$ mice of the β_2 microglobulin [$\beta\text{-}_2$m (−/−)] gene, which blocks expression of significant levels of MHC class I or functional $CD8^+$ T cells, abrogates resistance to TMEV demyelinating disease, allowing TMEV persistence and demyelination in the CNS but without the development of neurologic signs (10, 28, 31). These results indicate that MHC class I and/or $CD8^+$ T cells are required for virus clearance and clinical manifestation of the disease but not for demyelination. The unexpected survival of β_2m (−/−) mice has also been demonstrated in lymphocytic choriomeningitis virus infection (8). Instead of the usually acute and fatal disease, lymphocytic choriomeningitis virus-infected β_2m (−/−) mice developed persistent infection characterized clinically by chronic wasting and low mortality. In addition, β_2m (−/−) mice clear vaccinia, influenza A, and parainfluenza type I (Sendai) viruses with almost normal kinetics (2, 9, 14, 44). These results suggest that there is a class I- and $CD8^+$ T cell-independent mechanism of virus clearance (possibly by $CD4^+$ T cells).

In this study, we used mice lacking MHC class II molecules [$H\text{-}2^b(Ab^o)$] (6) to investigate the role of these immune response gene products in CNS virus persistence, demyelination, and neurologic clinical disease following TMEV infection. The experiments demonstrate the persistence of high titers of TMEV in the CNS, demyelination, and neurologic impairment in normally resistant $H\text{-}2^b$ mice lacking MHC class II molecules and functional $CD4^+$ T cells.

MATERIALS AND METHODS

Virus. The Daniel's strain of TMEV was used. Tissue culture growth and production of the virus have been described previously (34). Purified virus for an anti-TMEV immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) and a delayed-type hypersensitivity (DTH) response test was prepared

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from infected BHK-21 cells by ultracentrifugation on sucrose and cesium chloride gradients as described previously (34).

Mice. Mice heterozygous for the targeted Ab^o gene (6) were generously provided by Chris Benoist, Strasbourg, France. Class II-deficient ($H\text{-}2^b$)Ab^o and normal C57BL/6 or C57BL/10 mice were bred at the Mayo Immunogenetics Mouse Colony. Mice (4 to 6 weeks old) were inoculated intracerebrally with 2×10^5 PFU of TMEV in a 10-μl volume. Handling of all animals conformed to National Institutes of Health and Mayo Clinic institutional guidelines.

Virus plaque assay. Virus titers were determined with brain and spinal cord homogenates of mice at 7, 21, 45, and 90 days p.i. The plaque assay was carried out as previously described (35). Briefly, a 10% (wt/vol) homogenate was prepared in Dulbecco modified Eagle medium, sonicated three times for 20 s each time, clarified by centrifugation, and stored at −70°C before the plaque assay. All plaque assays were done without the identity of the mouse strain being known and were performed in duplicate.

Preparation of tissue for light microscopy and quantitative morphology. Mice were sacrificed by intraperitoneal administration of 0.2 ml of pentobarbital and perfused by intracardiac puncture with Trump's fixative (40). The spinal cords were removed and embedded in glycol-methacrylate plastic as described elsewhere. Detailed morphologic analysis for gray matter inflammation, meningeal inflammation, and demyelination was carried out on 12 to 15 coronal sections per mouse stained with a modified erichrome stain for myelin of osmicated tissue (26). A maximum score of 100 represented the presence of gray matter disease, meningeal inflammation, or demyelination in every spinal cord quadrant of every mouse examined. Selected spinal cord blocks were embedded in Araldite and prepared for electron microscopy.

Fresh frozen sections. Frozen tissue sections for immunostaining and in situ hybridization were prepared from etherized mice at 7, 45, and 90 days p.i. Coronal brain sections and longitudinal spinal cord sections were frozen in OCT embedding compound (Miles Inc., Elkhart, Ind.). Sections (10 μm thick) were cut with a Reichert-Jung Cryocut 1800 (Cambridge Instruments, Heidelberg, Germany).

In situ hybridization. In situ hybridization for TMEV RNA was carried out as previously described (24). Briefly, fixed sections were treated with 1 μg of proteinase K per ml, acetylated, and prehybridized for 4 h at room temperature with buffer containing deionized formamide, Denhardt's solution, sodium chloride, salmon sperm DNA, yeast total RNA, and yeast tRNA. Slides were then hybridized with ^{35}S-labeled 253-bp (nucleotides 3053 to 3305) and 363-bp (nucleotides 3306 to 3668) cDNA probes corresponding to VP1 of TMEV (DA strain) (23). The cDNA probes were obtained by double digesting the VP1 plasmid with *Kpn*I and *Sal*I restriction enzymes and radiolabeling the probes with between 0.5×10^8 to 0.8×10^8 cpm of [α-^{35}S]dATP per μg of DNA by nick translation. TMEV RNA-positive cells were counted by light microscopy at ×400 magnification. The CNS area was measured with the IBAS Image Analysis System (Kontron, Munich, Germany) attached to an Axiophot microscope (Carl Zeiss, Inc., Thornwood, N.Y.).

Immunohistochemical staining for TMEV and T-cell antigens. Fresh frozen spinal cord and brain sections from infected ($H\text{-}2^b$)Ab^o and infected nonmutant $H\text{-}2^b$ mice were fixed with cold acetone. The sections were immunostained with polyclonal rabbit anti-TMEV sera which reacted by Western blotting (immunoblotting) with all the capsid proteins of TMEV (34). In addition, selected spinal cord sections from infected Ab^o mice showing demyelination and inflammatory lesions were stained for CD4$^+$ and CD8$^+$ T cells. Purified rat anti-mouse CD4 and CD8 antibodies (Pharmingen, San Diego, Calif.) at 20 μg/ml followed by biotinylated anti-rat IgG were used. The antigens were detected by the avidin-biotin immunoperoxidase technique as previously described (28). All slides were lightly counterstained with Mayer's hematoxylin.

Cytotoxic T-lymphocyte assay. Brains and spinal cords of Ab^o, β_2m (−/−), and C57BL/6 mice infected with TMEV for 7 days were pooled by strain. The CNS-infiltrating lymphocytes were isolated as previously described (19) and resuspended to 2×10^6 lymphocytes per ml in RPMI medium with 5% fetal calf serum. Twofold serial dilutions were made to provide effector-to-target ratios of between 100:1 to 6.25:1. TMEV-infected L cells transfected with the $H\text{-}2D^b$ gene (and designated as L/D^b cells) were labeled with sodium [^{51}Cr]chromate (Amersham Life Science Corp., Arlington Heights, Ill.) and served as target cells (2×10^4 cells per ml). Equal volumes of target and effector cells were incubated for 5 h in 96-well round-bottom microtiter plates. The mean radioactivity values were calculated, and standard errors were determined from triplicate wells. Statistical comparisons for virus-specific percent cytotoxicity were performed by the unpaired Student's *t* test.

Simultaneous immunostaining and in situ hybridization. To identify those cells in which TMEV persisted, we did immunocytochemical staining, with cell markers for oligodendrocytes, astrocytes, and macrophages, and the staining was followed by in situ hybridization for TMEV RNA in spinal cord sections of Ab^o mice at 45 and 90 days p.i. The technique and antibodies sources were as described previously (27). Antibodies against myelin basic protein and glial fibrillary acidic protein were used to identify oligodendrocytes and astrocytes, respectively. Lectin *Bandeiraea simplicifolia* BS-1 and Mac-1 MAbs (Boehringer Mannheim, Indianapolis, Ind.) were used for macrophages. Immediately after the final rinse in distilled water, the sections were hybridized in situ for TMEV RNA as described elsewhere.

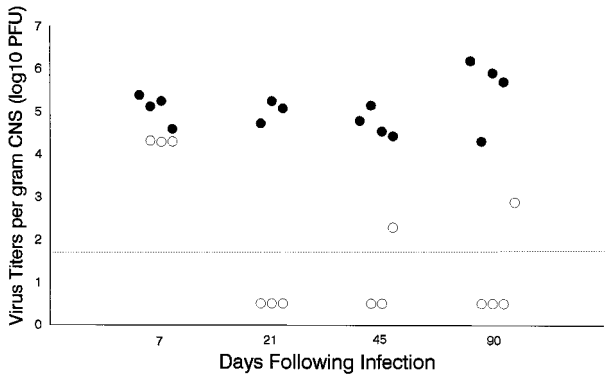

FIG. 1. Isolation of TMEV at 7, 21, 45, and 90 days p.i. from the CNSs of $H\text{-}2^b$ (open circles) and ($H\text{-}2^b$)Ab^o (solid circles) mice. Infectious virus levels ($\log_{10}$ PFU per gram of CNS tissue) were determined by viral plaque assays with L2 cells. Mice from which no virus was isolated are represented below the broken line (detection limit, 1.7 $\log_{10}$ PFU/g of CNS).

DTH responses to virus. TMEV-specific DTH responses were assessed in 20 Ab^o and 10 nonmutant $H\text{-}2^b$ mice between 44 and 88 days after virus infection. Four- to 6-week-old C57BL/6 mice were used as uninfected controls. Response was elicited in the ear by intradermal injection of 2.5 μg of UV-inactivated purified virus in a 10-μl volume. Twenty-four and 48 h after the challenge, the swelling of the ear compared with the prechallenge ear was recorded with a dial gauge micrometer (10^{-2} mm). Statistical analysis was done by unpaired Student's *t* test.

Anti-TMEV IgG ELISA. Total serum anti-TMEV IgGs were quantified by ELISA. Mice were bled by intracardiac puncture at the time of sacrifice, blood was allowed to clot, and serum was aliquoted and stored at −70°C. Polystyrene microtiter plates (Corning, Corning, N.Y.) were coated overnight with 0.5 μg of purified TMEV in 0.1 M carbonate buffer, pH 9.5, per well. Plates were blocked with 1% bovine serum albumin (BSA), and sera from individual mice were diluted fourfold (1:500 to 1:512,000) in 0.2% BSA and incubated on the coated plates for 4 h at room temperature. Bound IgGs were detected with biotinylated goat anti-mouse IgG and streptavidin conjugated to alkaline phosphatase (Jackson Immunoresearch, West Grove, Pa.). *p*-Nitrophenylphosphate substrate was added, and the A_{405} was read. The data are presented as the A_{405} of serial serum dilutions. Anti-TMEV titers from infected ($H\text{-}2^b$)Ab^o mice were compared with those from infected nonmutant $H\text{-}2^b$ mice and chronically infected SJL/J mice.

RESULTS

Disruption of MHC class II genes results in persistence in $H\text{-}2^b$ mice. We examined the amount of infectious virus in the CNS by plaque assay and localized viral antigen and RNA by immunostaining and in situ hybridization, respectively, following TMEV inoculation. At day 7 p.i., infectious virus titers for both class II-deficient [($H\text{-}2^b$)Ab^o] and infected nonmutant $H\text{-}2^b$ mice were comparable, ranging between 4.28 $\log_{10}$ and 5.38 $\log_{10}$ PFU per g of CNS tissue (Fig. 1). However, TMEV persisted at high titers in Ab^o mice throughout the 90-day experiment period (between 4.30 $\log_{10}$ to 6.18 $\log_{10}$ PFU), whereas titers in the infected nonmutant $H\text{-}2^b$ mice decreased below detectable levels by day 21 p.i., except in one mouse which had a titer of 2.28 $\log_{10}$ PFU at day 45 p.i. and one which had a titer of 2.87 $\log_{10}$ PFU at day 90 p.i. ($P < 0.0003$ by Fisher's exact test).

Immunostaining and in situ hybridization localized viral antigen and viral RNA in the brain (cortex, hippocampus, thalamus, and brain stem) and the spinal cord white and gray matter in the mutant Ab^o mice at 45 and 90 days p.i. (Fig. 2 and Table 1). At 7 days p.i., both the infected Ab^o and nonmutant $H\text{-}2^b$ mice demonstrated viral antigens and RNA in the brains and spinal cords. The number of TMEV RNA-positive cells in the brain stems and spinal cords of the TMEV-infected class II-deficient mice was greater than that in the nonmutant $H\text{-}2^b$ mice (Table 1) at the 7-day-p.i. time point. At days 45 and 90

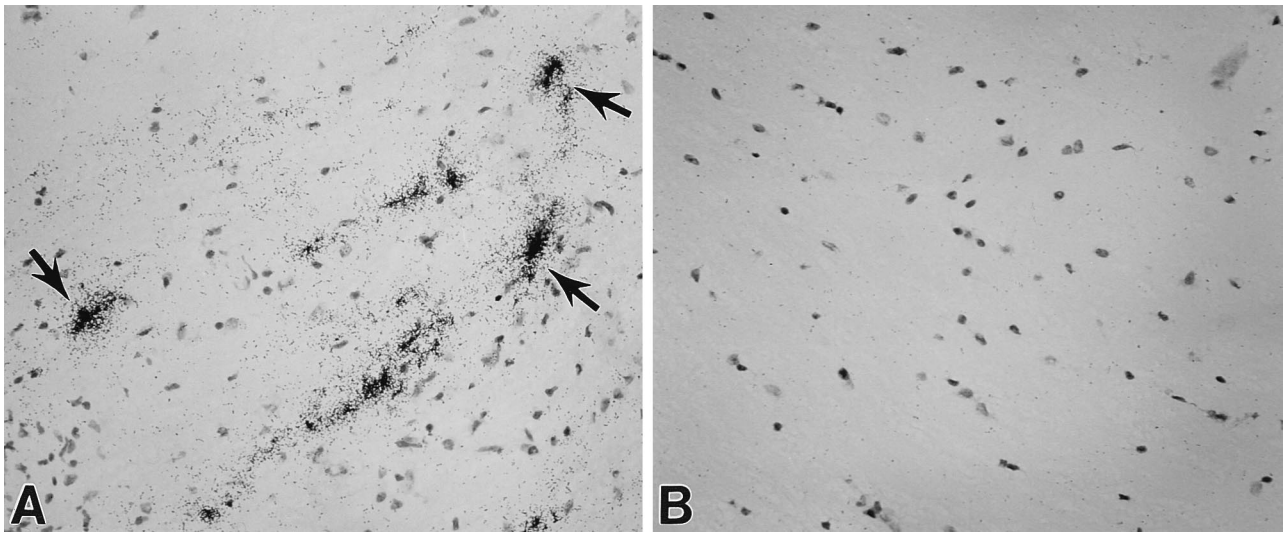

FIG. 2. Localization of TMEV RNA in the spinal cords of ($H\text{-}2^b$)Ab^o mice 90 days after infection by in situ hybridization. Frozen sections were hybridized with ^{35}S-labeled probe corresponding to the VP1 region of TMEV and exposed for 48 to 72 h. Dark grains indicate TMEV RNA localization. Sections were counterstained with Mayer's hematoxylin. (A) Infected ($H\text{-}2^b$)Ab^o mouse tissue positive for viral RNA (arrows). (B) Infected $H\text{-}2^b$ mouse tissue negative for TMEV RNA. Magnification, ×900.

p.i., viral RNA-positive cells averaged 1.9 cells per mm^2 and 4.2 cells per mm^2 in the spinal cord sections of the Ab^o mice, respectively, whereas none was detected in the sections from the infected nonmutant $H\text{-}2^b$ mice. Double labeling with immunoperoxidase for cell phenotypes (macrophages, astrocytes, and oligodendrocytes) followed by in situ hybridization for TMEV RNA localized persistent TMEV RNA in astrocytes (Fig. 3A) and oligodendrocytes (Fig. 3B) but not in macrophages (data not shown) within the demyelinating lesion.

Virus persistence in the CNS results in demyelination in Ab^o mice. Normally, $H\text{-}2^b$ mice clear TMEV by day 21 p.i. and do not develop demyelination. In the Ab^o mice, demyelination in the spinal cord was observed in 11 of 23 (47.8%) mice at 45 to 58 days p.i. and 6 of 9 (66.7%) mice at 90 days p.i. Demyelinating lesions were well circumscribed by parenchymal lymphocytic and macrophage inflammation, as illustrated by both light (Fig. 4A) and electron (Fig. 4B, C, D, and E) microscopy. Compared with the demyelinating lesions in the highly susceptible SJL/J mice, these lesions had better preservation of axons (30). Meningeal, white matter, and gray matter inflammation in the Ab^o mice was evident but was not as extensive as that observed in TMEV-infected susceptible SJL/J mice. Scores in animals with pathologic disease indicated that between 5.7 and 48.2% of the spinal cord quadrants in individual mice showed demyelination. Comparatively, minimal or no demyelination and inflammation disease were observed in the spinal cords of infected nonmutant $H\text{-}2^b$ mice ($n = 28$) ($P \leq 0.003$ by the Fisher exact test) at days 45 and 90 p.i. (Table 2).

TMEV-specific CD8$^+$ T cells in the CNS. Cytotoxic CD8$^+$ T cells reactive against TMEV antigens were demonstrated in the CNSs of both infected Ab^o and nonmutant $H\text{-}2^b$ mice (Fig. 5). There was between 23 to 25% specific lysis of TMEV-transfected cells in the Ab^o mice, compared with 29 to 31% lysis in nonmutant $H\text{-}2^b$ mice at the 100:1 dilution. No cytotoxic T-lymphocyte activity was observed in class I-deficient β_2m (−/−) mice. We selected several Ab^o mice showing demyelinating and inflammatory lesions and stained their spinal cord sections for T-cell subsets. There was heavy CD8$^+$ T-cell infiltration in the lesion (Fig. 6A). No CD4$^+$ T cells were observed in adjacent sections of the same mice (Fig. 6B).

Clinical demyelinating disease. The view that some component of the immune system is required for the development of neurologic disease in TMEV-induced demyelination was supported by the β_2m (−/−) mice, which showed extensive demyelination but no neurologic impairment (28, 31). To determine whether clinical signs associated with neurologic disease devel-

TABLE 1. TMEV RNA-positive cells localized by in situ hybridization in the brains and spinal cords of MHC class II-deficient mice[a]

Strain background	Days p.i.	No. of mice	Mean no. of cells (range)[b]			
			Cortex	Hippocampus and thalamus	Brain stem	Spinal cord
$H\text{-}2^b(Ab^o)$	7	3	>20.0[c]	>20.0[c]	8.5 (4.9–12.4)	6.9 (2.2–11.0)
$H\text{-}2^b$	7	2	>20.0[c]	>20.0[c]	0	1.3 (1.1–1.4)
$H\text{-}2^b(Ab^o)$	45	5	7.1 (0.6–20.3)	2.1 (0.2–6.3)	3.3 (0–8.5)	1.9 (0.2–5.0)
$H\text{-}2^b$	45	3	0	0	0	0
$H\text{-}2^b(Ab^o)$	90	3	2.1 (1.0–4.4)	0.4 (0–0.9)	0.2 (0–0.7)	4.2 (0–9.1)
$H\text{-}2^b$	90	2	0	0	0	0

[a] In situ hybridization with a ^{35}S-labeled probe corresponding to the VP1 region of TMEV was carried out with frozen sections of mouse brain and spinal cord. TMEV RNA-positive cells were counted by light microscopy, and the CNS area was measured with a quantitative image analysis system.
[b] RNA-positive cells are presented as mean numbers of cells per square millimeter, with the range being given in parentheses.
[c] The positive cells in these mouse sections, which were analyzed 7 days p.i., were too numerous to count.

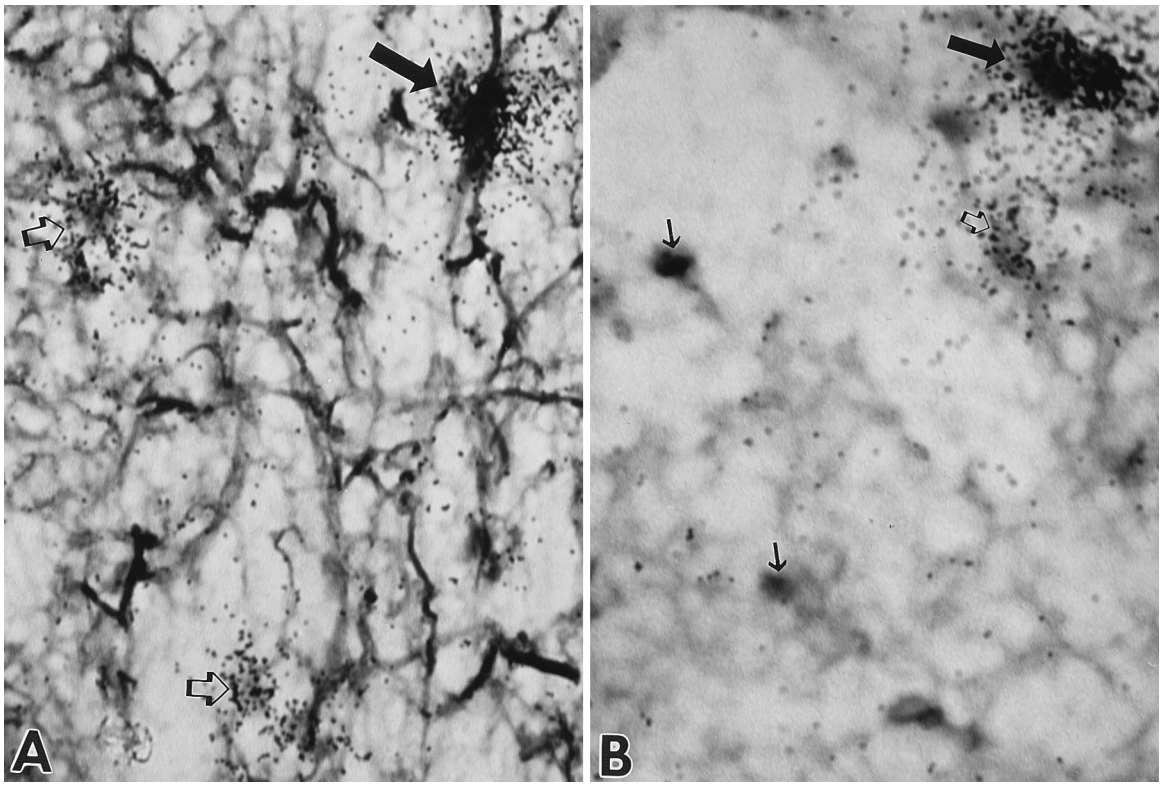

FIG. 3. Determination of the phenotypes of cells in which TMEV persists of Ab^o mice by simultaneous immunoperoxidase and in situ hybridization. Spinal cord sections were immunostained for astrocytes with antibodies to glial fibrillary acidic protein (A) and for oligodendrocytes with antibodies to myelin basic protein (B), and then in situ hybridization with ^{35}S-labeled cDNA probe for TMEV was carried out. Cells positive for both the phenotype marker and TMEV RNA are identified by dark staining together with the autoradiographic grain (large black arrows). Some cells were positive for viral RNA but not the phenotype marker (open arrows). Small arrows (B) indicate oligodendrocytes negative for viral RNA. Sections were counterstained with Mayer's hematoxylin. Magnification, ×4,200.

oped in Ab^o mice, animals were examined three times each week during the course of these experiments. Between days 35 and 45 p.i., stiffness, spasticity, and paralysis of at least one extremity was observed in 3 of 18 (16.7%) Ab^o mice. Between days 58 and 90 p.i., 7 of 14 (50%) had stiffness or paralysis of one to three extremities. In contrast, there were no neurologic signs in 19 TMEV-infected nonmutant $H\text{-}2^b$ mice sacrificed at days 45 and 90 p.i. ($P = 0.008$ by Fisher's exact test).

Lack of correlation between development of DTH response against TMEV and demyelination. Some studies have suggested a direct correlation among TMEV-specific DTH response, demyelination, and neurologic disease (4). Therefore, we evaluated the DTH response in 20 Ab^o (with demyelination) and 10 nonmutant $H\text{-}2^b$ mice (without demyelination) between 44 and 88 days p.i. The mean ear swelling for infected nonmutant $H\text{-}2^b$ mice at 48 h was significantly higher compared with that for infected Ab^o mice ($P < 0.001$) (Table 3). Compared with that for uninfected $H\text{-}2^b$ mice, the ear swelling for infected Ab^o mice was not significantly different ($P > 0.54$) (Table 3).

Absence of humoral immune response in class II-deficient mice. Recent studies have reported IgG responses in CD4-deficient mice (29). The studies have identified class II-restricted $CD4^-8^-$ $TcR\alpha\beta^+$ T cells responsible for the IgM-to-IgG switching. We measured anti-TMEV antibody responses in the class II knockout mice to confirm the absence of humoral immune response. Basal levels of anti-TMEV IgG were detected in the TMEV-infected Ab^o mice (Fig. 7). In contrast, high titers were observed in chronically infected nonmutant $H\text{-}2^b$ mice and susceptible SJL/J mice (Fig. 7). These results indicate that the TMEV-specific antibody response is dependent on MHC class II.

DISCUSSION

This study used class II-deficient mice to address two important questions. First, we investigated whether the absence of a class II-restricted immune response influenced virus clearance from the CNS. The results demonstrated that MHC class II determinants are required for resistance to TMEV infection,

FIG. 4. Micrographs of demyelination in the spinal cords of Ab^o mice 45 days after infection with TMEV. (A) Light microscopy showing multiple demyelination lesions (arrows) in a spinal cord embedded in glycol-methacrylate and stained with modified erichrome stain. Magnification, ca. ×160. (B) Electron micrograph showing the earliest stage of demyelination, with degeneration of the inner myelin sheath and complete preservation of the axon. An inflammatory cell (ic) is in intimate association with the degenerating myelin sheath. Magnification, ca. ×7,300. (C) Later stage of demyelination, showing complete dissolution of myelin and the preserved axon (ax). To the left is a macrophage (M) with ingested myelin debris, and to the right is a field of normally myelinated axons. Note the degeneration of the inner oligodendroglial loop (arrow) in the upper left corner, consistent with the concept of "dying-back oligodendropathy" (30). Magnification, ca. ×12,200. (D) The final consequence of TMEV infection, showing multiple denuded axons. Magnification, ca. ×12,600. (E) Macrophage (M) engulfing myelin debris in a scavenger function. Magnification, ca. ×4,400.

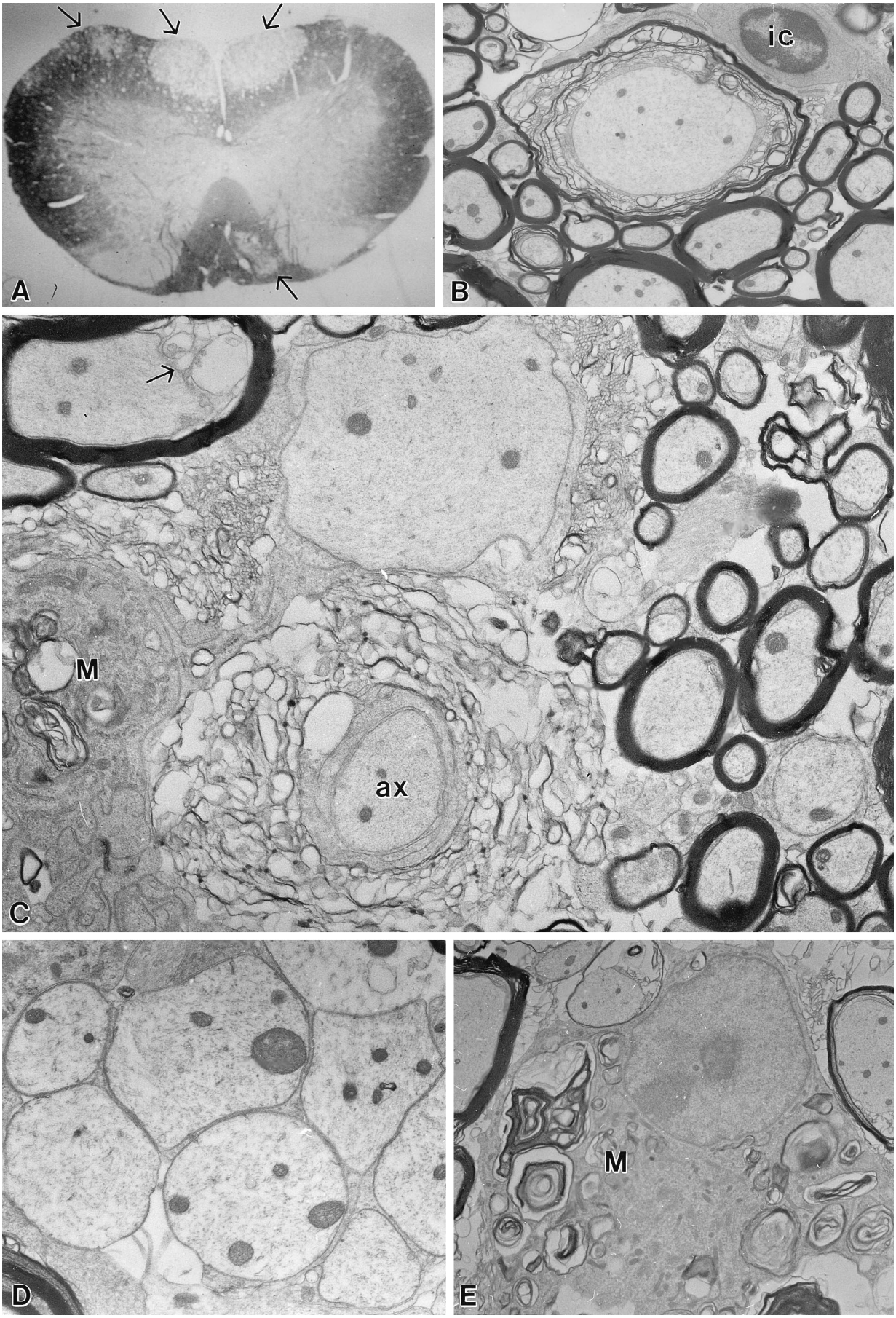
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TABLE 2. Effect of class II knockout on TMEV-induced CNS disease

Strain background	No. of mice	Days p.i.	Mean % of quadrants with disease ± SEM[a]		
			Gray matter inflammation	Meningeal inflammation	Demyelination[b]
$H\text{-}2^b$	4	7	1.6 ± 1.6	0.5 ± 0.5	0.0 ± 0.0
$H\text{-}2^b(Ab^o)$	5	7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
$H\text{-}2^b$	13	35–45	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
$H\text{-}2^b(Ab^o)$	18	45	0.3 ± 0.2	1.1 ± 0.7	4.5 ± 2.7
$H\text{-}2^b$	10	83–90	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
$H\text{-}2^b(Ab^o)$	6	90	1.3 ± 0.6	0.0 ± 0.0	9.8 ± 7.5

[a] Each quadrant of 10 to 15 spinal cord cross-sections from each mouse was scored for the presence and absence of gray matter inflammation, meningeal inflammation, and demyelination. The data represent the percentages of quadrants showing the indicated conditions.

[b] Statistical analyses by Fisher's exact test compared the presence and absence of demyelination in infected $H\text{-}2^b$ mice and infected $H\text{-}2^b(Ab^o)$ mice from 35 to 90 days p.i. ($P \leq 0.0026$).

characterized by virus clearance from the CNS and the absence of demyelinating lesions in $H\text{-}2^b$ mice. Virus titers in Ab^o mice (days 21 and 90 p.i.) were 10- to 100-fold higher than those observed in the highly susceptible infected SJL/J mice (20, 35). Compromised virus-specific immune responses in class II-deficient mice have also been reported for influenza virus but not Sendai virus infection (15, 45). In influenza virus infection, there was diminished cytotoxic T-cell activity and a substantial decrease in cytokine production (45), suggesting that the $CD4^+$ T cell-helper function was both direct and via cytokines. Because the mechanism of TMEV persistence in susceptible strains is unknown, we can only speculate on how resistance to TMEV was abrogated by the absence of class II glycoproteins.

Class II MHC and $CD4^+$ T cells may be functioning specifically to clear TMEV infection from the CNS by the secretion of cytokines. Gamma interferon (11, 39), tumor necrosis factor alpha (25), and interleukin-6 (40) have all been shown to play a critical role in resistance to TMEV-induced demyelinating disease. Alternatively, class II MHC and $CD4^+$ T cells may be functioning to provide help for B cells and CD8 cytotoxic T cells. The absence of TMEV-specific antibodies in Ab^o mice may have compromised their ability to clear TMEV. Suppressed humoral immunity in *xid* gene-deficient mice and by treatment with anti-IgM results in more extensive TMEV-induced demyelination (32). Neutralizing antibodies (21) and TMEV-specific B lymphocytes (3) are regularly found in the CNS of TMEV-infected mice, and passive administration of neutralizing MAbs enhances the survival of athymic mice after TMEV infection (13). In vitro functional assays of CNS-infiltrating lymphocytes recovered from Ab^o mice showed normal cytotoxic T-lymphocyte activity, suggesting that the anti-TMEV activity of $CD8^+$ T cells is effective. Unknown is whether in the absence of $CD4^+$ T cells, the $CD8^+$ T cells in Ab^o mice would function effectively in vivo to clear virus infection from the CNS.

Second, this study examined the role of the class II-restricted immune response in demyelinating disease. Although the precise mechanism of TMEV-induced demyelination has not been determined, several hypotheses have been proposed (reviewed in reference 38). The first hypothesis envisions demyelination as the result of direct viral cytolytic injury to oligodendrocytes, the cells that produce myelin. This hypothesis is based on in vivo and in vitro studies that have demonstrated productive infection of oligodendrocytes (1, 34, 35) and have localized TMEV antigen within the inner and outer oligodendroglial loops which connect directly with the myelin sheath (30, 34). In Ab^o mice, virus persistence was demonstrated in oligodendrocytes and astrocytes but not macrophages, even though in other studies, TMEV persistence has been shown in all of the three cell types (1, 5). The high levels of virus persistence in glial cells and the degenerative changes within oligodendroglial loops of Ab^o mice support a direct viral cytopathological mechanism of demyelination. The second hypothesis is that TMEV-induced demyelination is the result of an autoimmune attack on a myelin component, as occurs in experimental allergic encephalitis (17). Since experimental allergic encephalitis is a class II-restricted $CD4^+$ cell-mediated process, the demonstration of demyelination in class II-deficient mice argues against a similar mechanism for TMEV-induced demyelination. The bystander hypothesis suggests that myelin is destroyed by a nonspecific mechanism stemming from DTH immune-inflammatory processes induced by TMEV infection (4, 5). Myelin damage, according to this hypothesis, is caused by cytokines and phagocytic cells recruited to the infection sites. Because class II-deficient mice develop demyelination despite the absence of a TMEV-specific DTH response, these results argue against the bystander hypothesis.

We favor the hypothesis that demyelination results from a specific host immune response directed against viral or virally induced antigen on oligodendrocytes or myelin (38). This hypothesis envisions a chronic infection of oligodendrocytes, providing antigenic targets for a class I- or class II-restricted T cell-mediated attack. Following TMEV infection, novel antigens not associated with normal oligodendrocytes, either of viral origin or previously masked cellular antigens, may be expressed on the surface and become a target of immune attack, leading to demyelination. Oligodendroglial cell virus persistence and damage to the inner glial loop are observed chronically in Ab^o mice. The fact that demyelination in Ab^o

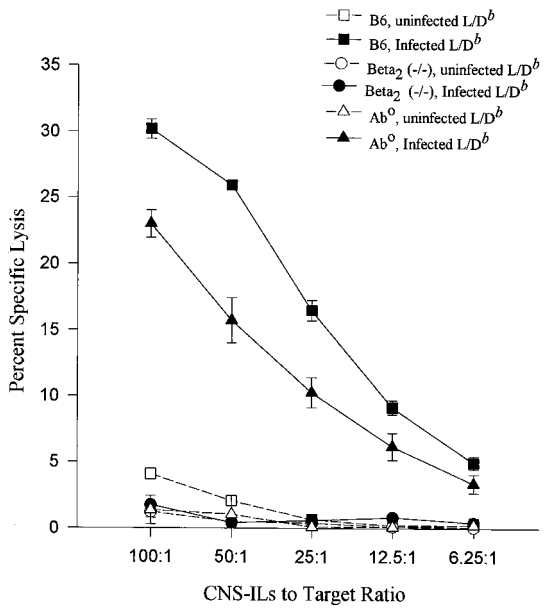

FIG. 5. Cytotoxicity against TMEV by CNS-infiltrating lymphocytes (CNS-ILs) isolated from Ab^o, β_2m (−/−), and C57BL/6 mice 7 days p.i. Cytotoxicity against TMEV was assayed with virus-infected L/D^b cells and noninfected L/D^b cells. The results represent the pooled activity of CNS-infiltrating lymphocytes isolated from 10 mice of each strain. Percent specific lysis of L/D^b cells by the CNS-infiltrating lymphocytes of nonmutant $H\text{-}2^b$ and Ab^o mice was significantly greater than that for β_2m (−/−) mice ($P < 0.01$ for an effector-to-target ratio of 100:1 by Student's t test).

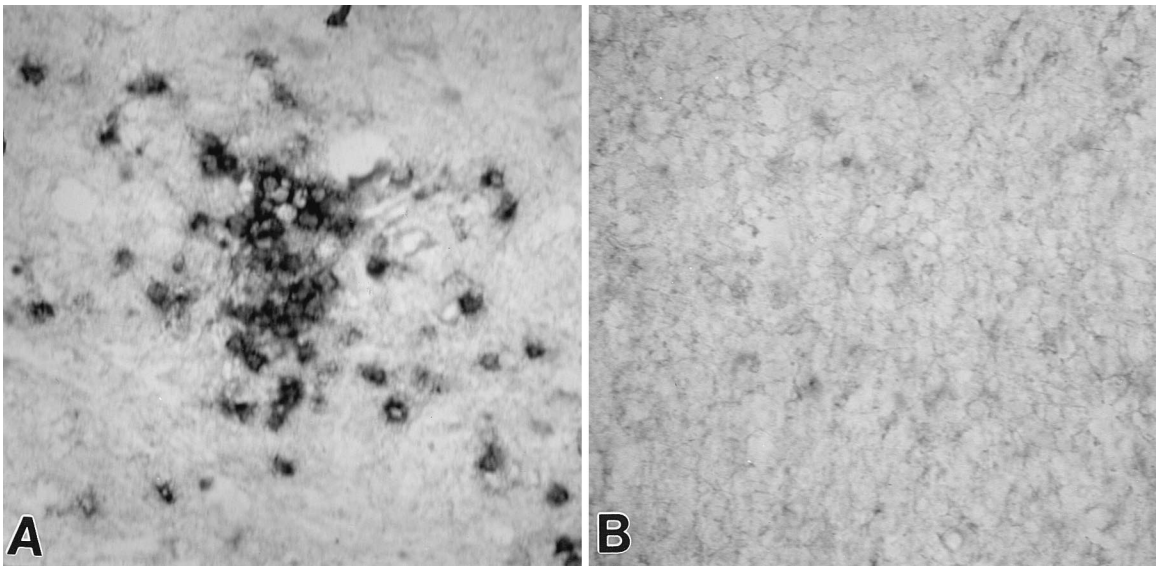

FIG. 6. Immunodetection by the avidin-biotin immunoperoxidase technique of $CD4^+$ and $CD8^+$ T cells in the spinal cord of an ($H\text{-}2^b$)Ab^o mouse infected for 45 days with TMEV. Dark-stained cells are immunoreactive. Sections were counterstained with Mayer's hematoxylin. (A) Multiple $CD8^+$ T cells in a demyelinated lesion. (B) Absence of $CD4^+$ T cells in the adjacent section of the same lesion area as that shown in panel A. Magnification, ×1,200.

mice, as in all susceptible strains, was observed in the chronic (45 and 90 days p.i.) but not acute (before 21 days p.i.) stages of the disease argues against direct TMEV cytopathology as the sole mechanism for demyelination. An immune-mediated model of virus-induced diabetes has also been reported in class II-deficient mice (18).

The role of class I-restricted $CD8^+$ and class II-restricted $CD4^+$ T cells in the demyelination process is unclear. Depletion of $CD4^+$ and $CD8^+$ T cells by MAb treatment decreases the number and extent of demyelinating lesions in susceptible SJL/J mice (42, 46), whereas similar treatment in resistant $H\text{-}2^b$ mice results in small areas of demyelination (37). The demyelinating lesions observed in the Ab^o mice were less extensive than those observed in the β_2m (−/−) mice (31). On average, approximately 4.5 and 9.8% of the spinal cord quadrants of Ab^o mice at 45 and 90 days p.i. showed demyelination, compared with 14.8 and 27.5% of those of β_2m (−/−) mice. These results suggests that class I and II immune determinants independently can contribute to the development of demyelination. Given that mice with the $H\text{-}2^b$ background normally do not develop TMEV-induced demyelination, the exact role of class I or II genes in demyelination in susceptible strains cannot be derived from the result of this study and the β_2m (−/−) studies. Disruption of MHC class I, class II, CD4, and CD8 genes in susceptible haplotypes will define the roles of these genes in demyelination more precisely.

The most important finding of this study was that neurologic clinical signs can develop in the absence of class II gene products. We have confirmed these results with another study in which 17 of 19 Ab^o mice infected with TMEV developed severe neurologic clinical signs between 35 and 120 days p.i., whereas 6 infected nonmutant $H\text{-}2^b$ mice did not develop clinical disease. Taken together with the results for β_2m (−/−) mice in which no clinical signs were observed despite extensive lesions, the findings suggest that MHC class I determinants (probably $CD8^+$ T cells) but not MHC class II determinants are sufficient for the development of clinical demyelinating disease. A possible explanation for the absence of clinical signs in the β_2m

TABLE 3. DTH responses to TMEV in chronically infected Ab^o mice[a]

Strain or background	No. of mice	Days p.i.	Mean ear swelling (10^{-2} mm) ± SEM (P)[b]	
			24 h	48 h
B6	9	0	4.89 ± 0.72	3.56 ± 0.53
$H\text{-}2^b(Ab^o)$	20	44–88	5.30 ± 0.58 (0.68)	4.45 ± 0.95 (0.546)
B6 or B10	10	44–88	12.70 ± 0.92 (<0.0001)	13.3 ± 1.02 (<0.0001)

[a] $H\text{-}2^b(Ab^o)$ and nonmutant $H\text{-}2^b$ (B6 or B10) mice infected with TMEV were assessed for DTH responses at 44 to 88 days p.i. For the DTH assay, 2.5 μg of purified TMEV antigens was injected intradermally in the ear, and swelling was assessed against preinjection measurements.

[b] Ear swelling in Ab^o mice was compared with that in uninfected controls, and ear swelling in nonmutant $H\text{-}2^b$ mice was compared with that in Ab^o mice.

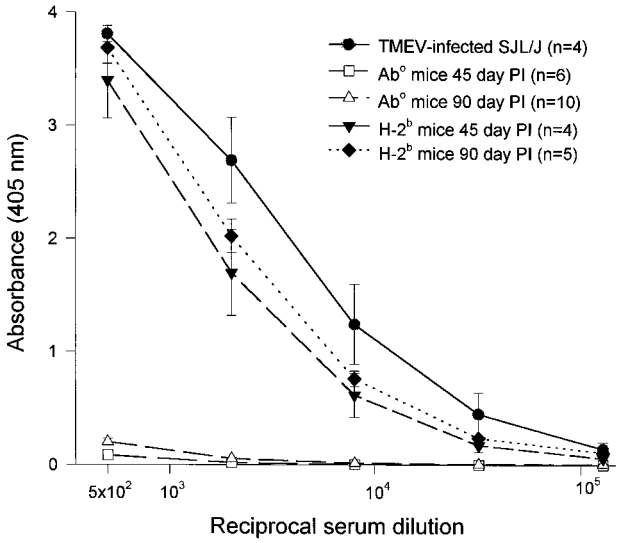

FIG. 7. TMEV-specific IgG responses in Ab^o, $H\text{-}2^b$, and SJL/J mice infected with Theiler's virus. The data represent means ± standard errors of the means for 4 to 10 mice per group. PI, postinfection.

(−/−) mice was that a critical threshold of cumulative demyelinating lesions required for the development of clinical disease was not reached. However, the observation of severe clinical deficits in the Ab^o mice which had less extensive lesions than the β_2m (−/−) mice argues against this possibility. A more likely possibility is that factors secreted by $CD8^+$ T cells interfere with axonal conduction in the presence of a previously demyelinated lesion.

This study supports earlier results indicating that the DTH response to TMEV is predominantly class II mediated but argues against the suggested correlation between DTH-specific immune response and demyelinating disease (4). DTH responses to TMEV were observed in $CD8^+$ T cell-deficient β_2m (−/−) mice chronically infected with TMEV, indicating that the $CD4^+$ T cells are responsible for the response (9), but these mice did not develop clinical disease. In contrast, there was no DTH response in the Ab^o mice. Since we observed demyelination and clinical disease, but no DTH responses against TMEV in Ab^o mice, and a strong DTH response in infected nonmutant $H\text{-}2^b$ mice that rapidly cleared virus and did not develop demyelination, no correlation between DTH-specific immune response and demyelinating disease is evident.

In conclusion, the high titers of persistent virus and the presence of demyelinating lesions in the CNS of Ab^o mice with a normally resistant $H\text{-}2^b$ haplotype indicate that class II genes are critical for clearance of TMEV infection and resistance to demyelination. In contrast, the presence of clinical disease in class II- but not in class I-deficient mice with the same haplotype and bearing comparable pathologic lesions argues against a causal role for MHC class II genes in clinical disease.

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REFERENCES

1. **Aubert, C., M. Chamorro, and M. Brahic.** 1987. Identification of Theiler's virus infected cells in the central nervous system of the mouse during demyelinating disease. Microb. Pathog. **3:**319–326.
2. **Bender, B. S., T. Croghan, L. Zhang, and P. A. Small, Jr.** 1992. Transgenic mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. J. Exp. Med. **175:**1143–1145.
3. **Cash, E. A., A. Bandeira, S. Chirinian, and M. Brahic.** 1989. Characterization of B lymphocytes present in the demyelinating lesions induced by Theiler's virus. J. Immunol. **143:**984–988.
4. **Clatch, R. J., R. W. Melvold, S. D. Miller, and H. L. Lipton.** 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.
5. **Clatch, R. J., S. D. Miller, R. Metzner, M. C. Dal Canto, and H. L. Lipton.** 1990. Monocyte/macrophages isolated from the mouse central nervous system contain infectious Theiler's murine encephalomyelitis virus (TMEV). Virology **176:**244–254.
6. **Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur, C. Benoist, and D. Mathis.** 1991. Mice lacking MHC class II molecules. Cell **66:**1051–1066.
7. **Doherty, P. C.** 1993. Virus infections in mice with targeted gene disruptions. Curr. Opin. Immunol. **5:**479–483.
8. **Doherty, P. C., S. Hou, and P. Southern.** 1993. Lymphocytic choriomeningitis virus induces a chronic wasting disease in mice lacking class I MHC glycoproteins. J. Neuroimmunol. **46:**11–17.
9. **Eichelberger, M., W. Allan, M. Zijlstra, R. Jaenisch, and P. C. Doherty.** 1991. Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted CD8+ T cells. J. Exp. Med. **174:**875–880.
10. **Fiette, L., C. Aubert, M. Brahic, and C. P. Rossi.** 1993. Theiler's virus infection of β_2-microglobulin-deficient mice. J. Virol. **67:**589–592.
11. **Fiette, L., C. Aubert, U. Muller, S. Huang, M. Aguet, M. Brahic, and J. F. Bureau.** 1995. Theiler's virus infection of 129SV mice that lack the interferon alpha/beta or interferon gamma receptors. J. Exp. Med. **181:** 2069–2076.
12. **Friedman, A., G. Frankel, Y. Lorch, and L. Steinman.** 1987. Monoclonal anti-I-A antibody reverses chronic paralysis and demyelination in Theiler's virus-infected mice: critical importance of timing of treatment. J. Virol. **61:**898–903.
13. **Fujinami, R. S., A. Rosenthal, P. W. Lampert, A. Zurbriggen, and M. Yamada.** 1989. Survival of athymic (*nu*/*nu*) mice after Theiler's murine encephalomyelitis virus infection by passive administration of neutralizing monoclonal antibody. J. Virol. **63:**2081–2087.
14. **Hou, S., P. C. Doherty, M. Zijlstra, R. Jaenisch, and J. M. Katz.** 1992. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. J. Immunol. **149:**1319–1325.
15. **Hou, S., L. Hyland, and P. C. Doherty.** 1995. Host response to Sendai virus in mice lacking class II major histocompatibility complex glycoproteins. J. Virol. **69:**1429–1434.
16. **Kaufmann, S. H. E.** 1994. Bacterial and protozoal infections in genetically disrupted mice. Curr. Opin. Immunol. **6:**518–525.
17. **Lampert, P.** 1967. Electron microscopic studies on ordinary and hyperacute experimental allergic encephalomyelitis. Acta Neuropathol. **9:**99–126.
18. **Laufer, T. M., M. G. von Herrath, M. J. Grusby, M. B. A. Oldtstone, and L. H. Glimcher.** 1993. Autoimmune diabetes can be induced in transgenic major histocompatibility complex class II-deficient mice. J. Exp. Med. **178:**589–596.
19. **Lin, X., R. L. Thiemann, L. R. Pease, and M. Rodriguez.** 1995. VP1 and VP2 capsid proteins of Theiler's virus are targets of H-2D-restricted cytotoxic lymphocytes in the central nervous system of B10 mice. Virology **214:**91–99.
20. **Lipton, H. L.** 1975. Theiler's virus infection in mice: an unusual biphasic disease leading to demyelination. Infect. Immun. **11:**1147–1155.
21. **Lipton, H. L.** 1978. Central nervous system immunity in mice infected with Theiler's virus. 1. Local neutralizing antibody response. J. Infect. Dis. **137:** 145–151.
22. **Lipton, H. L., and M. C. Dal Canto.** 1976. Theiler's virus-induced demyelination: prevention by immunosuppression. Science **192:**62–64.
23. **Ohara, Y., S. Stein, J. Fu, L. Stillman, L. Klaman, and R. P. Roos.** 1988. Molecular cloning and sequence determination of DA strain of Theiler's murine encephalomyelitis viruses. Virology **164:**245–255.
24. **Patick, A. K., E. L. Oleszak, J. L. Leibowitz, and M. Rodriguez.** 1990. Persistent infection of a glioma cell line generates a Theiler's virus variant which fails to induce demyelinating disease in SJL/J mice. J. Gen. Virol. **71:** 2123–2132.
25. **Paya, C. V., P. J. Leibson, A. K. Patick, and M. Rodriguez.** 1990. Inhibition of Theiler's virus-induced demyelination in vivo by tumor necrosis factor alpha. Int. Immunol. **2:**909–913.
26. **Pierce, M., and M. Rodriguez.** 1989. Erichrome stain for myelin on osmicated tissue embedded in glycol methacrylate plastic. J. Histotechnol. **12:** 35–36.
27. **Prayoonwiwat, N., and M. Rodriguez.** 1993. The potential for oligodendrocyte proliferation during demyelinating disease. J. Neuropathol. Exp. Neurol. **52:**55–62.
28. **Pullen, L. C., S. D. Miller, M. C. Dal Canto, and B. S. Kim.** 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.
29. **Rahemtulla, A., T. M. Kündig, A. Narendran, M. F. Bachman, M. Julius, C. J. Paige, P. S. Ohashi, R. F. Zinkernagel, and T. W. Mak.** 1994. Class II major histocompatibility complex-restricted T cell function in CD4-deficient mice. Eur. J. Immunol. **24:**2213–2218.
30. **Rodriguez, M.** 1985. Virus induced demyelination in mice: "dying back" of oligodendrocytes. Mayo Clin. Proc. **60:**433–436.
31. **Rodriguez, M., A. J. Dunkel, R. L. Thiemann, J. Leibowitz, M. Zijlstra, and R. Jaenisch.** 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.
32. **Rodriguez, M., J. J. Kenny, R. L. Thiemann, and G. E. Woloschak.** 1990. Theiler's virus-induced demyelination in mice immunosuppressed with anti-IgM and in mice expressing the *xid* gene. Microb. Pathog. **8:**23–35.
33. **Rodriguez, M., W. P. Lafuse, J. L. Leibowitz, and C. S. David.** 1986. Partial suppression of Theiler's virus-induced demyelination in vivo by administration of monoclonal antibodies to immune-response gene products (Ia antigens). Neurology **30:**964–970.
34. **Rodriguez, M., J. L. Leibowitz, and P. W. Lampert.** 1983. Persistent infection of oligodendrocytes in Theiler's virus-induced encephalomyelitis. Ann. Neurol. **13:**426–432.
35. **Rodriguez, M., J. L. Leibowitz, H. C. Powell, and P. W. Lampert.** 1983. Neonatal infection with the Daniel strain of Theiler's murine encephalomyelitis virus. Lab. Invest. **49:**672–678.
36. **Rodriguez, M., and M. Lindsley.** 1992. Immunosuppression promotes central nervous system remyelination in chronic virus-induced demyelinating disease. Neurology **42:**348–357.
37. **Rodriguez, M., M. D. Lindsley, and M. L. Pierce.** 1991. Role of T cell resistance to Theiler's virus infection. Microb. Pathog. **11:**269–281.

38. **Rodriguez, M., E. Oleszak, and J. Leibowitz.** 1987. Theiler's murine encephalomyelitis: a model of demyelination and persistence of virus. Crit. Rev. Immunol. **7:**325–365.
39. **Rodriguez, M., K. Pavelko, and R. L. Coffman.** 1995. Gamma interferon is critical for resistance to Theiler's virus-induced demyelination. J. Virol. **69:** 7286–7290.
40. **Rodriguez, M., K. D. Pavelko, C. W. McKinney, and J. L. Leibowitz.** 1994. Recombinant human IL-6 suppresses demyelination in a viral model of multiple sclerosis. J. Immunol. **153:**3811–3821.
41. **Rodriguez, M., and J. Quddus.** 1986. Effect of cyclosporin A, silica quartz dust and macrophage protease inhibitors on virus-induced demyelination. J. Neuroimmunol. **13:**159–174.
42. **Rodriguez, M., and S. Sriram.** 1988. Successful therapy of Theiler's virus-induced demyelination (DA strain) with monoclonal anti-lyt-2 antibody. J. Immunol. **140:**2950–2955.
43. **Roos, R. P., S. Firestone, R. Wollman, D. Variakojis, and B. G. W. Arnason.** 1982. The effect of short term and chronic immunosuppression on Theiler's virus demyelination. J. Neuroimmunol. **2:**223–234.
44. **Spriggs, M. K., B. H. Koller, T. Sato, P. J. Morrissey, W. C. Fanslow, O. Smithies, R. F. Voice, M. B. Widmer, and C. R. Malizewski.** 1992. β_2-Microglobulin, CD8+ T cell-deficient mice survive inoculation with high doses of vaccinia virus and exhibit altered IgG responses. Proc. Natl. Acad. Sci. USA **89:**6070–6074.
45. **Tripp, A., S. R. Sarawar, and P. C. Doherty.** 1995. Characteristics of influenza virus-specific CD8+ T cell response in mice homozygous for disruption of the H-2I-A^b gene. J. Immunol. **155:**2955–2959.
46. **Welsh, C. J. R., P. Tonks, A. A. Nash, and W. F. Blakemore.** 1987. The effect of L3T4 T cell depletion on the pathogenesis of Theiler's murine encephalomyelitis virus infection in CBA mice. J. Gen. Virol. **68:**1659–1667.