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Class II-restricted T cell responses in Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease

III. Failure of neuroantigen-specific immune tolerance to affect the clinical course of demyelination

Stephen D. Miller^{1,4}, Sheila J. Gerety¹, Mary K. Kennedy¹, Jeffrey D. Peterson⁴,
John L. Trotter⁵, Vincent K. Tuohy⁶, Carl Waltenbaugh¹, Mauro C. Dal Canto²
and Howard L. Lipton^{3,4}

Departments of ¹ Microbiology-Immunology, ² Pathology, ³ Neurology, and ⁴ the Program in Neuroscience, Northwestern University Medical School, Chicago, IL 60611, U.S.A., ⁵ Department of Neurology, Washington University Medical School, St. Louis, MO 63110, U.S.A., and ⁶ Department of Biochemistry, E.K. Shriver Center, Waltham, MA 02115, U.S.A.

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Summary

Intracerebral inoculation of Theiler's murine encephalomyelitis virus (TMEV) into susceptible mouse strains produces a chronic demyelinating disease in which mononuclear cell-rich infiltrates in the central nervous system (CNS) are prominent. Current evidence strongly supports an immune-mediated basis for myelin breakdown, with an effector role proposed for TMEV-specific, major histocompatibility complex (MHC) class II-restricted delayed-type hypersensitivity (DTH) responses in which lymphokine-activated macrophages mediate bystander demyelination. The present study examined the possibility that concomitant or later-appearing neuroantigen-specific autoimmune T cell responses, such as those demonstrated in chronic-relapsing experimental allergic encephalomyelitis (R-EAE), may contribute to the demyelinating process following TMEV infection. T cell responses against intact, purified major myelin proteins (myelin

Address for correspondence: Dr. Stephen D. Miller, Department of Microbiology-Immunology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611, U.S.A.

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Abbreviations: CMI, cell-mediated immunity; CNS, central nervous system; DTH, delayed-type hypersensitivity; i.c., intracerebral; MBP, myelin basic protein; MDO, mean day of onset; MHC, major histocompatibility complex; MS, multiple sclerosis; MSCH, mouse spinal cord homogenate; MSCH-SP, MSCH-coupled splenocytes; PCFIA, particle concentration fluorescence immunoassay; pfu, plaque-forming unit; p.i., post-infection; PLP, proteolipid apoprotein; R-EAE, chronic-relapsing experimental allergic encephalomyelitis; rfu, relative fluorescence units; TMEV, Theiler's murine encephalomyelitis virus; T_{DH}, delayed-type hypersensitivity T cell(s); Tprlf, T cell proliferation.

basic protein (MBP) and proteolipid protein (PLP)), and against altered myelin constituents were readily demonstrable in SJL/J mice with R-EAE, but were not detectable in SJL/J mice with TMEV-induced demyelinating disease. TMEV-infected mice also did not display T cell responses against the peptide fragments of MBP(91–104) and PLP(139–151) recently shown to be encephalitogenic in SJL/J mice. In addition, induction of neuroantigen-specific tolerance to a heterogeneous mixture of CNS antigens, via the i.v. injection of syngeneic SJL/J splenocytes covalently coupled with mouse spinal cord homogenate, resulted in significant suppression of clinical and histologic signs of R-EAE and the accompanying MBP- and PLP-specific DTH responses. In contrast, neuroantigen-specific tolerance failed to alter the development of clinical and histologic signs of TMEV-induced demyelinating disease or the accompanying virus-specific DTH and humoral immune responses. These findings demonstrate that TMEV-induced demyelinating disease can occur in the apparent absence of neuroantigen-specific autoimmune responses. The relationship of the present results to the immunopathology of multiple sclerosis is discussed.

Introduction

Following intracerebral (i.c.) inoculation with Theiler's murine encephalomyelitis viruses (TMEV), certain mouse strains develop a chronic, inflammatory demyelinating disease that begins 1–2 months post-infection (p.i.) (Lipton, 1975; Dal Canto and Lipton, 1976; Lehrich et al., 1976). This pathologic process is related to persistent infection wherein low levels of virus can be recovered from the central nervous system (CNS) for virtually the lifetime of the mouse (Lipton, 1975; Lehrich et al., 1976; Lipton et al., 1984). We have recently proposed a model (Clatch et al., 1986) wherein virus-specific delayed-type hypersensitivity (DTH) within the CNS results in demyelination by a terminal nonspecific bystander response mediated by activated macrophages. This hypothesis is based on our recent studies showing that disease susceptibility correlated with the temporal development of chronic, high levels of TMEV-specific, major histocompatibility complex (MHC) class II-restricted DTH responses, but not with virus-specific serum antibody or splenic T cell proliferative (Tprlf) responses, or with CNS virus titers (Clatch et al., 1985, 1986, 1987). Our hypothesis would account for the characteristic mononuclear cell-rich infiltrates observed in areas of primary demyelination (Dal Canto and Lipton, 1976; Lehrich et al., 1976) and for the increased numbers of macrophages (in which TMEV appears to primarily persist) within the CNS (Dal Canto and Lipton, 1982; Rodriguez et al., 1983). The hypothesis is also consistent with previous

findings that susceptibility to TMEV-induced demyelinating disease is controlled by at least two unlinked genes, one of which maps to the MHC (Clatch et al., 1985, 1987; Rodriguez and David, 1985; Rodriguez et al., 1986) and another of which is closely linked to the constant region genes encoding the β -chain of the T cell receptor (Melvold et al., 1987).

TMEV-induced demyelinating disease is considered one of the best experimental animal models of multiple sclerosis (MS) due to histopathologic and genetic similarities and by epidemiologic studies favoring a viral etiology of MS (Nathanson and Miller, 1978; Kurtke and Hyllested, 1986). Due to its histopathologic similarities with experimental allergic encephalomyelitis (EAE), a possible role for autoimmune neuroantigen-specific T cell-mediated immune (CMI) responses has been hypothesized in MS (Waksman, 1984). One could also envision autoimmune mechanisms contributing to the demyelinating process following TMEV infection: (a) as a direct consequence of CNS virus infection; (b) secondary to TMEV-specific immune-mediated CNS damage; or (c) as a result of cross-reactivity between TMEV and CNS antigens (i.e., molecular mimicry).

Our previous studies did not support a role for autoimmune responses against myelin antigens in TMEV-induced demyelinating disease as neuroantigen-specific CMI responses against the major myelin proteins, myelin basic protein (MBP) and proteolipid protein (PLP), were not detected in TMEV-infected SJL/J mice (Miller et al., 1987). In contrast, such responses were easily demonstra-

ble in mice with chronic-relapsing EAE (R-EAE) (Kennedy et al., 1987; Miller et al., 1987). However, these previous studies did not examine T cell responsiveness specific for the encephalitogenic regions of MBP and PLP nor did they exclude a possible role for autoimmune responses against minor myelin components, altered myelin antigens, or other nonmyelin-associated CNS antigens. In the present study, examination of mice with TMEV-induced demyelinating disease failed to reveal T cell responses specific for the defined encephalitogenic peptides of either MBP (Kono et al., 1988) or PLP (Tuohy et al., 1989), or against spinal cord homogenate fractions, containing a heterogeneous mixture of both myelin and nonmyelin-associated CNS antigens. In addition, induction of neuroantigen-specific immune tolerance in SJL/J mice via the i.v. injection of syngeneic splenocytes covalently coupled with a crude mouse spinal cord homogenate (MSCH-SP) (Kennedy et al., 1988) had no effect on the development of TMEV-specific CMI responses or the clinical and histologic signs of TMEV-induced demyelinating disease, but severely inhibited the induction of clinical and histologic R-EAE and accompanying neuroantigen-specific autoimmune responses. These results demonstrate that chronic TMEV-induced demyelination occurs in the apparent absence of neuroantigen-specific autoimmune responses.

Materials and methods

Mice

Female SJL/J mice, 4–5 weeks old, were purchased from Jackson Laboratories, Bar Harbor, ME, U.S.A. All mice were housed in the Northwestern animal care facility and, except during periods of severe paralysis, were maintained on standard laboratory chow and water ad libitum. Severely paralyzed mice were afforded easier access to food and water. Within each experiment, mice were age-matched (6–10 weeks) and from the same shipment.

Virus production and purification

After plaque purification, working stocks of the BeAn 8386 strain of TMEV were prepared by

passage in BHK-21 cells. The virus was purified by isopycnic centrifugation in Cs_2SO_4 gradients (Lipton and Friedman, 1980).

Neuroantigens

Mouse myelin basic protein (Mouse MBP) was prepared and purified according to the method of Swanborg et al. (1974). Water-soluble human proteolipid apoprotein (Hu PLP) was prepared and purified according to the method of Lees and Sakura (1978). Synthetic peptides of murine PLP 139–151S (HSLGKWLGHDPKF — containing a serine for cysteine substitution at residue 140) (Tuohy, 1989) and murine MBP 91–104S (VTPRTPPPSQGKGR) (Kono et al., 1988), both of which have been shown to be encephalitogenic for the SJL/J mouse, were prepared by the simultaneous multiple peptide synthesis ('teabag') method of Houghten et al. (1985, 1986) exactly as previously described (Tuohy, 1989). Lyophilized preparations of mouse spinal cord homogenate (MSCH) were prepared following homogenization of freshly obtained SJL/J spinal cords in distilled water. Prior to use in DTH and Tprlf assays, MSCH suspensions (2–3 mg/ml) in PBS were sonicated for 40 min at 4°C in a Branson 1200 sonic water bath and then subjected to 3700 R irradiation (^{137}Cs source).

Preparation of mouse spinal cord homogenate-coupled splenocytes

Erythrocyte-free (Tris- NH_4Cl -treated) splenocytes were coupled with nonsonicated MSCH using an ECDI-coupling procedure (Miller et al., 1979). Briefly, saline-washed SJL/J splenocytes were pelleted in 50 ml centrifuge tubes and resuspended to a final concentration of 2.5×10^8 cells/ml in saline containing 1 mg/ml of MSCH. Sham-coupled cells were prepared without MSCH in the reaction mixture. The coupling reaction was initiated by the addition of 0.171 ml of freshly prepared carbodiimide HCl (100 mg/ml saline; Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) per ml of cell suspension (final concentration of carbodiimide: 14.6 mg/ml). Following a 1 h incubation at 4°C, the cells were washed 3 times with balanced salt solution (BSS) and maintained at 4°C until use.

Induction of neuroantigen-specific tolerance

Tolerance was induced by the i.v. injection of MSCH-coupled splenocytes (MSCH-SP) in 0.5 ml BSS. Mice were tolerized on day -7 relative to immunization on days 0 and +7.

Induction of chronic-relapsing experimental allergic encephalomyelitis

Seven- to 10-week-old mice were immunized with MSCH in complete Freund's adjuvant (hereafter referred to as MSCH/CFA) on days 0 and +7 according to the method of Brown and McFarlin (1981), with slight modifications as previously described (Kennedy et al., 1987). Mice were observed for clinical signs of disease every 1-3 days and were scored according to their clinical severity as follows: grade 0, no abnormality; grade 1, limp tail; grade 2, limp tail and hind limb weakness (waddling gait); grade 3, partial hind limb paralysis; grade 4, complete hind limb paralysis; and grade 5, moribund. A clinical relapse was scored when a mouse developed a new neurologic deficit (increase in clinical severity of at least 1 grade) after a period of stabilization or improvement of its clinical score.

Peripheral immunization with TMEV and neuroantigen peptides

SJL/J mice were immunized s.c. at the tail base with 25 µg of purified UV-inactivated TMEV, 25 µg of PLP(139-151), or 25 µg of MBP(91-104) in 0.1 ml of 1:1 CFA emulsion.

Induction of TMEV-induced demyelinating disease

Seven- to 10-week-old SJL/J female mice were anesthetized with methoxyflurane and inoculated in the right cerebral hemisphere with 2.9×10^6 plaque-forming units (PFU) of stock BeAn virus in 30 µl. Control mice received 20 µl of Dulbecco's modified Earle's medium (DMEM). All animals were examined several times per week for development of neurological signs, particularly the chronic gait abnormality indicative of TMEV-induced demyelination (Lipton and Dal Canto, 1976). We have found that a clinical disease endpoint is a reliable marker for severe demyelinating disease (Clatch et al., 1985, 1987).

Histologic evaluation

Two to four mice per experimental group were anesthetized with sodium pentobarbital and

sacrificed by total body perfusion through the left ventricle with chilled 3% glutaraldehyde in phosphate buffer, pH 7.3. Spinal cords were removed by dissection and cut into 1 mm thick cross-sections which were post-fixed in 1% OsO₄, dehydrated and embedded in Epon as previously described (Dal Canto and Lipton, 1976). Toluidine blue-stained sections from 10-11 segments per mouse were read blindly.

Measurement of antigen-specific DTH responses

DTH responses were elicited in mice with R-EAE 12-14 days after the first injection of MSCH/CFA; in TMEV-infected mice 45-90 days p.i.; and 7 days post-priming in mice peripherally immunized with UV-inactivated TMEV, or the encephalitogenic peptides, PLP(139-151) and MBP(91-104). Responses were quantitated using an ear swelling assay as previously described (Clatch et al., 1985). Pre-challenge ear thickness was determined using a Mitutoyo model 7326 engineer's micrometer (Schlesinger's Tools, Brooklyn, NY, U.S.A.). Immediately thereafter, DTH responses were elicited by injecting 20 µg of sonicated spinal cord homogenates, 5 µg of mouse MBP, MBP(91-104), human PLP, PLP(139-151), or UV-inactivated TMEV (all in 10 µl saline) into the dorsal surface of the ear using a 100 µl Hamilton syringe fitted with a 30-gauge needle. 24 h after challenge, the increase in ear thickness over pre-challenge measurements was determined and the results were expressed in units of 10^{-4} inches. Ear swelling responses were the result of mononuclear cell infiltration and showed typical DTH kinetics (i.e., minimal swelling at 4 h, maximal swelling at 24-48 h).

In vitro Tprlf

Following DTH measurement, spleen (from mice inoculated i.c. with viable virus) or draining lymph node cells (from R-EAE mice and mice peripherally immunized with UV-inactivated TMEV or encephalitogenic neuroantigen peptides) were pooled and cultured in vitro as previously described to determine antigen-specific Tprlf responses (Clatch et al., 1985). 5×10^5 viable cells were cultured for 96 h in 96-well flat-bottom microwell plates in 0.2 ml of modified Click's medium (Corradin et al., 1977) containing $5 \times$

10^{-5} M 2-mercaptoethanol (2-ME), 0.01 M NaHCO_3 , and supplemented with 0.5% fresh syngeneic mouse serum. Cultures were stimulated with 0.5–5.0 μg /well of UV-inactivated TMEV, PLP, PLP(139–151), MBP, or MBP(91–104) and with 10 μg /well of sonicated spinal cord homogenates. The cultures were pulsed with 1.0 μCi of [^3H]thymidine ([^3H]TdR, 6.7 Ci/mmol, Research Products International Corp., Mount Prospect, IL, U.S.A.) for the final 24 h. Cultures were harvested onto glass fiber filters with an automated sample harvester and radioactive measurements of trichloroacetic acid (TCA)-insoluble material were determined in a Beckman liquid scintillation counter. Cultures were run in triplicate and results expressed as delta counts per minute (Δcpm) = (mean cpm of stimulated cultures) – (mean cpm of control cultures) and as stimulation indices = (mean cpm of stimulated cultures)/(mean cpm of control cultures). Proliferative responses under these conditions have been shown to be I-A-restricted and mediated by Lyt-1^+2^- , L3T4^+ T cells (Clatch et al., 1986; Kennedy et al., 1987).

Assay of TMEV-specific serum antibody titers

Serum antibody levels to the BeAn strain of TMEV were quantitated using a modified particle concentration fluorescence immunoassay (PCFIA) as previously described (Peterson et al., 1989). Briefly, the assay is performed in fluid phase, allowing the binding of serial dilutions of serum antibodies to fluorescein isothiocyanate (FITC)-labeled TMEV. The resulting antibody-antigen complexes are captured by a solid-phase matrix consisting of affinity-purified, goat anti-mouse Ig-coated polystyrene particles. Unbound immunoglobulin is removed by washing and the solid-phase-associated fluorescence is concentrated to the bottom of the assay wells for measurement of relative fluorescence units (rfu). Antibody concentrations (mg/ml) were determined by logarithmic linear regression analysis of serial dilution curves in comparison to a hyperimmune serum standard.

Statistical analyses

The statistical significance of DTH and serum antibody responses between experimental groups was analyzed using a one-tailed analysis of vari-

ance. Group means were compared using the Scheffé multiple comparison test (Armitage, 1971). Comparisons of the percentage of animals showing clinical disease between any two groups of mice were analyzed by χ^2 . *P* values < 0.05 were considered significant.

Results

TMEV-infected SJL/J mice fail to exhibit T cell responses to the encephalitogenic determinants of MBP and PLP

Our previous studies indicated that TMEV-infected mice failed to exhibit DTH or Tprlf responses upon challenge with the purified major myelin proteins, MBP and PLP, while such responses were detectable in mice with R-EAE (Miller et al., 1987). To determine whether potential anti-myelin responses may have gone undetected due to an insufficient molar concentration of the relevant encephalitogenic epitopes upon challenge with the *intact* myelin proteins, mice (three per group) infected with viable TMEV or peripherally immunized with UV-inactivated TMEV emulsified in CFA were challenged with the encephalitogenic peptides PLP(139–151) and MBP(91–104). As seen in Fig. 1, both SJL/J mice infected with TMEV (60 days p.i.) and mice peripherally immunized with TMEV exhibited significant DTH (left panel) and Tprlf responses (right panel) upon challenge with UV-inactivated TMEV, but failed to respond to PLP, MBP, or their encephalitogenic peptides. Similar results were seen in mice infected with TMEV 90 days previously. As controls, SJL/J mice immunized with 25 μg of PLP(139–151) (four per group) responded significantly to challenge with the homologous PLP peptide and intact PLP, but failed to respond to TMEV or to the MBP(91–104). Similarly, mice immunized with 25 μg of MBP(91–104) (three per group) responded weakly, but significantly, to challenge with MBP(91–104), but did not respond to challenge with TMEV or PLP(139–151). These data confirm that in our earlier report which showed lack of T cell responses specific for intact MBP and PLP in TMEV-infected mice and further indicate lack of re-

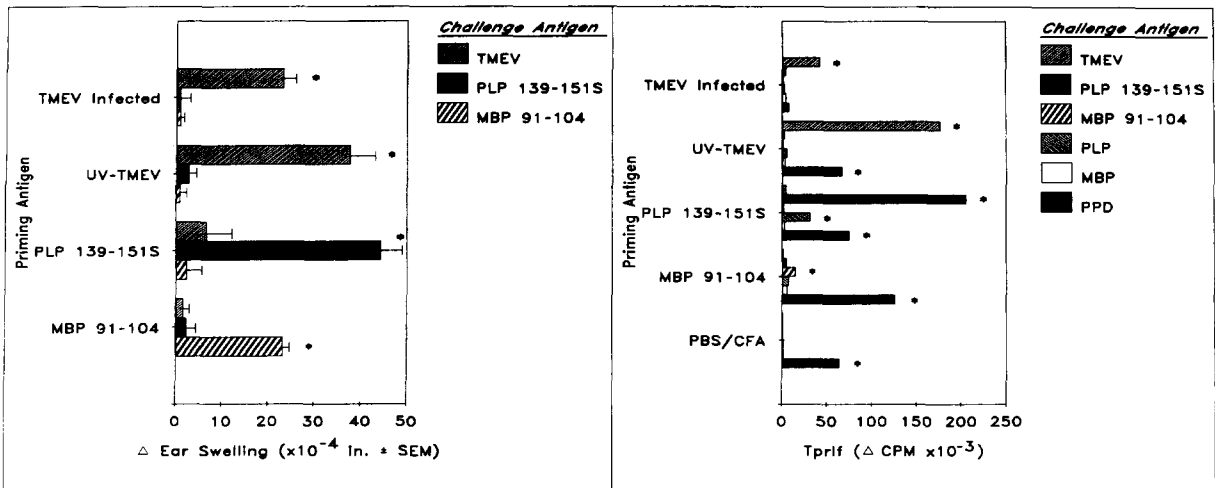


Fig. 1. TMEV-infected SJL/J mice fail to exhibit T cell responses to the encephalitogenic determinants of MBP and PLP. Left panel: Female SJL/J mice (nine per group) infected 60 days previously with TMEV or immunized 7 days previously with 25 μ g of UV-inactivated TMEV, 25 μ g of PLP(139-151), or 25 μ g of MBP(91-104) were divided into three groups (three per group) and ear challenged with 5 μ g of UV-TMEV, PLP(139-151), and MBP(91-104). Results are expressed as Δ 24 h ear swelling responses (background responses of negative control mice subtracted) in units of 10^{-4} inches \pm SEM. Background ear swelling responses in unimmunized mice were 3.3 ± 2.0 for UV-TMEV, 8.2 ± 2.2 for PLP(139-151), and 4.7 ± 2.0 for MBP(91-104). Asterisks indicate responses which were significantly different ($P < 0.001$) from the corresponding unimmunized controls. Right panel: following 24 h ear measurement, 3-4 mice from each group were sacrificed and 4×10^5 viable spleen (from TMEV-infected mice) or LN (from peripherally immunized mice) cells were cultured with 5 μ g/well of the indicated antigens. Cultures were pulsed with 1.0 μ Ci of [3 H]TdR at 72 h and harvested 24 h later. Results are expressed as Δ cpm (no antigen cpm subtracted). Asterisks indicate responses significantly above background ($SI \geq 2.00$).

sponsiveness to high concentrations of the encephalitogenic epitopes of MBP and PLP.

TMEV-infected SJL/J mice fail to exhibit T cell responses to spinal cord homogenate

To address a possible role for autoimmune responses against minor myelin components, altered myelin antigens, or other nonmyelin-associated CNS antigens in the pathogenesis of TMEV-induced demyelinating disease, we next asked if TMEV-infected mice exhibited T cell responsiveness against spinal cord homogenate fractions, containing a heterogeneous mixture of both myelin and nonmyelin-associated CNS antigens. Homogenates prepared from the spinal cords of normal mice (Normal MSCH) or from the spinal cords of mice undergoing active R-EAE (EAE MSCH) or TMEV-induced demyelinating disease (TMEV MSCH) were tested. The latter two preparations were used in an attempt to determine if TMEV-infected mice would respond to neuroantigenic determinants which might have been modified or

altered as a consequence of active immune-mediated demyelination. As seen in Fig. 2, mice (three per group) infected with TMEV 60 days previously failed to respond by either DTH or Tprlf responses to challenge with MBP or any of the MSCH preparations (panel B). In contrast, mice (3-4 per group) sensitized with MSCH/CFA (panel A) responded vigorously to challenge with MBP as well as to each of the MSCH preparations. Thus, mice undergoing active TMEV-induced demyelinating disease fail to respond to a heterogeneous mixture of both myelin and nonmyelin-associated CNS antigens derived from either normal or damaged spinal cord tissue.

Effect of neuroantigen-specific tolerance induction on the clinical signs of R-EAE- and TMEV-induced demyelinating disease

We recently reported (Kennedy et al., 1988) that the i.v. injection of SJL/J mice with syngeneic spleen cells covalently coupled with MSCH led to the profound suppression of clinical and

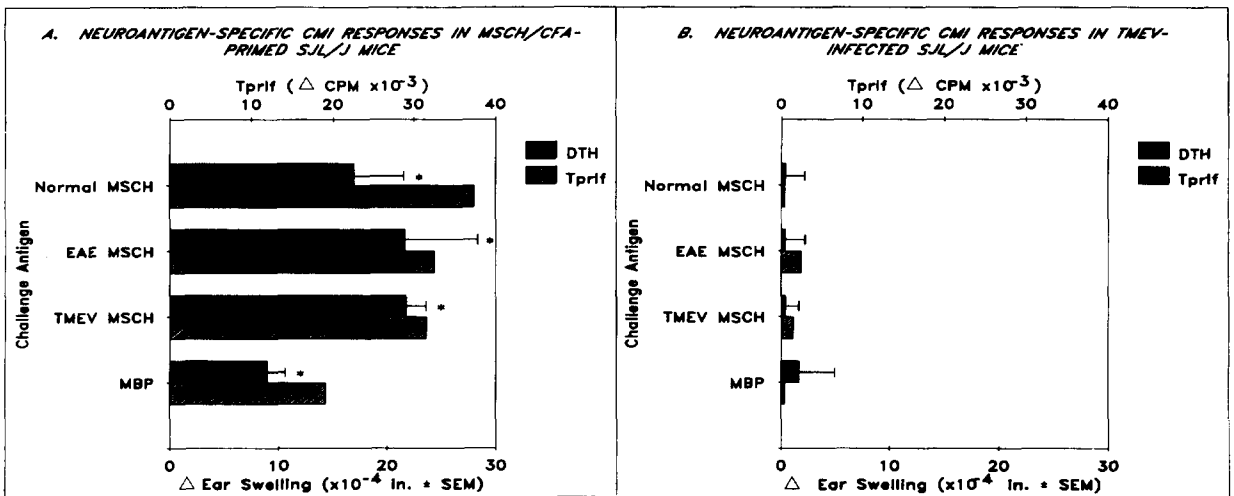


Fig. 2. TMEV-infected SJL/J mice fail to exhibit T cell responses to spinal cord homogenate. Groups of female SJL/J mice 12 days post-sensitization on days 0 and +7 with MSCH/CFA (panel A) or 60 days post-i.c. infection with TMEV (panel B) were tested for DTH responses (three mice per group) upon challenge with 5 μ g of mouse MBP, or 20 μ g of spinal cord homogenates prepared from normal mice (Normal MSCH), mice with active R-EAE (EAE MSCH), and mice with TMEV-induced demyelinating disease (TMEV MSCH). Results are expressed as Δ 24 h ear swelling responses (background responses of unimmunized mice subtracted) in units of 10^{-4} inches \pm SEM. Background ear swelling responses in unimmunized mice were 10.0 ± 1.4 for Normal MSCH, 4.4 ± 1.4 for EAE MSCH, 5.0 ± 1.5 for TMEV MSCH, and 4.0 ± 2.3 for Mouse MBP. Asterisks indicate responses which were significantly different ($P < 0.01$) from the corresponding unimmunized controls. Following 24 h ear measurement, 3–4 mice from each group were sacrificed and 4×10^5 viable LN (from MSCH/CFA-primed mice) or spleen (from TMEV-infected mice) cells cultured with 5 μ g/well of MBP or 10 μ g/well of the indicated MSCH antigens. Cultures were pulsed with 1.0 μ Ci of [3 H]TdR at 72 h and harvested 24 h later. Results are expressed as Δ cpm (no antigen cpm subtracted). Stimulation indices ranged between 2.4 and 5.5 in the cultures of MSCH/CFA-primed LN cells.

histopathologic R-EAE induced by the s.c. injection of MSCH/CFA. Inhibition of clinical R-EAE following MSCH-SP tolerization correlated with the induction of antigen-specific tolerance of DTH to the two major myelin proteins, MBP and PLP. To further test the hypothesis that neuroantigen-specific autoimmune responses may play a contributory role in the pathogenesis of TMEV-induced demyelinating disease, we asked if neuroantigen-specific tolerance to MSCH proteins (containing a heterogeneous mixture of neuroantigens) would affect the development of clinical signs of TMEV-induced demyelination. In two separate experiments, a total of 44 SJL/J mice were injected with 7.5×10^7 sham-coupled spleen cells (Sham-SP) and 45 mice with 7.5×10^7 MSCH-SP on day -7. On days 0 and +7, 25 mice from each treatment group were immunized with MSCH/CFA and observed for development of clinical R-EAE for 60 days (Fig. 3A). The remaining 19–20 mice from each treatment group were inoc-

ulated i.c. with virus and observed for clinical signs of TMEV-induced demyelinating disease for 65 days (Fig. 3B). In agreement with our recent results (Kennedy et al., 1988), i.v. tolerization with MSCH-SP led to a significant ($P < 0.01$) decrease in clinical R-EAE (7/25 = 28% affected) compared to controls injected with Sham-SP (17/25 = 68% affected). Average clinical scores of affected mice in the Sham-SP-injected group were severe. Of 17 affected mice, one died, one recovered completely, and 15 remained chronically affected with six of those exhibiting clinical relapses. The average clinical scores in affected MSCH-SP-injected mice were generally less severe than in the sham-tolerized controls, and 6/7 affected animals completely recovered after the initial clinical episode.

In contrast to the suppression of R-EAE, neuroantigen-specific tolerance had no effect on the development of clinical signs of TMEV-induced demyelinating disease (Fig. 3B). 16/19 mice (84.2%) tolerized with Sham-SP exhibited clinical

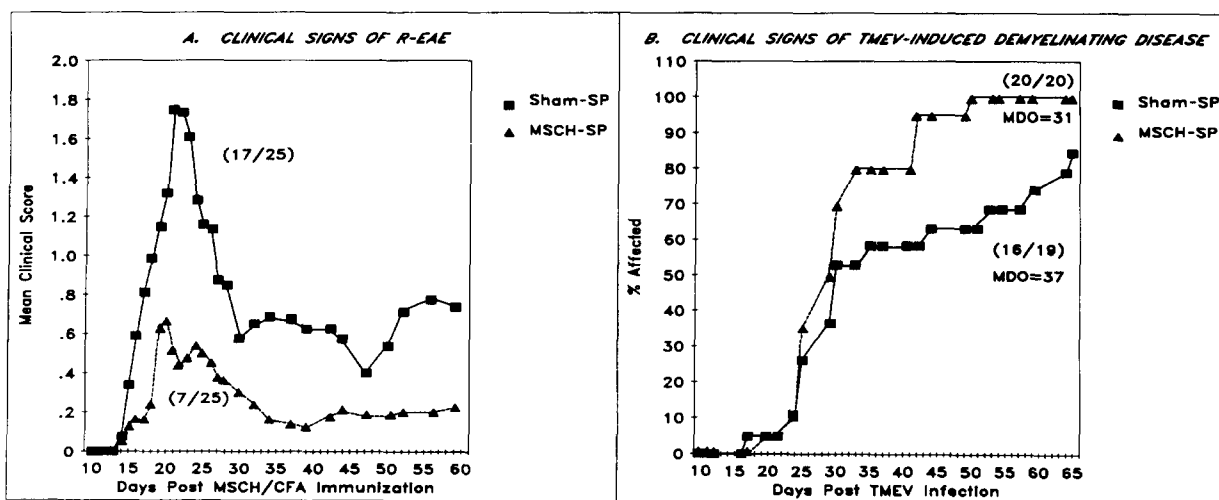


Fig. 3. Effect of neuroantigen-specific tolerance induction on the clinical signs of R-EAE- and TMEV-induced demyelinating disease. Groups of female SJL/J mice were tolerized on day -7 with 7.5×10^7 sham-coupled splenocytes (Sham-SP, $n = 44$) or 7.5×10^7 MSCH-coupled splenocytes (MSCH-SP, $n = 45$). The data shown is the composite of two separate experiments. Panel A: On days 0 and $+7$, 25 mice from each treatment group were sensitized with MSCH/CFA and observed and scored (see Materials and Methods) for clinical signs of R-EAE for 60 days post-immunization. The number of mice showing clinical signs of disease at any time during the observation period out of the total mice in each group is indicated in parentheses. Panel B: On day 0, 19 Sham-SP-tolerized mice and 20 MSCH-SP-tolerized mice were inoculated i.c. with 1.3×10^6 pfu of TMEV and observed for clinical signs of disease for 60 days p.i. The number of mice showing clinical signs of disease at any time during the observation period is shown in parentheses and the mean day of onset (MDO) of clinical signs is indicated.

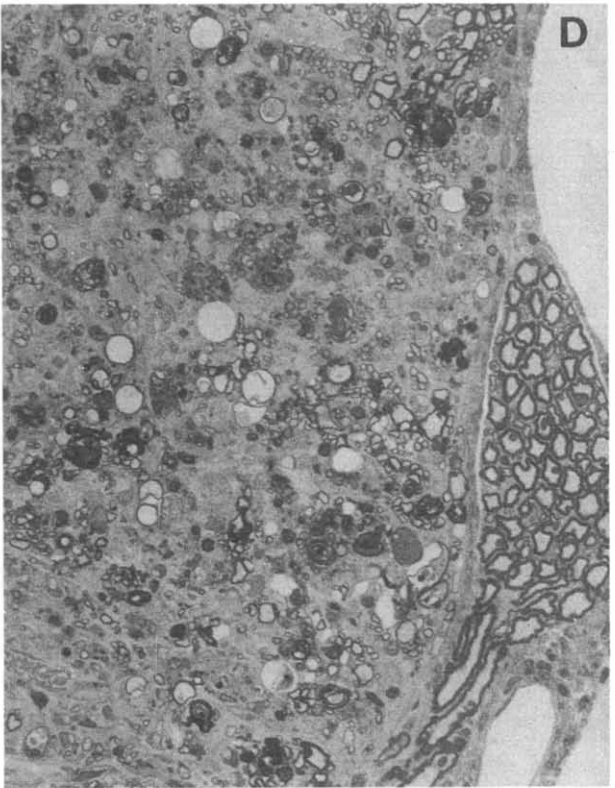
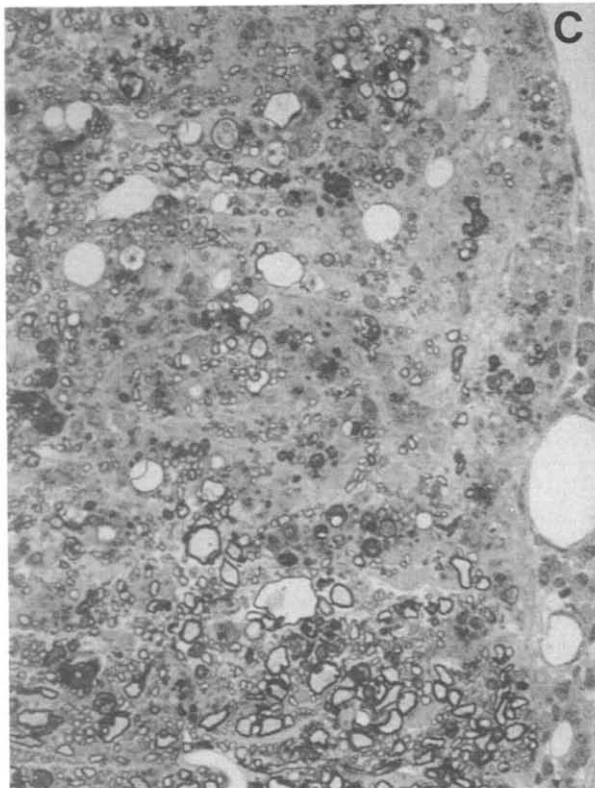
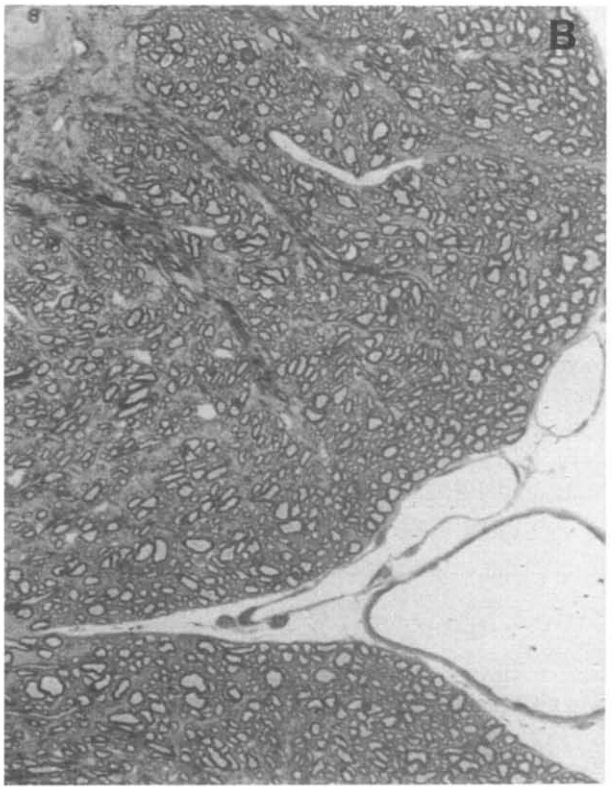
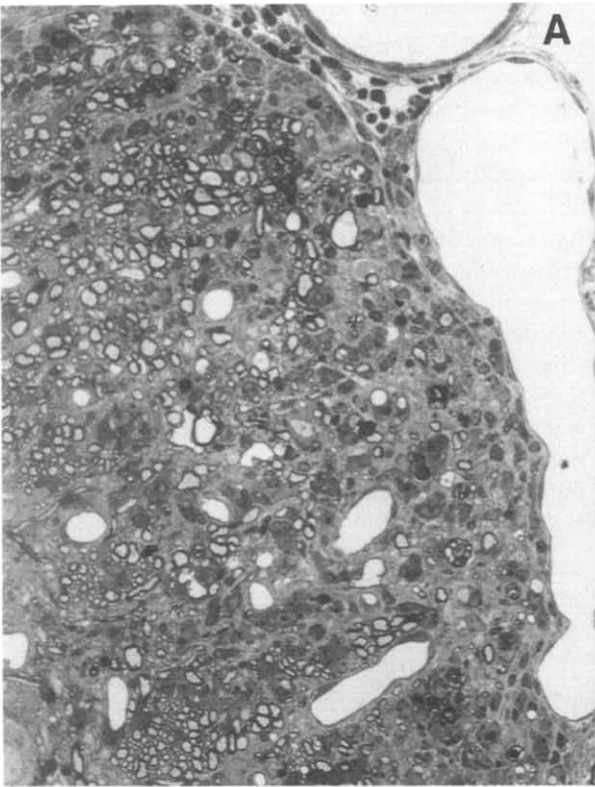
signs with a mean day of onset (MDO) of 37 days, while 100% (20/20) of MSCH-SP-tolerized mice developed clinical signs (MDO = 31 days). Although in both experiments disease developed slightly earlier in MSCH-SP-tolerized, TMEV-infected mice, the differences were not statistically significant.

Effect of neuroantigen-specific tolerance induction on histopathologic signs of R-EAE- and TMEV-induced demyelinating disease

Microscopic examination of spinal cords (day $+17$) from sham-tolerized mice sensitized with

MSCH/CFA showed the classical features of murine EAE. The disease was characterized by the presence of inflammatory infiltrates surrounded by areas of demyelination, as well as axonal destruction (Fig. 4A). In contrast, animals tolerized with MSCH-SP 7 days prior to sensitization with MSCH/CFA were devoid of demyelinating lesions, and only occasional small numbers of mononuclear cells were observed around meningeal vessels (Fig. 4B). Spinal cord sections from TMEV-infected animals (day 39 p.i.) showed no differences between sham-tolerized and MSCH-SP-tolerized animals. Both groups developed in-

Fig. 4. Effect of neuroantigen-specific tolerance induction on histopathological signs of R-EAE- and TMEV-induced demyelinating disease. Spinal cord sections were prepared on day $+17$ from Sham-SP- and MSCH-SP-tolerized SJL/J mice primed with MSCH/CFA and on day $+39$ from Sham-SP- and MSCH-SP-tolerized SJL/J mice infected with TMEV. Panel A: Section of spinal cord from a mouse sham-tolerized and sensitized with MSCH/CFA. Mononuclear cell infiltration and extensive subpial and perivascular demyelination are observed. $1 \mu\text{m}$ thick Epon-embedded section stained with toluidine blue. $\times 230$. Panel B: Section of spinal cord from a mouse tolerized with MSCH-SP and sensitized with MSCH/CFA. No inflammation nor demyelination are observed. $1 \mu\text{m}$ thick Epon-embedded section stained with toluidine blue. $\times 230$. Panel C: Section of spinal cord from a mouse sham-tolerized and infected with TMEV. Mononuclear cell infiltration and extensive demyelination are present 45 days after infection. $1 \mu\text{m}$ thick Epon-embedded section stained with toluidine blue. $\times 230$. Panel D: Section of spinal cord from a mouse tolerized with MSCH-SP and infected with TMEV. Mononuclear cell infiltrates and extensive demyelination are observed 45 days after infection. $1 \mu\text{m}$ thick Epon-embedded section stained with toluidine blue. $\times 230$.



flammatory demyelinating lesions characteristic of this viral infection (Fig. 4C and D).

Effect of neuroantigen-specific tolerance induction on specific immune responses

We have previously shown that the onset of initial clinical signs of R-EAE correlated with the development of MBP- and PLP-specific, MHC class II-restricted DTH responses (Kennedy et al., 1987). To determine if the inhibition of clinical signs of demyelination in MSCH-SP-tolerized, MSCH/CFA-primed mice correlated with induction of neuroantigen-specific T cell tolerance, the same mice used for assessment of clinical signs were tested on day +12 for DTH reactivity to MBP and PLP. As seen in Fig. 5A, mice injected

i.v. with MSCH-SP exhibited significantly suppressed MBP- and PLP-specific DTH responses as compared to Sham-SP-injected controls. Thus, these results confirm our earlier observation that inhibition of clinical R-EAE following neuroantigen-specific tolerance induction correlated with abrogation of neuroantigen-specific DTH reactivity (Kennedy et al., 1988). In contrast, neuroantigen-specific tolerance induction did not affect the level of TMEV-specific DTH or splenic T cell proliferative responses in TMEV-infected animals (Fig. 5B). Similarly, TMEV-specific serum antibody responses, as measured by a PCFIA assay, were similar ($P = 0.27$) in both sham- (0.62 ± 0.11 mg/ml) and MSCH-SP-tolerized (0.73 ± 0.14 mg/ml) mice (data not shown).

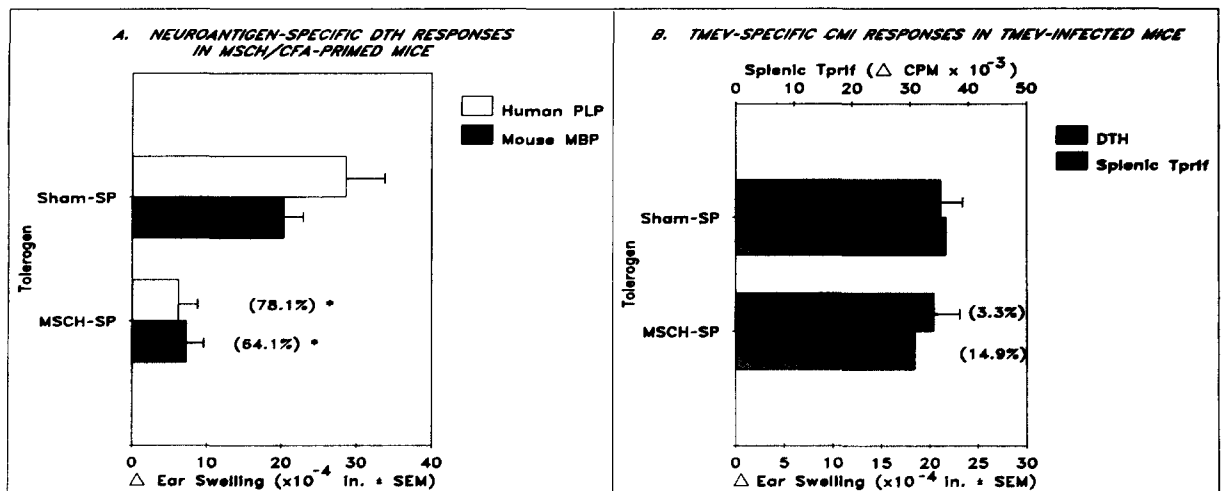


Fig. 5. Effect of neuroantigen-specific tolerance induction on T cell-mediated immune responses. The same groups of Sham-SP- and MSCH-SP-tolerized mice described in Fig. 3 were tested for antigen-specific DTH and/or splenic T cell proliferative responses. Panel A: Eight Sham-SP- and eight MSCH-SP-tolerized mice were tested for neuroantigen-specific DTH responses on day +12 relative to MSCH/CFA sensitization on days 0 and +7, by ear challenge with 5 μ g of mouse MBP in the left ear (open bars) and 5 μ g of human PLP in the right ear (solid bars). Results are expressed as Δ 24 h ear swelling responses (background responses of negative control mice subtracted) in units of 10^{-4} inches \pm SEM. Background ear swelling responses in unimmunized mice were 2.3 ± 1.5 for mouse MBP and 3.2 ± 0.9 for human PLP. Asterisks indicate responses which were significantly different ($P < 0.01$) from the corresponding responses of the Sham-SP-tolerized controls. Percent suppression of DTH responses of the MSCH-SP-tolerized mice relative to the sham-tolerized controls is indicated in parentheses. Panel B: Seven Sham-SP-tolerized mice and eight MSCH-SP-tolerized mice were tested for TMEV-specific CMI (DTH and splenic Tprlf) responses 37 days p.i. DTH was elicited by ear challenge with 5 μ g of UV-inactivated TMEV. Results are expressed as Δ 24 h ear swelling responses (background responses of negative control mice subtracted) in units of 10^{-4} inches \pm SEM. Background ear swelling in five unimmunized mice was 6.7 ± 1.3 units. Following 24 h ear measurement, 3-4 mice from each group were sacrificed and 4×10^5 viable spleen cells were cultured with 1 μ g/well of UV-inactivated TMEV. Cultures were pulsed with 1.0 μ Ci of [3 H]TdR at 72 h and harvested 24 h later. Results are expressed as Δ cpm (no antigen cpm subtracted). Stimulation indices were 5.1 for Sham-SP-tolerized mice and 4.8 for MSCH-SP-tolerized mice. Present suppression of DTH and Tprlf responses of the MSCH-SP-tolerized mice relative to the sham-tolerized controls is indicated in parentheses.

Discussion

Current evidence strongly suggests an immune-mediated basis for myelin breakdown in TMEV-induced demyelinating disease. This evidence includes studies showing that clinical signs of TMEV-induced demyelinating disease are inhibited by immunosuppressive agents (Lipton and Dal Canto, 1976; Roos et al., 1982) and by the *in vivo* administration of monoclonal antibodies to Ia antigens (Rodriguez et al., 1986; Friedmann et al., 1987) and the L3T4 determinant (Welsh et al., 1987; S.D. Miller et al., unpublished). Based on the correlation of susceptibility to demyelinating disease with the development of high levels of MHC class II-restricted, TMEV-specific DTH (Clatch et al., 1985, 1986, 1987), we have proposed a model (Clatch et al., 1986) wherein TMEV-specific effector T_{DH} cells play a central role in CNS demyelination. According to our hypothesis, local lymphokine production by virus-specific T_{DH} cells within the CNS effects recruitment, accumulation, and activation of macrophages which then mediate a terminal nonspecific bystander response resulting in stripping of myelin lamellae (Wisniewski and Bloom, 1975).

In addition to virus-specific CMI, it is also possible that an autoimmune CMI effector component, such as that demonstrated in EAE, could contribute to the demyelinating process. Activation of neuroantigen-specific CMI responses could occur as a consequence of CNS viral infection via at least three mechanisms. First, viral replication could lead to altered neuroantigen metabolism with the subsequent release of native or altered CNS antigens either locally or into the periphery resulting in auto sensitization. Within the CNS, this may be influenced by inappropriate presentation of self antigens by CNS astrocytes (Londei et al., 1984) which have been induced to express nonphysiologic levels of Ia antigens (Fontana et al., 1984). Second, virus-specific immune-mediated damage may similarly lead to release of negative or altered CNS antigens and subsequent auto sensitization. In support of these first two possibilities, sensitization to MBP has been shown to accompany two different CNS viral infections. Lymphocytes from rats with subacute demyelinating encephalomyelitis induced by *i.c.* infection

with the coronavirus, JHM, have been stimulated to proliferate *in vitro* in response to MBP. Furthermore, adoptive transfer of these cells into normal recipients has led to mild clinical disease and CNS lesions resembling EAE (Watanabe et al., 1983). Similarly, T cell lines specific for MBP, isolated from rats with subacute measles encephalomyelitis, transferred EAE-like symptoms to normal recipients (Liebert et al., 1988). However, no apparent cross-reactivity between either the MBP-specific T cells or serum antibody with measles virus has been demonstrated. A third mechanism leading to auto sensitization could occur if virus-specific immune responses cross-react with an antigenic determinant(s) normally expressed on CNS tissue (i.e., molecular mimicry) (Notkins et al., 1984; Oldstone and Notkins, 1986). In support of molecular mimicry, approximately 4% of monoclonal anti-viral antibodies have been shown to cross-react with host determinants expressed on uninfected tissues (Srinivasappa et al., 1986). Similarly, potential cross-reactive antigens capable of stimulating CMI responses have been identified by sequence homology with viral structural proteins. A synthetic peptide, the composition of which was based on an area of sequence homology between the hepatitis B virus polymerase and the encephalitogenic region of rabbit MBP, has been reported to induce a mild perivascular infiltration of the CNS reminiscent of EAE in rabbits. This peptide also induced humoral and CMI responses which cross-reacted with MBP (Fujinami and Oldstone, 1985).

In contrast to the JHM and measles virus models, our previous studies have not supported a role for neuroantigen-specific autoimmune responses in TMEV-induced demyelinating disease. Neither clinical nor histopathological signs of demyelination were transferred by serum or lymphoid cells from TMEV-infected donors to normal, non-TMEV-infected recipients suggesting a lack of neuroantigen-specific autoimmune responses (Barbano and Dal Canto, 1984). In addition, serum and lymphoid cells from TMEV-infected donors failed to injure myelinating isogeneic organotypic cultures *in vitro* (Barbano and Dal Canto, 1984). As a more direct measure, neuroantigen-specific DTH and splenic Tprlf responses specific for the major myelin proteins, MBP and PLP, were not

detected in SJL/J mice at any time following i.c. inoculation with TMEV (Miller et al., 1987). This included time points prior to the onset of clinical signs of disease (day 23 p.i.), shortly after the onset of disease (day 69 p.i.), and 8–12 weeks after the onset of disease (day 127 p.i.). In contrast, MBP- and PLP-specific DTH and Tprlf responses were readily demonstrable in SJL/J mice directly before the first appearance of clinical R-EAE (day 14 after immunization with MSCH/CFA) indicating that CMI responses against myelin components are readily detectable in mice with R-EAE (Kennedy et al., 1987; Miller et al., 1987). In addition, peripheral immunization of SJL/J mice with either UV-inactivated TMEV or rat MBP emulsified in CFA failed to reveal cross-reactive CMI responses upon secondary stimulation of primed lymph node cells in vitro (Miller et al., 1987). The lack of neuroantigen reactivity in TMEV-infected mice, as measured by functional CMI analyses, was supported by the absence of significant amino acid identity between the TMEV capsid proteins (VP1, VP2, or VP3) and MBP or PLP (Miller et al., 1987). Thus these data were consistent with our hypothesis (Clatch et al., 1985, 1986) that TMEV-specific CMI responses constitute the major effector mechanism of CNS demyelination following TMEV infection. Lack of reactivity to the major myelin proteins was confirmed in the present report and, in addition, present data (Fig. 1) failed to demonstrate T cell responses specific for determinants on PLP (Tuohy et al., 1989) and MBP (Kono et al., 1988) which have recently been shown to be encephalitogenic in SJL/J mice. The failure of MBP(91–104) (VTPTPPPSQKGR) to elicit responses in TMEV-infected SJL/J mice is particularly relevant. The RTPPP pentapeptide sequence contained within this peptide corresponds to the only area of any sequence homology between mouse MBP and the TMEV polyprotein, mapping to the *nonstructural* 2B protein of TMEV (Miller et al., 1987).

Although the previous data do not support a major role for class II-restricted autoimmune T cell responses against MBP and PLP in the TMEV-induced demyelinating process, we could not exclude a possible role for autoimmune responses against minor myelin components, such as

myelin-associated glycoprotein, myelin oligodendrocyte glycoprotein, or galactocerebroside (Rapport et al., 1964; Quarles et al., 1985; Lington and Lassmann, 1987), against altered neuroantigenic determinants, or against other nonmyelin-associated neuroantigens. In an attempt to address these possibilities, we employed two different experimental approaches. First, we asked if mice with TMEV-induced demyelinating disease exhibited T cell responses to spinal cord homogenate fractions containing a heterogeneous mixture of both myelin and nonmyelin-associated CNS antigens. Homogenates prepared from the spinal cords of normal mice and mice undergoing active R-EAE or TMEV-induced demyelinating disease (which might be expected to contain neuroantigenic determinants which had been modified or altered as a consequence of active immune-mediated demyelination) stimulated T cell responses in mice with R-EAE, but not in TMEV-infected mice (Fig. 2).

We next determined the effect of inducing neuroantigen-specific tolerance on the course of TMEV-induced demyelinating disease. Splenocytes covalently coupled with mouse spinal cord homogenate were used to induce specific tolerance to a heterogeneous mixture of CNS antigens. Studies from our and other laboratories have shown that specific inhibition of DTH and antibody responses occurs following the i.v. injection of antigen-coupled syngeneic splenocytes (Miller and Claman, 1976; Bach et al., 1978; Greene et al., 1978; Miller et al., 1979) via the clonal inhibition of T cells and the activation of antigen-specific suppressor T cells (Miller et al., 1977). We have previously shown (Kennedy et al., 1988) that MSCH-SP-tolerized mice failed to develop neuroantigen-specific DTH responses and had a significantly reduced incidence of clinical and histologic signs of R-EAE for at least 4–5 weeks after tolerization.

The present results confirm our previous findings regarding the ability of MSCH-SP-induced tolerization of adult, immunocompetent mice to significantly suppress the development of R-EAE and the causal MBP- and PLP-specific DTH responses. In contrast, the data show that the neuroantigen-specific tolerance failed to affect development of clinical and histologic signs of de-

myelinating disease and the accompanying virus-specific CMI and antibody responses induced by TMEV infection (Figs. 3, 4, and 5). Thus, the present results confirm and extend the non-quantitative results of Lang et al. (1985) which showed that treatment of immunoincompetent neonatal SJL/J mice with MSCH in incomplete Freund's adjuvant failed to affect development of demyelinating lesions in the CNS following infection with the DA strain of TMEV. Since our tolerance regimen involved the use of a heterogeneous mixture of neuroantigens (i.e., MSCH), it is likely that T cell-mediated immune responses to neuroantigens of minor or undefined encephalitogenic potential were tolerized in addition to DTH responses to MBP and PLP. The probable lack of responses to minor neuroantigens in TMEV-induced demyelinating disease is supported by the observation that CMI responses to whole MSCH were demonstrable in mice with R-EAE, but not in TMEV-infected mice (Fig. 2). It should be emphasized that the present results only address a lack of neuroantigen-specific autoimmune responses in the early stages (days 45–90 p.i.) of TMEV-induced demyelination. We are currently determining whether the induction of neuroantigen-specific tolerance *after* the first appearance of clinical signs of demyelination has any effect on the *chronic* course of the disease. It would be interesting to apply the current methodology of neuroantigen-specific tolerance induction to determine if MBP-specific immune responses play a primary or secondary pathologic role in the subacute demyelination observed in rats infected with JHM (Watanabe et al., 1983) and measles (Liebert et al., 1988) viruses.

We feel our present and previous results suggest that chronic demyelination can occur in the apparent absence of neuroantigen-specific autoimmune responses and are consistent with our proposed hypothesis that TMEV-specific CMI responses are the major effector mechanism of CNS demyelination following TMEV infection (Clatch et al., 1985, 1986). The current results may be particularly applicable to MS where there is circumstantial evidence favoring a viral etiology (Nathanson and Miller, 1978; Kurtke and Hyllested, 1986), and where there is a lack of convincing evidence that MS patients display elevated

humoral or CMI responses to major encephalitogenic neuroantigens such as MBP and PLP (Waksman, 1984; Waksman and Reynolds, 1984; Raine, 1985; Hafler et al., 1987). Future studies on the TMEV-induced demyelinating disease model will examine whether induction of specific tolerance to TMEV antigens will prevent or alter the course of disease.

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