

Functional Evidence for Epitope Spreading in the Relapsing Pathology of Experimental Autoimmune Encephalomyelitis

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Summary

The role of epitope spreading in the pathology of relapsing–remitting experimental autoimmune encephalomyelitis (R-EAE) was examined. Using peripherally induced immunologic tolerance as a probe to analyze the neuropathologic T cell repertoire, we show that the majority of the immunopathologic reactivity during the acute phase of R-EAE in SJL/J mice induced by active immunization with the intact proteolipid (PLP) molecule is directed at the PLP139–151 epitope and that responses to secondary encephalitogenic PLP epitopes may contribute to the later relapsing phases of disease. Intermolecular epitope spreading was demonstrated by showing the development of T cell responses to PLP139–151 after acute disease in mice in which R-EAE was initiated by the transfer of T cells specific for the non–cross-reactive MBP84–104 determinant. Intramolecular epitope spreading was demonstrated by showing that endogenous host T cells specific for a secondary encephalitogenic PLP epitope (PLP178–191) are demonstrable by both splenic T cell proliferative and *in vivo* delayed-type hypersensitivity responses in mice in which acute central nervous system damage was initiated by T cells reactive with the immunodominant, non–cross-reactive PLP139–151 sequence. The PLP178–191–specific responses are activated as a result of and correlate with the degree of acute tissue damage, since they do not develop in mice tolerized to the initiating epitope before expression of acute disease. Most importantly, we show that the PLP178–191–specific responses are capable of mediating R-EAE upon adoptive secondary transfer to naive recipient mice. Furthermore, induction of tolerance to intact PLP (which inhibits responses to both the initiating PLP139–151 epitope and to the PLP178–191 epitope) after the acute disease episode is sufficient to prevent relapsing disease. These results strongly support a contributory role of T cell responses to epitopes released as a result of acute tissue damage to the immunopathogenesis of relapsing clinical episodes and have important implications for the design of antigen-specific immunotherapies for the treatment of chronic autoimmune disorders in humans.

Relapsing experimental autoimmune encephalomyelitis (R-EAE)¹ is a CD4⁺ Th1-mediated demyelinating disease of the central nervous system (CNS) (1) that is inducible in genetically susceptible animals by immunization with myelin basic protein (MBP) or proteolipid protein (PLP) (2, 3) or synthetic peptides corresponding to the major encephalitogenic epitopes of MBP and PLP (4, 5). Alternatively, R-EAE

may be transferred to naive recipients with *in vitro*–activated MBP or PLP-specific CD4⁺ T cells (5–7). The relapsing–remitting course of paralysis is characterized by a moderate to severe initial acute phase of disease followed by recovery and one or more relapses. This pattern of relapses and spontaneous recovery in experimental animal models, which occurs over a period of weeks to months, is very similar to the clinical signs of disease observed in multiple sclerosis (MS) patients over many years (8).

The immunologic mechanisms involved in the induction and regulation of clinical relapses are unclear. Increased MHC class II expression has been found in the CNS of mice during clinical relapses (9, 10) and may influence the pattern of local production of pro- and antiinflammatory cytokines during relapses or remissions (11). Several studies have also demonstrated that changes occur in class II restriction and antigen

The first two authors contributed equally to this work and should both be considered first authors.

¹ Abbreviations used in this paper: CNS, central nervous system; DTH, delayed-type hypersensitivity; ECDI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl; MBP, myelin basic protein; MMS, mean maximal disease severity; MS, multiple sclerosis; PLP, proteolipid protein; R-EAE, relapsing experimental autoimmune encephalomyelitis; SP, splenocyte.

specificity of neuroantigen-specific proliferative responses during the course of disease (12–15). Thus, reactivity to neuroepitopes other than that used to induce the initial clinical episode, a phenomenon termed epitope spreading (16), may contribute to the relapsing course of clinical R-EAE. Epitope spreading has also recently been proposed to contribute to the pathogenesis of spontaneous autoimmune diabetes in the nonobese diabetic mouse (17, 18). These reports provide intriguing evidence that the T cell repertoire is dynamic and that reactivity to additional specificities occurs during the course of both experimentally induced and spontaneous autoimmune diseases. This is of particular interest in MS, where B and T cell reactivity to MBP and PLP has been observed (19–21). The broad spectrum of lymphocyte reactivity against CNS antigens in MS may result from diversification of antibody and T cell reactivities that occur as the myelin sheath suffers progressive damage. Epitope spreading could occur regardless of whether the initial inflammatory response in MS is due to direct reactivity against a CNS resident pathogen such as a virus (22) or to cross-reactivity between a pathogen-encoded epitope and an epitope(s) on a myelin protein (16, 23).

While T cell specificities appear to change over the course of R-EAE, the significance of these events in disease progression is not known. Relapses could result from activation of T cells specific for endogenous myelin epitopes released during the acute phase of disease. Proliferative responses to additional encephalitogenic myelin epitopes have been reported to arise after the initial acute phase of EAE (16, 24, 25), but the functional consequences of these responses have not been addressed. In the current report, we demonstrate both intermolecular and intramolecular epitope spreading in two different models of myelin peptide-induced R-EAE. T cells reactive to a secondary encephalitogenic PLP epitope (PLP178-191) are found in the spleens of mice in which acute EAE was induced by T cells reactive with the dominant, non-cross-reactive PLP139-151 epitope. T cell responses specific for the secondary epitope are activated as a result of and correlate with the degree of acute CNS tissue damage. Most importantly, these T cells possess encephalitogenic potential in that they can initiate EAE upon secondary adoptive transfer to naive recipients. These results strongly support a role for intramolecular epitope spreading in the relapsing pathogenesis of PLP139-151-induced EAE.

Materials and Methods

Mice. Female SJL/J mice, 6–7 wk old, were purchased from Harlan Bioproducts for Science, Inc. (Indianapolis, IN). All mice were housed in the Northwestern animal care facility and were maintained on standard laboratory food and water ad lib.

Antigens/Peptides. PLP was purified from bovine brain according to the method of Lees and Sakura (26), and purity was confirmed by SDS-PAGE. PLP and MBP peptides PLP139-151 (HSLGKWLGHDPKF), bovine PLP178-191B (NTWTTTCQSIAPSK), mouse PLP178-191M (NTWTTTCQSIAPFSK), PLP104-117 (KTTICGKGLSATVT), and MBP84-104 (VHFFKNIVTPRTPPPSQGKGR) were synthesized (RaMPS Multiple Peptide Synthesis System; New England Nuclear-DuPont, Wilmington, DE). Amino acid com-

position of these peptides was confirmed by the Northwestern University Biotechnology Center. PLP178-191M was insoluble in PBS and was dissolved in 0.035 M acetic acid (27).

Induction of R-EAE by Active Immunization with PLP and PLP139-151. Mice were immunized with PLP or PLP139-151 in adjuvant as previously described (5). Each mouse received 100 μ l of a CFA emulsion containing 200 μ g of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Inc., Detroit, MI) and 87 μ g of PLP139-151 or 100 μ g of intact PLP subcutaneously distributed over three spots on the flank. Initial clinical signs of disease were usually observed between days 12 and 20 after immunization.

Induction of R-EAE by Adoptive Transfer of PLP139-151- or MBP84-104-specific Lymph Node Cells. Mice were immunized with PLP139-151 or MBP84-104 according to previously described methods (5, 28). Each mouse received 100 μ l of an IFA emulsion containing 200 μ g of *M. tuberculosis* H37Ra and 25 μ g of PLP139-151 or 100 μ g of MBP84-104 subcutaneously distributed over three spots on the flank. 7–10 d after immunization, the inguinal, brachial, and axillary lymph nodes were removed from sensitized donors, and single-cell suspensions were prepared. The cells were adjusted to 8×10^6 /ml in complete DMEM containing 15–20 μ g/ml of the appropriate peptide and incubated at 37°C in a humidified atmosphere containing 5% CO₂. 4 d later, the cells were washed twice with buffered saline solution, counted, resuspended in 50% Percoll (Pharmacia Biotech, Inc., Piscataway, NJ), and centrifuged at 1,500 rpm for 15 min at room temperature. Lymphoblasts were removed from the gradient, washed, and injected intraperitoneally into naive mice.

Induction of R-EAE by Adoptive Serial Transfer of PLP139-151- or 178-191M-specific Splenocytes from Mice with PLP139-151-induced Disease. 40 d after active immunization with PLP139-151 or adoptive transfer of PLP139-151-specific lymph node cells, mice were killed and spleens were removed. Spleen cells were cultured with PLP139-151 or PLP178-191M as described above. After 4 d of culture, 3×10^7 – 5×10^7 PLP139-151-specific splenocytes or 5×10^7 PLP178-191M-specific splenocytes were transferred intraperitoneally into naive recipients.

Clinical Evaluation. Mice were observed daily for clinical signs of disease. Mice 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. The data are plotted as the mean daily clinical score for all animals in a particular treatment group. In some instances, the mean maximal clinical score per group is given as an indication of disease severity. A relapse was defined as an increase of at least one full grade in clinical score after the animal had previously improved at least a full clinical score and had stabilized.

Histologic Evaluation. Mice were anesthetized with sodium pentobarbital and killed 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 postfixated in 1% OsO₄, dehydrated, and embedded in Epon as previously described (29). Toluidine blue-stained sections from 10 to 11 segments per mouse were read blind and scored as follows: \pm , mild inflammation without demyelination; 1⁺, inflammation with focal demyelination; 2⁺, inflammation with multiple foci of demyelination; 3⁺, marked inflammation with bilateral, converging areas of demyelination; and 4⁺, extensive bilateral areas of demyelination and remyelination.

Ag-specific Delayed-type Hypersensitivity (DTH). DTH responses were quantitated using a 24-h ear swelling assay in mice challenged with 5 μ g of the relevant peptide (in 10 μ l saline) as previously

described (30). Results are expressed in units of 10^{-4} inches \pm SEM. 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 T Cell Proliferation Assay. T cell proliferative responses were assessed by incorporation of [3 H]thymidine. 5×10^5 viable draining lymph node (brachial, axillary, and inguinal) cells recovered from immunized mice were cultured in 96-well flat-bottom microculture plates (Falcon Labware, Becton Dickinson and Co., Oxnard, CA) in 0.2 ml of DMEM (Gibco Laboratories, Grand Island, NY) supplemented with 0.75% syngeneic mouse serum. Alternatively, 5×10^5 splenocytes from adoptive transfer recipients were cultured as described above. Triplicate cultures were stimulated with the indicated doses of antigen and incubated in a humidified atmosphere of 5% CO₂. After 72 h, culture wells were pulsed with 1.0 μ Ci of [3 H]thymidine (6.7 Ci/mM, ICN Biomedicals, Inc., Costa Mesa, CA). Cultures were harvested 24 h later with a semiautomated cell harvester, and measurements of radioactive material were determined in a scintillation counter. Results are expressed as (Δ cpm) = [(mean cpm of antigen-stimulated cultures) - (mean cpm of control cultures)] and/or as stimulation indices [(mean cpm of antigen-stimulated cultures)/(mean cpm of control cultures)].

Peripheral Tolerance Induction by Antigen-coupled Splenocytes. RBC-free (Tris-NH₄Cl-treated) splenocytes were coupled with antigen by the method of Miller et al. (31) using water-soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (ECDI, Calbiochem-Behring Corp., La Jolla, CA). 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 PLP or PLP139-151. The coupling reaction was initiated by the addition of 0.205 ml of freshly prepared ECDI (150 mg/ml in saline)/ml of cell suspension. Sham-coupled cells were prepared by incubation of splenocytes in saline containing ECDI but no Ag. After a 1-h incubation at 4°C, the cells were washed three times with BSS. At the indicated time relative to active immunization with PLP or PLP139-151 or to the adoptive transfer of PLP139-151-primed lymph node cells, 5×10^7 Ag-coupled splenocytes were injected intravenously into the lateral tail veins of recipient mice.

Statistical Analyses. Comparisons of the percentage of animals showing clinical disease between any two groups of mice were analyzed by chi-squared using Fisher's exact probability. Comparisons of the mean day of onset and mean maximal severity between any two groups of mice were analyzed by the Student's *t* test. *P* values <0.05 were considered significant.

Results

Evidence that PLP139-151 Is the Dominant Encephalitogenic PLP Epitope in SJL/J Mice. Data from our laboratory and from others have shown gain of T cell responses to additional myelin epitopes and switches in use of MHC class II I-A and I-E restriction elements during the course of EAE induced with intact MBP (13–15, 17). To approach the possibility of similar switches in antigen specificity through the course of PLP-induced EAE, we first determined the relative contributions of T cell responses to the two immunodominant encephalitogenic PLP epitopes, PLP139-151 (5, 32) and PLP178-191 (27), in R-EAE induced with intact PLP. As seen in Fig. 1, mice receiving sham-coupled cells and subsequently primed with intact PLP (A) underwent a normal relapsing-remitting disease course, with four out of seven (57%) of the mice exhibiting disease symptoms by day 17 after immunization. Sham-tolerized mice primed with PLP139-151/CFA (B) also underwent a relapsing disease course with 13/14 (93%) of the mice affected by day 14 after immunization. The reduced incidence and longer time of onset of PLP-induced vs PLP139-151-induced R-EAE may be due to lower molar concentrations of encephalitogenic epitopes in the whole PLP preparations. Prior tolerization with intact PLP resulted in total protection of the animals from both the initial and relapsing episodes of PLP-induced clinical disease (A) and PLP139-151-induced disease (data not shown). In contrast, although administration of PLP139-151-coupled splenocytes significantly delayed the onset of PLP-induced disease, four out of seven (57%) of these mice developed clinical disease between days 31 and 43 after immunization, and these animals experienced a much milder form of disease (mean maximal disease severity [MMS] = 1.0) as compared with the control group (MMS = 2.5). Interestingly, the timing of clinical symptoms in the PLP139-151-tolerized group (31–43 d) was similar to that seen for the initial relapse in the control group (days 31–38 in three out of four mice). These results cannot be explained by differences in the ability of PLP and PLP139-151 to induce tolerance, since PLP139-151-coupled splenocytes effectively inhibited PLP139-151-induced R-EAE (B). We have previously demonstrated that tolerance induced by

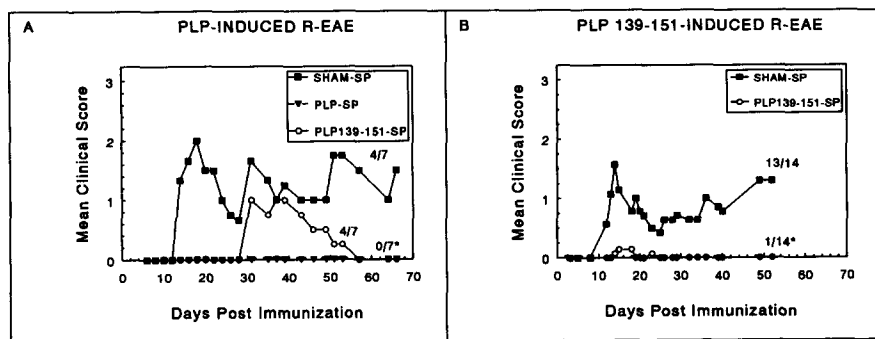


Figure 1. Differential ability of tolerance induced with PLP and PLP139-151 to inhibit active R-EAE induced with intact PLP or the immunodominant PLP139-151 epitope. Mice were tolerized by the intravenous injection of $5\text{--}6 \times 10^7$ ECDI-treated splenocytes coupled with either nothing (SHAM-SP), intact PLP (PLP-SP), or PLP139-151 (PLP139-151-SP) 7–10 d before induction of active R-EAE with either PLP/CFA (A) or PLP139-151/CFA (B). Animals were followed for clinical signs of disease, expressed as both the mean disease severity and as the incidence rate, for 55–65 d thereafter. *Disease incidence significantly less than that of the SHAM-SP injected controls, *P* <0.05.

antigen-coupled syngeneic splenocytes is antigen specific (33). Pretolerance with PLP inhibits the onset of PLP-induced disease (A) but not MBP-induced R-EAE (34). Collectively, these results indicate that the initial acute encephalitogenic response in PLP-immunized mice is directed primarily towards PLP139-151, and that this epitope is immunodominant when compared with PLP178-191 (27), responses to which possibly contribute to later phases of disease in mice primed with the intact, multideterminant PLP molecule.

Diversification of Proliferative Responses to PLP Epitopes during the Course of Adoptive R-EAE Induced with PLP139-151-specific T Cells. To examine directly the functional consequences of epitope spreading in the pathogenesis of relapsing EAE, we reasoned that the reliable relapsing-remitting disease induced by the major immunodominant PLP139-151 epitope would provide an ideal system, in that the initial acute disease would be mediated by T cells specific for a single encephalitogenic epitope. R-EAE was initiated by adoptive transfer of lymph node T cells specific for PLP139-151, and responses to this epitope and to other non-cross-reactive encephalitogenic PLP (PLP178-191 [27] and PLP104-117 [35]) and MBP (MBP84-104 [36]) epitopes were assessed (Fig. 2). On day 3 after transfer (before the appearance of acute EAE, MMS = 0) and on day 17 (concomitant with the peak of acute EAE, MMS = 3.5), splenic proliferative responses to PLP139-151, but not to the other PLP determinants or to MBP84-104, could be detected. However, on days 24 and 40 after transfer (during the remission from the acute disease episode), significant splenic proliferative responses were seen to both the inducing PLP139-151 epitope and to PLP178-191M, which further increased at day 60 after transfer (concomitant with the appearance of a clinical relapse in this group of mice, MMS = 4.0). The development of PLP178-191M-specific responses is reproducible in that it was noted in five separate experiments in mice with both active and adoptive PLP139-151-induced EAE (see also Table 1). Interestingly, no responses were seen at any time point upon stimulation with the immunodominant MBP84-104 epitope or with PLP104-117. These results show that intramolecular epitope spreading to an endogenous PLP epitope distinct from the disease-inducing determinant occurs during the course of PLP139-151-induced R-EAE.

Peptide Specificity of T Cell Responses in SJL Mice Primed with Intact PLP, PLP139-151, and PLP178-191M. PLP is a 276-amino acid structural myelin protein that is highly conserved across many species. The sequences of mouse, rat, and human PLP molecules are identical but differ from the bovine sequence at four positions, including an alanine for phenylalanine substitution at position 188 (37). To determine the fine specificity of T cell responses to the dominant and secondary PLP epitopes and to identify potential effects of the substitution at position 188 on T cell recognition of intact PLP, proliferative assays were performed on PLP, PLP139-151, and PLP178-191M-primed lymph node cells (Fig. 3). Before the onset of acute clinical symptoms (10 d after immunization), lymph node T cells from SJL/J mice primed with intact bovine PLP responded well to PLP139-151 and

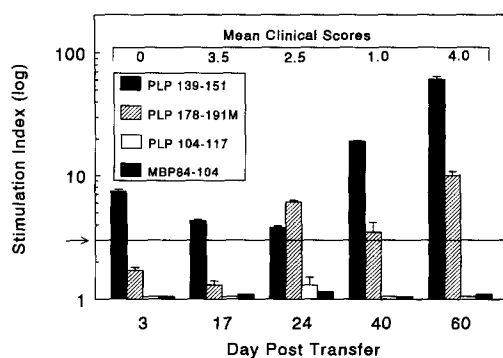


Figure 2. Specificity of T cell responses in SJL/J mice during the relapsing course of adoptive PLP139-151-specific EAE. R-EAE was induced in a large group of SJL/J mice via the adoptive transfer of 5×10^6 PLP139-151-specific lymph node T cell blasts on day 0. At the indicated times (days 3, 17, 24, 40, and 60) after transfer, recipient splenocytes were cultured with PLP139-151, PLP178-191M, PLP104-117, or MBP84-104 for 72 h, and [3 H]thymidine incorporation determined over an additional 24-h period. The data are presented as stimulation indices \pm SEM for two to three mice per time point. The mean clinical score of the animals sacrificed at the various time points is also indicated at the top of the chart. The horizontal line represents stimulation index = 3, which is considered the cutoff for significance. Background cpm in for all groups were between 3,833 and 13,392 cpm with the exception of day 60, which was 26,142 cpm.

to PLP178-191B, but not to PLP178-191M, indicating that the alanine at position 188 is critical for T cell recognition of the bovine PLP178-191 epitope. However, after development of acute disease (20 d after immunization), PLP-primed mice exhibited proliferative responses to both PLP178-191B and PLP178-191M. T cells from PLP139-151-primed mice responded only to PLP139-151, indicating that the epitope spreading was not a result of cross-reactivity between CD4+

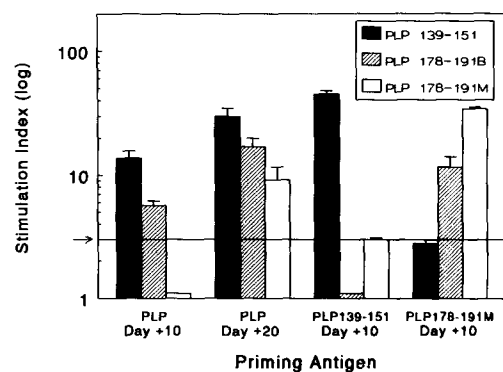


Figure 3. Peptide specificity of T cell responses in SJL/J mice primed with PLP and with the primary (PLP139-151) and secondary (PLP178-191) encephalitogenic PLP peptides. Three to four mice per group were primed with PLP, PLP139-151, or PLP178-191M in CFA on day 0. On day 10 or 20, draining lymph node cells were cultured with PLP139-151, PLP178-191M, or PLP178-191B for 72 h, and [3 H]thymidine incorporation was determined over an additional 24-h period. The data are presented as stimulation indices \pm SEM. The horizontal line represents stimulation index = 3, which is considered the cutoff for significance. Background cpm in for all groups were between 2,382 and 14,944 cpm.

T cells specific for the EAE-inducing PLP139-151 epitope and the recruited PLP178-191M epitope. Similarly, PLP178-191M did not induce proliferation in several PLP139-151-specific T cell lines and clones (data not shown). Interestingly, T cells primed to PLP178-191M cross-reacted to some extent with PLP178-191B, indicating that position 188 is most likely not a primary TCR contact residue for all T cell clones specific for PLP178-191M.

Intermolecular Epitope Spreading in MBP84-104-induced R-EAE. To determine if epitope spreading could be identified in a second peptide-induced model of R-EAE, disease was induced in SJL/J mice by the transfer of MBP84-104-specific T cells. Individually, these animals generally display a milder acute disease course than recipients of PLP139-151-specific T cells and an initial relapse that was more severe than the acute disease (Fig. 4 A). T cell reactivity, as measured by DTH in vivo (Fig. 4 B) and by T cell proliferation in vitro (not shown), to the immunodominant PLP139-151 epitope de-

veloped in these mice concomitant with the appearance of the first relapse. This has been a consistent observation in a number of experiments and is an example of intermolecular epitope spreading. The more severe relapse in these mice concurrent with the development of T cell reactivity to PLP139-151 is consistent with our earlier observation that this peptide is overwhelmingly the dominant encephalitogenic myelin epitope in the SJL/J mouse (38).

Functional Evidence that PLP178-191M-specific Splenic T Cells from Mice with PLP139-151-induced R-EAE Participate in Relapsing Pathology. To further delineate the encephalitogenic potential of recruited T cells with specificity for endogenous neuroepitopes, we asked if PLP178-191M-specific T cells from mice with R-EAE induced by PLP139-151 were capable of transferring EAE (Table 1). Splenocytes were collected from donor mice ~40 d after induction of both active (experiment 1) and adoptive (experiment 2) PLP139-151-specific EAE. The cells were activated in vitro with PLP139-151,

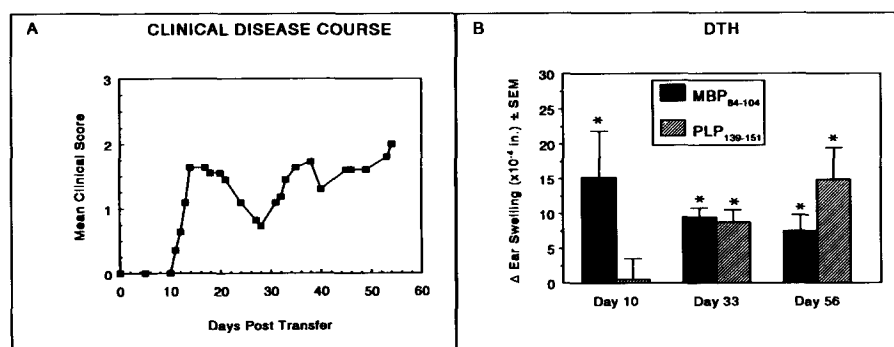


Figure 4. Intermolecular epitope spreading during the relapsing course of MBP84-104-specific R-EAE. R-EAE was induced in a large group of SJL/J mice via the adoptive transfer of 40×10^6 MBP84-104-specific lymph node T cell blasts on day 0. (A) A subset of the mice ($n = 11$) was observed for clinical signs of disease for 56 d after transfer. (B) At the indicated times (days 10, 33, and 56) after T cell transfer, four to six recipient animals were each challenged with $5 \mu\text{g}$ of the inducing peptide (MBP84-104) in the left ear and with $5 \mu\text{g}$ of PLP139-151 in the right ear, and increases in ear thickness as a measure of DTH reactivity were determined 24 h later. The data are expressed as the mean change in ear swelling

(background swelling in unimmunized controls subtracted) in units of 10^{-4} inches \pm SEM. Mean background swelling was 4.6 U for MBP84-104 and 3.8 U for PLP139-151. *DTH responses significantly greater than those of unimmunized controls, $P < 0.01$.

Table 1. Serial Transfer of EAE with PLP178-191-specific T Cells from SJL/J Mice Recovered from Acute PLP139-151-induced CNS Damage

Experiment no.	Day 0 inducing stimulus	Day 40 activation peptide*	Disease incidence	MDO [†]	MMS [§]
1	PLP139-151/CFA	PLP139-151	3/3 (100%)	11.0 ± 1.0	3.7 ± 0.3
		PLP178-191M	3/4 (75%)	17.7 ± 3.8	2.0 ± 0.6
		MBP84-104	0/2 (0%)	—	—
2	5×10^6 PLP139-151-specific T cells	PLP139-151	3/3 (100%)	7.7 ± 1.3	4.3 ± 0.3
		PLP178-191M	4/4 (100%)	14.3 ± 3.4	2.3 ± 0.5
		MBP84-104	0/1 (0%)	—	—

* On day 40 after recovery from the initial acute phase of clinical disease induced by either immunization with PLP139-151/CFA (experiment 1) or the transfer of 5×10^6 PLP139-151-specific lymph node T cell blasts (experiment 2), splenic T cells were cultured with the indicated peptides for 4 d, and 5×10^7 cells were transferred to naive, syngeneic recipients.

[†] MDO, mean day of onset \pm SEM for clinically affected mice in each group.

[§] MMS, mean maximal disease severity \pm SEM for clinically affected mice in each group.

^{||} Significant percentage of the recipient mice displayed clinical disease, $P < 0.05$.

PLP178-191M, or MBP84-104 and transferred to syngeneic naive recipients. All mice (six out of six combined from the two experiments) that received splenocytes reactivated with the inducing PLP139-151 epitope exhibited severe clinical R-EAE (MMS = 4.0). Splenocytes stimulated in vitro with PLP178-191M transferred disease to seven out of eight (88%) recipient mice with a significantly delayed mean day of onset when compared with PLP139-151-activated cells (15.7 vs 9.3 d). Discrepancies in disease onset may result from differences in the numbers of antigen-specific T cells transferred. However, transfer of equal numbers of activated PLP178-191M and PLP139-151-specific lymph node T cells suggests that PLP178-191M-induced R-EAE is consistently delayed when compared with PLP139-151 (data not shown). Recipients of the PLP178-191M-activated T cells also exhibited clinical scores (MMS = 2.1) that were significantly less severe than mice receiving PLP139-151-activated splenocytes (MMS = 4.0). Interestingly, splenocytes activated in vitro with MBP84-104 did not proliferate at any time point tested (Fig. 1) or transfer disease to naive recipients (Table 1). Collectively, these results suggest that PLP178-191M-specific Th1 cells are activated by recognition of the endogenous mouse PLP178-191 epitope during the course of both active and adoptive PLP139-151-induced R-EAE, and that these cells are most likely involved in the relapsing course of disease, as they have potent encephalitogenic activity.

The data in Fig. 1 indicated that responses to secondary PLP epitopes, including PLP178-191, played a role in the later phases of PLP-induced R-EAE. As a second functional test of the role of epitope spreading in the relapsing pathology of PLP139-151-induced R-EAE, we asked if tolerance to intact PLP induced after the acute phase of disease would be able to ameliorate clinical relapses. Thus, R-EAE was induced in SJL/J mice by active immunization with PLP139-151 in CFA. After recovery from the acute phase of disease (day 19 after priming), 12–13 mice were each tolerized by the intravenous injection of syngeneic splenocytes coupled with intact PLP and observed for an additional 50 d for the development of clinical relapses. As seen in Fig. 5, thirteen out of thirteen (100%) of the sham-tolerized mice exhibited clinical relapses during the observation period, but only five out of twelve (42%) of the PLP-tolerized mice relapsed, and these relapses were routinely mild (increase of only one clinical grade) as compared with those in the sham-tolerized group (increases of two or more clinical grades). Similar results were observed when mice were tolerized with a crude mouse spinal cord homogenate that contains a heterogeneous mixture of neuroantigens (data not shown). Since tolerization with intact PLP inhibits T cell responses to both PLP139-151 and PLP178-191 (Fig. 1 and additional data not shown) and has no effect on induction of active EAE induced with intact MBP (34), these results are consistent with a role for PLP178-191-specific responses, but a lack of MBP84-104-specific responses, in PLP139-151-induced R-EAE.

Peptide-specific Tolerance Prevents the Development of Epitope Spreading. Based on the temporal sequence of appearance of PLP178-191M responses (Fig. 2), the data to this point

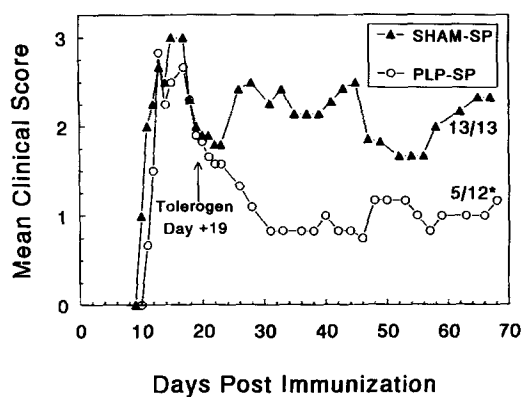


Figure 5. Effective treatment of clinical relapses in PLP139-151-induced R-EAE by specific tolerization with intact PLP after recovery from the acute disease. R-EAE was induced in SJL/J mice by active immunization with PLP139-151 in CFA. After recovery from the acute phase of disease (day 19 after priming), 12–13 mice were each tolerized by the intravenous injection of syngeneic splenocytes coupled with intact PLP and observed for an additional 50 d for the development of clinical relapses. The data are expressed as both the mean disease severity and as the relapse incidence. *Relapse incidence significantly less than that of the sham SP-injected controls, $P < 0.01$.

are consistent with the hypothesis that T cells with novel specificities are activated after CNS tissue damage has occurred. One would thus predict that inhibition of CNS damage may prevent priming of T cells with specificity for endogenous self epitopes. Therefore, we asked if induction of PLP139-151-specific tolerance after effector cell transfer but before the onset of the initial acute clinical episode of adoptive PLP139-151-induced R-EAE would prevent the development of T cell reactivity to the PLP178-191M epitope. The intravenous injection of PLP139-151-splenocytes (SP) on day 3 after adoptive transfer of PLP139-151-activated lymph node cells resulted in a significant diminution of clinical R-EAE (Fig. 6 A). In addition, these animals were tested for DTH responsiveness to both the inducing PLP139-151 epitope and to PLP178-191M on day 25 after transfer. As seen in Fig. 6 B, the four sham-SP-treated mice that developed a severe acute phase of EAE (MMS = 4.5) displayed significant DTH upon challenge with either PLP139-151 or PLP178-191M, indicating that epitope spreading can also be demonstrated by DTH, an in vivo readout of proinflammatory cytokine activity. The three out of six mice in the PLP139-151-tolerized group that developed very mild EAE (MMS = 1.0) also displayed DTH responses to both peptides that were significantly above background, but significantly less than those observed in the sham SP-treated mice. In contrast, the three tolerant mice that displayed no acute clinical disease symptoms did not respond to either the inducing peptide or to PLP178-191M. Pathological examination of these groups of animals was also performed (Fig. 7). The sham-tolerized mice showed the characteristic changes of EAE, consisting of florid mononuclear cell infiltrations in meninges and around parenchymal venules in the white matter columns of the spinal cord. Tolerized mice that displayed mild neurological impairment showed

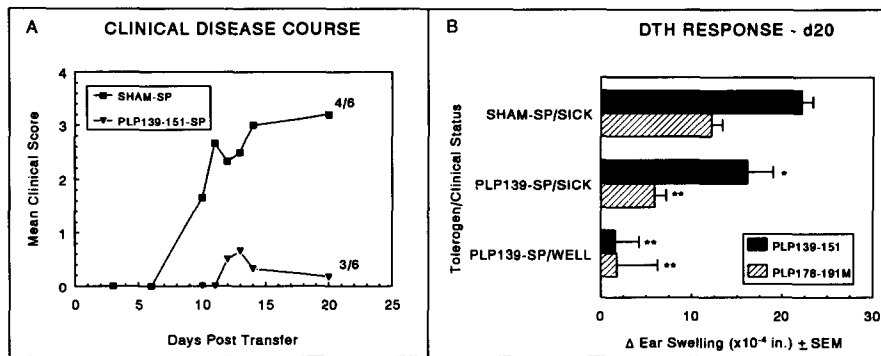


Figure 6. Effect of peptide-specific tolerance on the development of secondary peptide-specific DTH responses during the course of adoptive R-EAE induced by transfer of PLP139-151-specific T cells. R-EAE was induced by the intravenous transfer of 5×10^6 PLP139-151-activated T cell blasts on day 0. On day 3, before the onset of clinical signs of disease, half of the mice were tolerized with PLP139-151-coupled splenocytes and half with sham SP. (A) The mean clinical scores for all the mice in the two treatment groups and the disease incidence are shown. (B) 25 d after transfer, the recipient animals were challenged with $5 \mu\text{g}$ of the inducing peptide (PLP139-151) in the left ear and with $5 \mu\text{g}$ of PLP178-191M in the right

ear, and increases in ear thickness as a measure of DTH reactivity were determined 24 h later. The data are expressed as the mean change in ear swelling (background swelling in unimmunized controls subtracted) in units of 10^{-4} inches \pm SEM. Mean background swelling was 5.1 U for PLP139-151 and 2.1 U for PLP178-191M. The data for the PLP139-151-tolerized group is divided into two groups, those animals showing minimal clinical disease (MMS = 1.0) and those showing no clinical symptoms (MMS = 0). The four clinically affected mice in the sham SP-tolerized group exhibited a very severe acute clinical phase with MMS = 4.5. *DTH responses significantly less than those of the sham SP controls, $P < 0.05$; ** $P < 0.01$.

very minimal tissue involvement, consisting mainly of meningeal inflammatory infiltrates. Occasionally, one could observe a focal demyelinating lesion that remained confined to the immediate sub-pial layer of the white matter. In contrast, the tolerized animals that showed no clinical symptoms were devoid of any CNS lesions. These results indicate that the diversification of the immune response normally seen in mice with R-EAE correlates with the severity of both clinical and histological symptoms during the acute phase of disease. Most importantly, epitope spreading can be abrogated by induction of specific tolerance to the inducing epitope before acute tissue damage.

Discussion

Using antigen-specific, peripherally induced immunologic tolerance as a probe for analysis of the neuropathologic T cell repertoire, we have previously shown that PLP, not MBP, constitutes the immunodominant neuroantigenic myelin protein for the induction of R-EAE in the SJL/J mouse (23). Residues 139–151 and 178–191 of the PLP molecule have been previously described as being codominant encephalitogenic epitopes (27, 32). However, our examination of the contributions of T cell responses to these two PLP determinants revealed that the majority of immunopathologic reactivity during the acute phase of R-EAE in SJL/J mice primed with the intact PLP molecule is directed at the PLP139-151 epitope (Fig. 1). Thus, the current findings indicate that PLP139-151 is the dominant immunopathologic epitope of the PLP molecule in acute disease and suggest that responses to secondary PLP determinants, primarily to PLP178-191, may contribute to the later phases of disease in mice primed with the multideterminant PLP molecule. These results are similar to recent findings in the Lewis rat acute EAE system, where it has been shown that responses to a secondary encephalitogenic epitope (MBP87-99) arise considerably later than responses to the dominant MBP68-86 epitope (39), and that MBP-induced clinical disease is more efficiently regulated upon

tolerization with intact MBP than with the dominant MBP68-86 peptide (40, 41).

The immunodominance of PLP139-151 is also illustrated by the fact that active EAE can be induced by immunization with this peptide emulsified in CFA (5), whereas induction of active EAE with PLP178-191 requires the additional use of pertussis toxin (27). In addition, the clinical course of both active and adoptive PLP139-151-induced R-EAE is characterized by a severe acute clinical disease phase and multiple clinical relapses (Figs. 1 and 5), whereas PLP178-191M-induced disease is characterized by a relatively mild acute phase (Table 1 and additional data not shown). Lastly, analysis of proliferative responses in SJL/J mice primed with intact PLP has revealed that precursor frequency of PLP139-151-specific T cells is three- to fourfold higher than the frequency of PLP178-191-specific cells (McRae, B. L., K. M. Nikcevic, and S. D. Miller, manuscript in preparation). This is mirrored by the increased bulk of proliferative responses observed upon challenge of PLP-primed cells with PLP139-151 (Fig. 3).

T cell specificities appear to change during the course of R-EAE (12–15, 42), and this may also occur in MS. Clearly, the expression of these additional reactivities could be due to the activation of T cells specific for endogenous myelin epitopes released during the acute phase of CNS damage. Although some of the specificities that arise have been shown to encompass determinants previously defined as encephalitogenic when administered with complete adjuvant, the significance of these events in disease progression (i.e., whether T cells specific for these recruited epitopes contribute to the immunopathology of relapsing disease) is largely unknown.

To determine directly if new antigen specificities arise during the course of R-EAE, we used a model of relapsing EAE wherein disease was initiated, either actively or adoptively, by T cells specific for the immunodominant PLP139-151 epitope. T cell reactivity to other reported encephalitogenic PLP epitopes and to the major encephalitogenic epitope of MBP (MBP84-104) was then assessed. After the initial acute phase

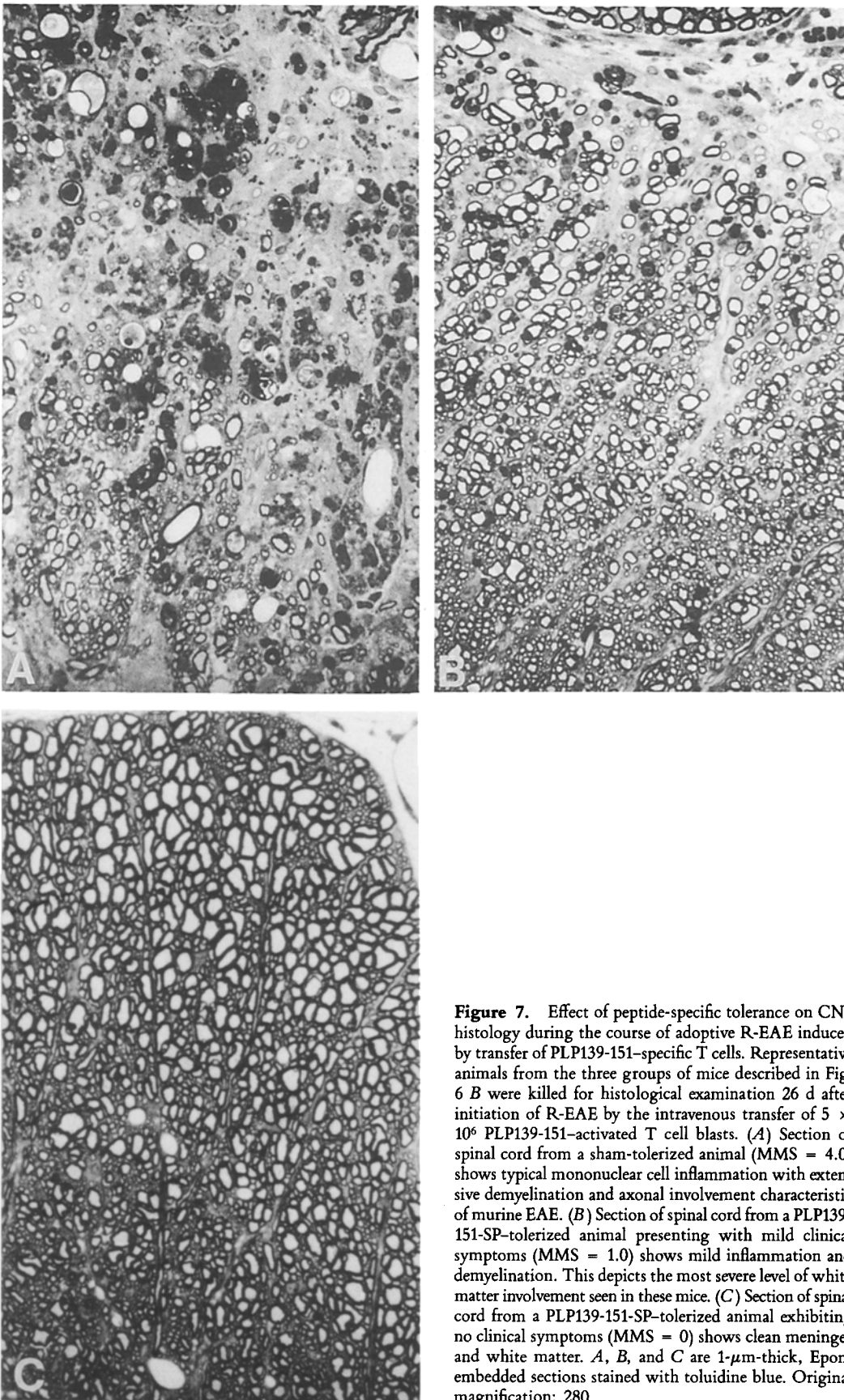


Figure 7. Effect of peptide-specific tolerance on CNS histology during the course of adoptive R-EAE induced by transfer of PLP139-151-specific T cells. Representative animals from the three groups of mice described in Fig. 6 B were killed for histological examination 26 d after initiation of R-EAE by the intravenous transfer of 5×10^6 PLP139-151-activated T cell blasts. (A) Section of spinal cord from a sham-tolerized animal (MMS = 4.0) shows typical mononuclear cell inflammation with extensive demyelination and axonal involvement characteristic of murine EAE. (B) Section of spinal cord from a PLP139-151-SP-tolerized animal presenting with mild clinical symptoms (MMS = 1.0) shows mild inflammation and demyelination. This depicts the most severe level of white matter involvement seen in these mice. (C) Section of spinal cord from a PLP139-151-SP-tolerized animal exhibiting no clinical symptoms (MMS = 0) shows clean meninges and white matter. A, B, and C are 1- μ m-thick, Epon-embedded sections stained with toluidine blue. Original magnification: 280.

of disease (days 24, 40, and 60), significant proliferative (Fig. 2) and DTH responses (Fig. 6) to the subdominant PLP178-191M epitope were detected. Several observations indicate that this response is due to the activation of endogenous host T cells. First, there is no cross-reactivity observed at the clonal (data not shown) or polyclonal T cell levels (Fig. 3) between PLP139-151 and PLP178-191M, indicating that the response is not due to the transferred effector cell population. Second, this response is specific for the mouse 178-191 sequence, since the cells from mice with disease initiated by PLP139-151 do not respond to bovine sequence (PLP178-191B), which contains an alanine for phenylalanine substitution at position 188. Lastly, the induction of PLP139-151-specific tolerance in recipients of PLP139-151-activated T cells prevents clinical disease and the induction of DTH reactivity to PLP178-191M (Fig. 6), indicating that CNS tissue damage must precede the development of the recruited response. Interestingly, the histopathological severity of the acute phase of PLP139-151-induced clinical disease roughly correlated with the magnitude of the DTH response to PLP178-191M observed 25 d after disease initiation (Fig. 6). Our ability to prevent epitope spreading by tolerizing to the initiating epitope confirms recent findings that induction of tolerance to glutamate decarboxylase before the onset of insulinitis in nonobese diabetic mice prevents the development of diabetes and the accompanying immune responses to other pancreatic β cell antigens such as carboxypeptidase H, insulin, and Hsp65 (17, 18).

Descriptions of epitope spreading in EAE have largely involved descriptions of additional proliferative specificities of T cells from various lymphoid compartments in mice undergoing EAE induced by intact MBP (12-14) or an encephalitogenic MBP peptide (24). However, it is possible that the emergence of new specificities during R-EAE may simply be secondary events in disease progression that do not influence the pathology or clinical expression of disease. For example, T cell lines and clones that proliferate to defined encephalitogenic epitopes but do not mediate tissue destruction upon transfer to naive recipients, presumably because of lack of production of proinflammatory cytokines such as lymphotoxin (43), have been described. The only functional evidence to date that T cells specific for secondary epitopes might contribute to clinical relapses comes from tolerance studies showing that, after recovery from MBP-induced R-EAE, tolerization with mouse spinal cord homogenate (containing a heterogeneous mixture of neuroantigens) only, but not with MBP, provided an effective means of inhibiting the subsequent clinical relapses (15, 33). These studies provide a direct functional indication that the response to that particular myelin protein or epitope contributes to disease pathology. The involvement of intermolecular epitope spreading in SJL/J mice with R-EAE induced by immunization with MBP84-104 is supported directly by the present observation that PLP139-151-specific responses appear to contribute to the initial severe clinical relapse seen in this disease model (Fig. 4).

In the current study, we took direct approaches to the question of the clinical significance of the development of the PLP178-191M-specific T cells in mice with PLP139-151-in-

duced R-EAE. First, we showed that PLP178-191M-reactive cells recovered from the spleens, but not the peripheral lymph nodes (data not shown), of mice 40-50 d after transfer of PLP139-151-specific T cells or active immunization with PLP139-151 mediate EAE after *in vitro* activation and transfer to naive recipients (Table 1). It is also significant that PLP178-191M-specific responses occurred after both active and adoptive induction of disease, indicating that prolonged peripheral antigenic stimulus from a bolus of PLP139-151/CFA is not necessary to invoke epitope spreading. A second functional test of the role of epitope spreading in the relapsing pathology of PLP139-151-induced R-EAE involved the demonstration that tolerance induction to determinants within the PLP molecule alone after the acute phase of disease was sufficient to ameliorate clinical relapses (Fig. 5). Taken together with the data shown in Figs. 1 and 2, these results are compatible with a model wherein responses to PLP178-191 and possibly to other secondary or cryptic PLP epitopes (intramolecular epitope spreading), but not to the MBP84-104 epitope (intermolecular epitope spreading), contribute to the relapsing pathology of R-EAE induced with the immunodominant PLP139-151 peptide.

Collectively, the results demonstrate that new T cell specificities to epitopes on the same and/or different myelin proteins emerge after acute CNS damage in SJL/J mice with peptide-induced R-EAE and are capable of mediating clinical disease. It is possible that local CNS presentation of endogenous neuroepitopes after acute CNS damage could be mediated by infiltrating inflammatory macrophages or by endothelial cells and/or astrocytes that have been shown to up-regulate expression of MHC class II (10, 44), certain adhesion molecules (45), and B-7 costimulatory molecules (Nikcevic, K. M., J. A. Bluestone, and S. D. Miller, manuscript in preparation) in response to proinflammatory cytokines. Alternatively, Mamula et al. (46) recently demonstrated that activated B cells can present multiple determinants from a single protein in an immunogenic form to overcome T cell tolerance. Antigen presentation by B cells has been postulated to be important in the pathogenesis of SLE, in which T cell-dependent IgG1 autoantibodies are produced against small nuclear ribonucleoproteins (46, 47). Although B cells are not necessary for the transfer of R-EAE (48), the role of activated B cells in diversification of autoimmune responses by presenting antigen to naive T cells is yet to be determined. It is interesting to speculate that epitope spreading may play a role in the pathogenesis of chronic and/or relapsing-remitting human autoimmune diseases such as SLE, rheumatoid arthritis, and MS. In MS, responses to a broad spectrum of myelin proteins could occur as a result of CNS damage initiated by T cell reactivity against a CNS-resident pathogen (22) or by a cross-reactive response between a pathogen-encoded epitope and an epitope on a neuroantigen (16, 23). Regardless of the initiating event, the current findings have important implications for designing effective antigen-specific immunotherapies for treatment of chronic autoimmune disorders.

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