

# A Predictable Sequential Determinant Spreading Cascade Invariably Accompanies Progression of Experimental Autoimmune Encephalomyelitis: A Basis for Peptide-Specific Therapy After Onset of Clinical Disease

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## Summary

The development of autoimmune disease is accompanied by the acquired recognition of new self-determinants, a process commonly referred to as determinant spreading. In this study, we addressed the question of whether determinant spreading is pathogenic for progression of chronic-relapsing experimental autoimmune encephalomyelitis (EAE), a disease with many similarities to multiple sclerosis (MS). Our approach involved a systematic epitope mapping of responses to myelin proteolipid protein (PLP) as well as assaying responses to known encephalitogenic determinants of myelin basic protein (MBP 87-99) and myelin oligodendrocyte glycoprotein (MOG 92-106) at various times after induction of EAE in (SWR×SJL) $F_1$  mice immunized with PLP 139-151. We found that the order in which new determinants are recognized during the course of disease follows a predictable sequential pattern. At monthly intervals after immunization with p139-151, responses to PLP 249-273, MBP 87-99, and PLP 173-198 were sequentially accumulated in all mice examined. Three lines of evidence showed that determinant spreading is pathogenic for disease progression: (a) spreading determinants mediate passive transfer of acute EAE in naive (SWR×SJL) $F_1$  recipients; (b) an invariant relationship exists between the development of relapse/progression and the spreading of recognition to new immunodominant encephalitogenic determinants; and (c) after EAE onset, the induction of peptide-specific tolerance to spreading but not to nonspreading encephalitogenic determinants prevents subsequent progression of EAE. Thus, the predictability of acquired self-determinant recognition provides a basis for sequential determinant-specific therapeutic intervention after onset of the autoimmune disease process.

Experimental autoimmune encephalomyelitis (EAE)<sup>1</sup> is an animal model commonly used in multiple sclerosis (MS) research. EAE is induced by immunization of animals with myelin proteins, and clinical disease develops when primed CD4<sup>+</sup> T cells enter the central nervous system (CNS) and recognize encephalitogenic peptide determinants presented in the context of class II MHC molecules. Perivascular inflammation often leads to clinical paralysis and demyelination. In SJL/J, SWR/J, and (SWR×SJL) $F_1$  mice, recovery from the primary attack is followed by a relapsing–remitting pattern of disease with a subsequent chronic progression of disability reminiscent of MS. How-

ever, the underlying cause for relapse and chronicity in both MS and EAE are presently unclear.

Recent studies have suggested that acquired recognition of new self-determinants, a process commonly referred to as determinant spreading, may be implicated in relapse and disease progression. McCarron et al. (1) showed that during relapse, (SJL×PL) $F_1$  (H-2<sup>s,u</sup>) mice acquire a new class II MHC-restricted response to myelin basic protein (MBP) that was not apparent during the primary inflammatory attack. Clayton et al. (2) found that B10.PL (H-2<sup>u</sup>) mice made neonatally tolerant to the immunodominant IA<sup>u</sup>-restricted p1-9 determinant of MBP remain susceptible to disease induction with intact MBP containing a nondominant IE<sup>u</sup>-restricted second determinant, p35-47. Furthermore, Lehmann et al. (3) showed that immunoreactivity to the nondominant determinants MBP 35-47, 81-100, and 121-140 occurs during the development of chronic EAE in

<sup>1</sup>Abbreviations used in this paper: CNS, central nervous system; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PLP, proteolipid protein.

(SJL×B10.PL)<sub>F</sub><sub>1</sub> (H-2<sup>s,u</sup>) mice immunized exclusively with the immunodominant IA<sup>a</sup>-restricted determinant p1-11. Intermolecular spreading to proteolipid protein (PLP) was demonstrated by Perry et al. (4) in mice immunized with MBP, while Cross et al. (5) showed that such intermolecular spreading generates encephalitogenic T cells with newly acquired antigen specificity. Most recently, McRae et al. (6) inhibited the progression of p139-151-induced EAE by inducing tolerance to whole PLP after recovery from the primary attack. These data suggest that spreading to new PLP determinants may provide a basis for peptide-specific therapy after disease onset.

In this study, we have extended these earlier findings by systematically assessing the pattern of intra- and intermolecular determinant spreading as a function of time in (SWR×SJL)<sub>F</sub><sub>1</sub> mice immunized with the immunodominant PLP 139-151 encephalitogen. We report that the order in which new determinants are recognized during the course of disease follows an ordered, predictable, sequential pattern. Moreover, an invariant relationship exists between the development of relapse/progression and spreading of recognition to new immunodominant encephalitogenic determinants. Finally, we show that peptide-specific treatment with the primary spreading determinant after disease onset prevents subsequent chronic progression of EAE. These data support the view that determinant spreading is a pathogenic process that leads to relapse and chronic progression of autoimmune disease, and that the predictable sequential pattern of spreading provides the basis for peptide-specific therapy after the onset of clinical disease.

## Materials and Methods

**Mice.** Female (SWR×SJL)<sub>F</sub><sub>1</sub> (H-2<sup>s,u</sup>) mice were bred at the Research Institute of the Cleveland Clinic Foundation by mating SWR/J (H-2<sup>d</sup>) females with SJL/J (H-2<sup>s</sup>) males purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were immunized at 7–12 wk of age. All protocols for animal research met with prior approval of the Animal Research Committee of the Cleveland Clinic Foundation in compliance with the Public Health Service policy on humane care and use of laboratory animals.

**Bulk Peptide Synthesis.** The PLP peptides 104-117 KTTIC-GKGLSATVT, 139-151 HSLGKWLGHDPDKF (serine for cysteine at residue 140), 178-191 NTWTTCSIAFPSK, and 258-273 IAA-TYNFAVLKLMGRG as well as MBP 87-99 VHFFKNIVTPRTP and MOG 92-106 DEGGYTCFFRDHSYQ were either purchased commercially (Bio-Synthesis, Lewisville, TX) or were synthesized manually as previously described (7) with standard solid-phase methodology using side chain-protected t-Boc amino acids (Peninsula Laboratories Inc., Belmont, CA). All peptides were purified >90% by reverse-phase HPLC using a 22 × 250-mm C-18 column (Vydac Separations Group, Hesperia, CA). The identity of purified peptides was confirmed by amino acid analysis.

**Active EAE Induction.** EAE was induced as previously described (7). (SWR×SJL)<sub>F</sub><sub>1</sub> mice were immunized by subcutaneous injection in the abdominal flanks on day 0 with 100 nm of PLP 139-151 peptide (154 µg) and 400 µg *Mycobacterium tuberculosis* H37RA (Difco Laboratories Inc., Detroit, MI) in 200 µl of an emulsion of equal volumes of water and IFA (Difco Laboratories

Inc.). On days 0 and 3, each mouse also received  $0.6 \times 10^{10}$  i.v. *Bordetella pertussis* bacilli (Michigan Department of Public Health, Lansing, MI). In this study, all mice developed clinical EAE within 30 d after immunization.

**Clinical and Histologic Evaluation.** All mice were weighed and examined daily for neurologic signs as previously described (7) according to the following criteria: 0, no disease; 1, decreased tail tone or slightly clumsy gait; 2, tail atony and/or moderately clumsy gait and/or poor righting ability; 3, limb weakness; 4, limb paralysis; 5, moribund state. The presence of relapse was determined when mice showed an increase in observed neurologic disability of at least one clinical score unit. The increased neurologic deficit was typically accompanied by an abrupt and substantial ( $\geq 7\%$ ) weight loss. Brains and spinal cords were fixed in 10% phosphate-buffered formalin, and paraffin-embedded sections were stained with luxol fast blue and counterstained with hematoxylin and eosin. The presence of inflammatory foci, cellular infiltrates, and demyelination in CNS meninges and parenchyma was determined under light microscopy.

**PLP Pin Peptides.** A PLP pin peptide series (see Fig. 1) effectively representing a walkthrough of the entire 276-amino acid primary sequence of mouse PLP (8, 9) was purchased from Chiron Mimotopes (San Diego, CA). A total of 265 overlapping 12-mers were synthesized on high density polyethylene rod tips assembled into holders designed in 96-well microtiter plate format (10). When Fmoc biochemistry is used in the synthesis, cleavage of one peptide per microtiter well can occur (11, 12). Similar pin series have been used successfully to map T cell determinants of hen egg white lysozyme (12), MBP (3, 13), and glutamic acid decarboxylase (14). Upon arrival, 1 µg of each PLP pin peptide was dissolved in 500 µl of a solution of 40% acetonitrile (Aldrich Chemical Co., Milwaukee, WI) in 10 mM Hepes buffer (GIBCO BRL, Gaithersburg, MD). Working aqueous concentrations of pin peptides were prepared at 150 µg/ml in PBS, pH 7.2, and 20 µl of each working solution was distributed sequentially into individual wells of 96-well flat-bottomed microtiter Falcon plates (Becton Dickinson & Co., Lincoln Park, NJ). The plates were stored at  $-20^{\circ}\text{C}$  until ready for use.

**Determinant Spreading during EAE.** At days 7, 28, 56, and 84 after immunization of (SWR×SJL)<sub>F</sub><sub>1</sub> mice with p139-151, spleen cells were tested for proliferative responses to the 265 overlapping PLP pin peptides, to MBP 87-99, an immunodominant encephalitogenic determinant for both SJL/J (15, 16) and SWR/J (17) mice, and to myelin oligodendrocyte glycoprotein (MOG) 92-106, an immunodominant encephalitogen for SJL/J mice (18). Mononuclear cells were separated from single-cell suspensions by centrifugation on Lympholyte-M (Accurate Chemical & Science Corp., Westbury, NY) for 20 min at 2,500 rpm. Cells collected from the interface were washed three times in HBSS (GIBCO BRL) and resuspended in serum-free HL-1 media (Hycor, Irvine, CA) supplemented with 2 mM fresh L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 30 mM Hepes buffer (GIBCO BRL). Each well contained  $2 \times 10^5$  spleen cells with 15 µg/ml pin peptide in a total volume of 200 µl. Triplicate wells containing 15 µg/ml MBP 87-99 and MOG 92-106 were also tested. Triplicate positive control wells contained anti-mouse CD3 antibody at 10 µg/ml (PharMingen, San Diego, CA) or 20 µg/ml *M. tuberculosis* H37RA (Difco Laboratories Inc.), whereas negative control wells contained either no peptide or 1 of 30 irrelevant pin peptides of myohemerythrin, a protein having minimal sequence homology with myelin proteins (19). Dose responses to whole PLP (0.1–100 µg/ml) were also assessed in each experiment. The PLP was prepared from a washed total lipid ex-

tract of bovine white matter (20) and was purified and converted to aqueous form as previously described (7). Cultures were incubated at 37°C in humidified air containing 5% CO<sub>2</sub>. At 4 d, cultures were pulsed with [methyl-<sup>3</sup>H]thymidine (1.0 μCi/well, specific activity 6.7 Ci/mmol; New England Nuclear, Boston, MA), and the cells were harvested after 16 h by aspiration onto glass fiber filters. Levels of incorporated radioactivity were determined by scintillation spectrometry. Test wells containing 15 μg/ml of peptide were considered positive when their stimulation index was at least two times both the SD and the mean proliferation of control wells without peptide. Identification of PLP antigenic determinants required that positive proliferative responses be generated by at least three adjacent overlapping 12-mers. Responses to irrelevant myohemerythrin peptides were never observed, and responses to PLP peptides did not occur in naive mice or in control mice immunized with BSA. Responses to whole PLP were inconsistent between and within different preparations and often did not accompany marked responses to immunodominant PLP determinants.

**T Cell Lines and T Cell Assays.** To determine the activation profile of the novel spreading determinant PLP 258-273, short-term (SWR×SJL)F<sub>1</sub> T cell lines were generated to the immunodominant p139-151 and to the nonencephalitogenic p258-273 as previously described (21). LN cells from day 10 peptide-primed mice were cultured at 2.5 × 10<sup>6</sup> cells/ml in 24-well flat-bottomed plates (Falcon Labware, Becton Dickinson & Co.) in a total volume of 2.0 ml/well of DMEM (GIBCO BRL) supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT), 2 mM fresh L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 30 mM Hepes buffer, 5 × 10<sup>-5</sup> M 2-ME, and 20 μM peptide. After 3–4 d of stimulation, cells were centrifuged on Lympholyte-M, washed and rested without antigen in culture media supplemented with 20 U/ml recombinant mouse IL-2 (PharMingen) and 2 × 10<sup>6</sup>/ml x-irradiated (2,000 rads) syngeneic spleen cells. Cells at 4–5 × 10<sup>5</sup>/ml were rested in 24-well plates for 10–14 d receiving fresh IL-2 every other day. The rested cells were harvested, centrifuged on Lympholyte-M, and restimulated with 20 μM peptide. For adoptive transfer, 5 × 10<sup>5</sup> cells/well were cultured with 4 × 10<sup>6</sup> x-irradiated (2,000 rads) (SWR×SJL)F<sub>1</sub> spleen feeder cells in 24-well plates. At 3 d, cells were harvested, centrifuged on Lympholyte-M, and injected into tail veins of naive x-irradiated (450 rads) (SWR×SJL)F<sub>1</sub> recipients. Supernates from some cultures were removed at 48 h for cytokine ELISA assays. For T cell assays, dose-response studies were performed using 5 × 10<sup>4</sup> line cells/microtiter well with 5 × 10<sup>5</sup> x-irradiated (2,000 rads) spleen feeder monolayers from either SWR/J, SJL/J, or (SWR×SJL)F<sub>1</sub> mice. Cells were pulsed at 48 h, harvested 16 h later, and processed for scintillation spectrometry as described above. Splenocyte proliferative responses to p139-151 and MBP 87-99 were determined in (SWR×SJL)F<sub>1</sub> mice tolerized after EAE onset by intraperitoneal immunization with either the spreading MBP 87-99 determinant or the nonspreading PLP 104-117 determinant. Spleen cells at 3 × 10<sup>5</sup>/well were cultured in 96-well flat-bottomed microtiter plates in DMEM supplemented as described above. The final volume for each culture was 200 μl/well. Dose-response profiles to each peptide were determined. At 72 h, cultures were pulsed with tritiated thymidine and harvested 16 h later for scintillation spectrometry as described above.

**ELISAs.** Purified anti-mouse cytokine capture/detection antibody pairs were purchased from PharMingen. The capture/detection antibody pairs used in this study included anti-mouse IFN-γ (R4-6A2 and biotin-XMG1.2), anti-mouse IL-2 (JES6-

1A12 and biotin-JES6-5H4), anti-mouse IL-4 (BVD4-1D11 and biotin-BVD6-24G2), and anti-mouse IL-10 (JES5-2A5 and biotin-SXC-1). 50 μl of coating buffer (0.1 M NaHCO<sub>3</sub>, pH 8.2) containing capture antibody at 4 μg/ml was added to each microtiter well in 96-well enhanced protein binding ELISA plates (Corning Glass Inc., Corning, NY). After overnight incubation at 4°C, plates were washed with PBS/Tween (0.5%) and blocked by adding 200 μl/well of PBS containing 3% BSA. Plates were incubated for 2 h at room temperature and washed. 100 μl of recombinant cytokine standards (PharMingen) at multiple concentrations and 100 μl of each test sample were added to duplicate wells. After incubation for 4 h at room temperature and washing, 100 μl of appropriate detection antibody at 2 μg/ml was added to duplicate wells for 45 min at room temperature. After washing, 100 μl of a 1:400 dilution of avidin peroxidase (Sigma Chemical Co., St. Louis, MO) was added to each well and incubated for 30 min at room temperature. Plates were washed, and each well received 100 μl of fresh substrate buffer containing 300 μg/ml of the substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma Chemical Co.) and 0.03% H<sub>2</sub>O<sub>2</sub> in 0.1 M citric acid at pH 4.35. After 12 min, the absorbance at 405 nm was measured using an ELISA microplate autoreader (Bio-Tek Instruments, Inc., Winooski, VT). Standard values were plotted as absorbance (optical density) vs cytokine concentration, and sample cytokine concentrations were determined as values within the linear part of the standard curve.

**Passive Transfer of EAE with Spreading Determinants.** 50 d after immunization with p139-151, spleen cells from (SWR×SJL)F<sub>1</sub> EAE mice were stimulated *in vitro* with 20 μM of either p139-151 or the spreading determinant MBP 87-99. 3 d after activation, the cells were centrifuged on Lympholyte-M, washed, and injected into tail veins of naive x-irradiated (450 rads) (SWR×SJL)F<sub>1</sub> mice. Mice were assessed daily for clinical signs of EAE as described above.

**Hemispplenectomy.** 28 d after immunization with p139-151, (SWR×SJL)F<sub>1</sub> mice were anesthetized by subcutaneous injection of 200 μg/kg ketamine (Fort Dodge Laboratories, Fort Dodge, IA) and 10 μg/kg xylazine (Miles Laboratories, Shawnee Mission, KS), and an incision was made in the shaved left abdominal flank under sterile conditions. Half of the spleen was removed for immunologic testing using a battery operated cauterizer (Jorgensen Laboratories, Loveland, CO), which kept bleeding to a minimum. Care was taken to avoid obstruction to blood flow through the splenic artery and vein. The abdominal wall was closed with 4.0 silk suture, and the skin was closed with wound clips. Hemispplenectomized mice were weighed and examined daily for neurologic signs and were killed on day 84 for retesting autoreactive immune responses using the remaining half of the spleen.

**Induction of Peptide-specific Tolerance.** 10 d after onset of EAE, (SWR×SJL)F<sub>1</sub> mice were tolerized to either PLP 104-117 or MBP 87-99 by one of two methods: (a) tail vein injection of 300 μg of peptide in 0.2 ml PBS according to previously described procedures (22, 23); or (b) intraperitoneal injection of 100 μg peptide in 0.2 ml of an emulsion containing equal volumes of aqueous peptide and IFA. The latter method has been described previously (2, 24) and has been recently shown in adult mice to induce a splenic CD4<sup>+</sup> Th2 Ag-specific response involving the production of IL-5 and IL-10 (25). All mice were subsequently weighed and evaluated daily by an investigator blinded to the tolerance protocols.

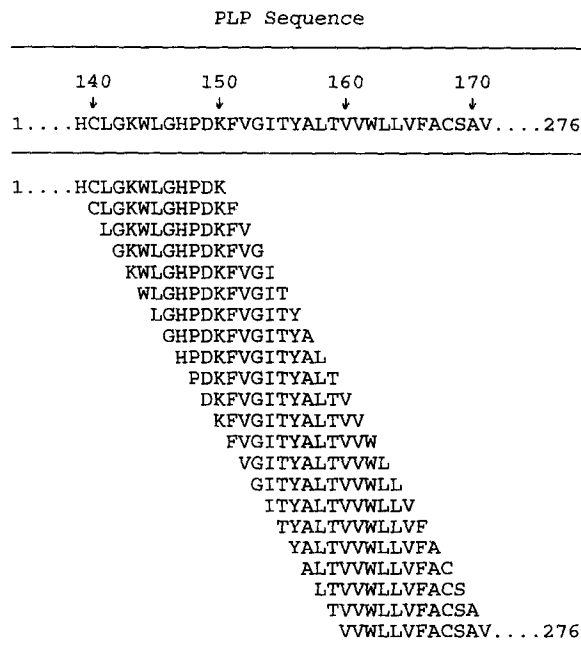
**Delayed-type Hypersensitivity (DTH) Ear Swelling.** Determinant-specific DTH reactions were quantitated as previously described (26). At 56 d after EAE onset, (SWR×SJL)F<sub>1</sub> mice tolerized to

PLP 104-117 and MBP 87-99 were challenged by injecting 10  $\mu$ l PBS containing 10  $\mu$ g of p139-151 in the dorsal surface of the ear using a 100- $\mu$ l Hamilton syringe and a 30-gauge needle. Contralateral ears were injected similarly with PBS containing 10  $\mu$ g of MBP 87-99. Ear thickness was measured three times before injection and three more times 24 h after challenge using an engineer's micrometer (Mitutoyo model 7326; Schlesinger's Tools, Brooklyn, NY). Increases in peptide-specific ear swelling were determined and expressed in units of  $10^{-4}$  inches  $\pm$  SE.

*Statistical Analysis.* Student's *t*-test was used to analyze differences in DTH, proliferation, and mean clinical scores between PLP 104-117- and MBP 87-99-tolerized mice.

## Results

*Sequential Pattern of Determinant Spreading during EAE Progression.* Determinant spreading was evaluated in (SWR  $\times$  SJL) $F_1$  mice immunized with the immunodominant PLP 139-151 determinant. In three independently performed experiments, spleens were removed from one mouse on days 7, 28, 56, and 84 after p139-151 immunization and tested for proliferative responses to a pin series of PLP peptides. The pin series consisted of 265 overlapping 12-mers of PLP, each differing by one additional amino acid at the COOH-terminus and one deleted amino acid at the NH<sub>2</sub>-terminus (Fig. 1). Intermolecular determinant spreading was evaluated by measuring responses to the immunodominant determinants MBP 87-99 (15-17) and MOG 92-106 (18).



**Figure 1.** Epitope-mapping pin peptide series for PLP. An overlapping series of 12-mer peptides effectively representing a walkthrough of the entire 276 amino acid primary sequence of mouse PLP was used to measure determinant spreading during the course of EAE in (SWR  $\times$  SJL) $F_1$  mice. In addition, dose-response profiles were also determined for reactivity to the SJL/J encephalitogenic determinants MBP 87-99 and MOG 92-106.

Responses to the priming determinant p139-151 were readily apparent in all mice on all days tested and were often characterized by plasticity of determinant recognition involving reactivity to peptide regions directly adjacent to the defined p139-151 immunogen (Table 1). On day 7, two of three mice responded to PLP 152-174, a region directly adjacent to the COOH-terminus of p139-151, whereas on day 84 two of three mice responded to a peptide region flanking the NH<sub>2</sub>-terminus of p139-151.

Clinical EAE occurred within 19-30 d after p139-151 immunization. All mice tested after day 7 had clinical EAE, and all mice tested after day 28 developed at least one clinical EAE exacerbation. On days 28, 56, and 84 a sequential accumulation of responses developed to PLP 249-273, MBP 87-99, and PLP 173-198, respectively (Table 1). In addition, one mouse responded to PLP 47-60 ETYFSKN-YQDYEYL on day 56. The failure to observe reactivity to this determinant before or at any other time thereafter suggests that such ephemeral determinant recognition may reflect aborted autoreactivity. A response to MOG 92-106 was observed in one mouse on day 84 only. Plasticity of recognition was also apparent in reactivity to spreading determinants, particularly in response to flanking regions of the defined p178-191 encephalitogen (27) on day 84. Thus, broad regional recognition of core and flanking determinants appears to be a characteristic of early self-recognition events initiated by exogenous priming or endogenous spreading. The combined results of intra- and intermolecular determinant spreading are summarized in Table 2.

*PLP 249-273 Is a Nonencephalitogenic Determinant Involved in Spreading.* The observed autoreactivity to p249-273 was novel and was further examined. PLP 258-273 IAATYN-FAVLKLMGRG was synthesized and used as an immunogen in (SWR  $\times$  SJL) $F_1$  mice. Despite extensive efforts, we were unable to induce histologic or clinical EAE by either active immunization or passive transfer with p258-273-primed T cells (data not shown). A similar observation regarding an overlapping PLP sequence was made by Greer et al. (28) in the parental SJL/J strain.

The failure to observe p258-273-mediated disease may be due to inherent activation defects since primed LNC activated by p258-273 showed deficient production of inflammatory Th1 cytokines when compared with production by p139-151-activated primed LNC (Fig. 2 A). Responses to p258-273 were restricted by both MHC<sup>a</sup> and MHC<sup>b</sup>, and proliferation of p258-273-primed (SWR  $\times$  SJL) $F_1$  T cell lines with (SWR  $\times$  SJL) $F_1$  APC appeared to comprise the additive responses of cultures containing splenic APC from parental SWR/J (H-2<sup>a</sup>) and SJL/J (H-2<sup>b</sup>) mice (Fig. 2 B). Thus, p258-273 appears to be a nondominant, nonencephalitogenic promiscuous determinant of PLP consistently involved in determinant spreading but incapable by itself of generating a pathogenic level of Th1 inflammatory cytokines.

*Passive Transfer of EAE with Spreading Determinants.* To determine the pathogenicity of consistent intermolecular spreading to MBP 87-99, the ability to transfer EAE into naive recipients was evaluated. 50 d after immunization of



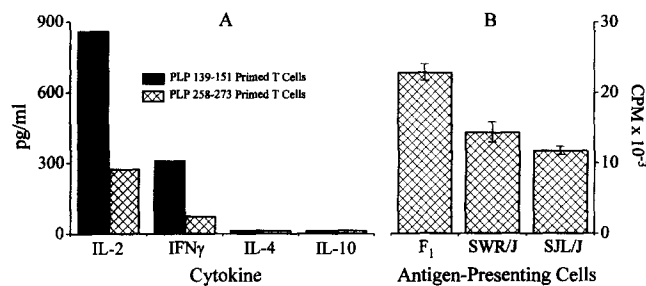
**Table 2.** Summary of Intra and Intermolecular Sequential Determinant Spreading in (SWR×SJL)<sub>F</sub><sub>1</sub> Mice Immunized with PLP 139-151

Sequence	Day after immunization with PLP 139-151			
	7	28	56	84
PLP 139-151	+++	+++	+++	+++
PLP 249-273	---	++-	+++	+++
MBP 87-99	---	---	+++	+++
PLP 173-198	---	---	---	+++

Summary table showing determinant-spreading patterns from three independently performed experiments in which spleens were removed from one (SWR×SJL)<sub>F</sub><sub>1</sub> mouse on days 7, 28, 56, and 84 after p139-151 immunization and tested for proliferative responses to a pin series of PLP peptides (shown in detail in Table 1) and to the immunodominant encephalitogenic determinants MBP 87-99 and MOG 92-106. + vs - indicates response vs no response for a given mouse on each day indicated.

(SWR×SJL)<sub>F</sub><sub>1</sub> mice with p139-151, spleen cells were removed from EAE mice and stimulated *in vitro* with either MBP 87-99 or PLP 139-151. Adoptive transfer of  $5.0 \times 10^7$  cells resulted in acute EAE in three out of five naive (SWR×SJL)<sub>F</sub><sub>1</sub> mice receiving MBP 87-99-stimulated spleen cells compared with six out of six recipients receiving p139-151-activated cells (Table 3). The severity of disease and time of onset were comparable in both groups of mice and demonstrated the pathogenic rather than protective nature of determinant spreading to the immunodominant MBP 87-99.

*Invariant Relationship between EAE Relapse/Progression and Determinant Spreading.* To determine whether the observed spreading pattern occurs within a given animal, spleen cell



**Figure 2.** T cell activity in response to PLP 258-273. (A) Deficient inflammatory cytokine production by PLP 258-273 T cell lines. High levels of Th1 cytokines are generated by (SWR×SJL)<sub>F</sub><sub>1</sub> T cell lines specific for the encephalitogenic p139-151 determinant but not for the non encephalitogenic p258-273. Cytokine levels were assayed by ELISA on supernates 48 h after peptide activation. (B) T cell lines generated to p258-273 in (SWR×SJL)<sub>F</sub><sub>1</sub> mice show promiscuous MHC restriction and respond ( $\pm$ SE) when peptide is presented by APC from either parent. Proliferation in response to 20  $\mu$ M p258-273 with (SWR×SJL)<sub>F</sub><sub>1</sub> spleen feeder cells appears to represent the additive responses of cultures containing APC of the individual parents. All background counts were  $\leq 2,000$  cpm.

**Table 3.** Passive Transfer of EAE by Spleen Cells Activated with the Spreading Determinant MBP 87-99

Priming peptide	Activating peptide	Number of mice with clinical EAE	Mean day of EAE onset (range)	Mean clinical score
PLP 139-151	PLP 139-151	6/6	12.3 (10-14)	3.5
PLP 139-151	MBP 87-99	3/5	15.7 (13-17)	3.5

(SWR×SJL)<sub>F</sub><sub>1</sub> mice were immunized with PLP 139-151. At 50 d after immunization, spleen cells were activated *in vitro* with either the p139-151 immunogen or with the spreading determinant MBP 87-99.  $5 \times 10^7$  cells were injected intravenously into naive x-irradiated (450 rads) (SWR×SJL)<sub>F</sub><sub>1</sub> mice.

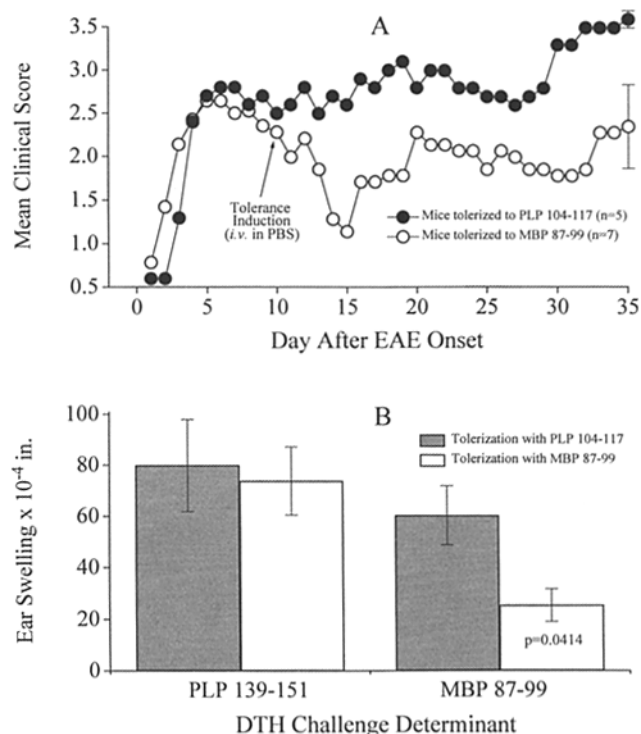
responses to PLP pin peptides, MBP 87-99, and MOG 92-106 were assessed at two time points within the same mouse. Mice were hemisplenectomized for immune testing 28 d after p139-151 immunization. After recovery from surgery, the mice were weighed and evaluated daily for neurologic impairment, and at day 84 the remaining half of each spleen was retested for responses to all peptides. Five of eight mice developed at least one clinical relapse and showed progression of clinical disease (Table 4). Moreover, all five relapsing-progressive mice showed a determinant spreading pattern virtually identical to that described for nonsurgically treated mice (Table 2). In contrast, the three mice that did not develop clinical relapse and progression also failed to show spreading of recognition to the dominant encephalitogenic peptides MBP 87-99 and PLP 173-198. Thus, it appears that an invariant relationship exists between clinical relapse/progression and spreading of self-recognition to new immunodominant encephalitogenic determinants.

*Prevention of EAE Progression by Peptide-specific Inhibition of Determinant Spreading.* The predictable sequential spreading pattern in (SWR×SJL)<sub>F</sub><sub>1</sub> mice offered an opportunity for directly addressing the pathogenicity of determinant spreading. 10 d after onset of EAE, mice were tolerized by intravenous injection with either the primary spreading determinant MBP 87-99 or with PLP 104-117, an equally potent encephalitogenic determinant for (SWR×SJL)<sub>F</sub><sub>1</sub> mice (21, 29) not involved in the observed spreading pattern. The results obtained by blinded evaluation indicate that mice tolerized to the spreading MBP 87-99 determinant showed consistent improvement in mean clinical score and delayed progression of clinical disease, whereas mice tolerized to the non-spreading PLP 104-117 determinant continued their progression to disability (Fig. 3 A). The clinical improvement of MBP 87-99 tolerized mice was accompanied by a corresponding significant inhibition ( $P = 0.0414$ ) of MBP 87-99 DTH as measured by determinant-specific ear swelling (Fig. 3 B). The inhibition of DTH was antigen specific to the extent that no differences

**Table 4.** Determinant Spreading in Hemisplenectomized (SWR×SJL)<sub>F1</sub> Mice: Invariant Relationship between Spreading and Relapse/Progression

Hemisplenectomy 28 d after immunization with PLP 139-151	Proliferative responses to spreading determinants			
	PLP 139-151	PLP 258-273	MBP 87-99	PLP 173-198
Relapsing mice (n = 5)				
Day 28 responses	+++++	+++--	-----	-----
Day 84 responses	+++++	+++++	+++++	++++-
Nonrelapsing mice (n = 3)				
Day 28 responses	+++	+--	---	---
Day 84 responses	+++	+--	---	---

28 d after immunization with PLP 139-151, (SWR×SJL)<sub>F1</sub> mice were hemisplenectomized and tested for proliferation to a pin peptide series for PLP as well as to MBP 87-99 and MOG 92-106. All mice had clinical EAE by day 28. Hemisplenectomized mice were weighed and examined daily for neurologic deficit. On day 84 after immunization, the remaining spleens were removed and retested for proliferative responses to the same peptides. All five mice that developed clinical relapse and progression showed determinant spreading to MBP 87-99, and four out of five mice showed spreading responses to PLP 173-198. All three mice that failed to relapse and progress also failed to show spreading of the autoimmune response to either MBP 87-99 or PLP 173-198.



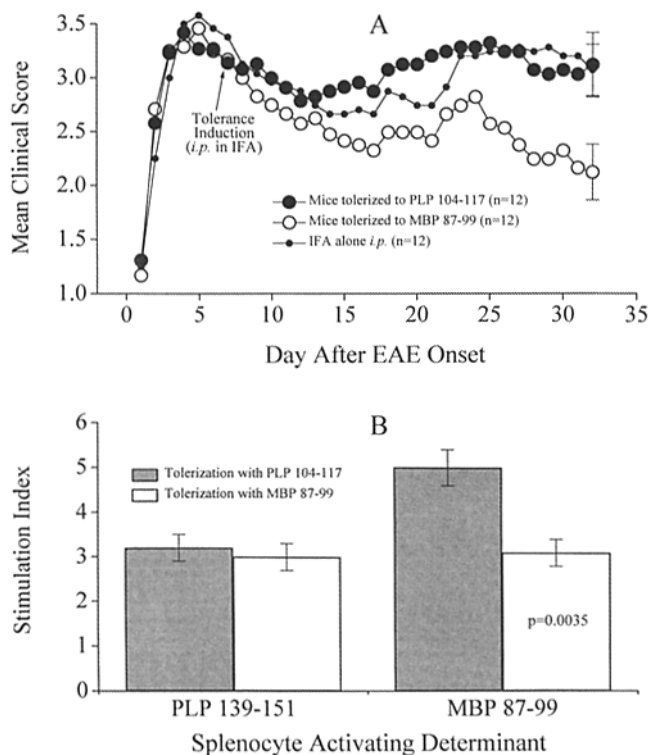
**Figure 3.** Inhibition of disease progression by intravenous induction of tolerance after onset of EAE to spreading vs nonspreading determinants. (A) 10 d after EAE onset, (SWR×SJL)<sub>F1</sub> mice were tolerized by intravenous injection of 300 μg of either the immunodominant spreading determinant MBP 87-99 or PLP 104-117, a highly encephalitogenic immunodominant determinant (21, 29) not involved in determinant spreading. Mice were examined daily by a person blinded to treatment protocols. Mice tolerized to the spreading determinant MBP 87-99 failed to show clinical progression of disease (±SE). (B) The clinical improvement directly correlated with inhibition of DTH responses (day 36–40 after onset) to the spreading determinant MBP 87-99. No difference in the DTH response to the priming immunogen PLP 139-151 was evident between both groups of tolerized mice. However, the DTH response (±SE) to the

were observed between both groups of tolerized mice in DTH responses to the priming p139-151 immunogen.

In addition to intravenous injection of peptide, (SWR×SJL)<sub>F1</sub> mice were tolerized 7 d after EAE onset by intraperitoneal immunization in an IFA emulsion containing either the spreading MBP 87-99 determinant, the nonspreading control encephalitogen PLP 104-117, or IFA emulsion alone. Induction of MBP 87-99 tolerance after EAE onset resulted in a marked therapeutic effect (Fig. 4 A). By day 32, a significant improvement in mean clinical score was evident in mice tolerized to MBP 87-99 compared with sham- ( $P = 0.0105$ ) or PLP 104-117-tolerized ( $P = 0.0204$ ) mice. No significant differences were apparent between PLP 104-117- and sham-tolerized mice. The clinical improvement in MBP 87-99-tolerized mice was accompanied by a significant inhibition ( $P = 0.0035$ ) in splenocyte proliferative responses to MBP 87-99, but not to the p139-151 priming determinant (Fig. 4 B).

**Histopathology in Peptide-tolerized EAE Mice.** At 32–35 d after EAE onset, peptide-tolerized (SWR×SJL)<sub>F1</sub> mice were killed, and the brains and spinal cords were examined for the appearance of inflammatory infiltrates and demyelination. Mice tolerized to the spreading MBP 87-99 determinant consistently showed inflammatory changes that were qualitatively less severe than those observed in mice tolerized to the nonspreading PLP 104-117 encephalitogen. MBP 87-99-tolerized mice had fewer CNS inflammatory infiltrates and markedly less severe demyelination compared with mice tolerized to PLP 104-117 (Fig. 5).

spreading determinant MBP 87-99 was significantly lower ( $P = 0.0414$ ) in mice tolerized to MBP 87-99 compared with mice tolerized to the nonspreading PLP 104-117.



**Figure 4.** Clinical improvement after peptide-specific inhibition of determinant spreading. (A) 7 d after EAE onset, (SWR×SJL)F<sub>1</sub> mice were immunized with 100 μg i.p. of either the spreading determinant MBP 87-99, the nonspreading determinant PLP 104-117, or sham injections with IFA emulsion alone. Mice were evaluated in a blinded manner as described in Materials and Methods. By day 32, a significant improvement in mean clinical score (±SE) was evident in mice tolerized to MBP 87-99 compared with sham- (P = 0.0105) or PLP 104-117-tolerized (P = 0.0204) mice. No significant clinical differences were apparent between PLP 104-117- and sham-tolerized mice. In contrast to the immediate therapeutic effectiveness observed after intravenous injection of MBP 87-99 (Fig. 3 A), intraperitoneal immunization with MBP 87-99 in IFA produced a more delayed effect consistent with immune priming. (B) The clinical improvement observed in MBP 87-99-tolerized mice correlated with a significant inhibition (P = 0.0035) in splenocyte proliferative responses (days 32–35 after onset) to MBP 87-99 compared with mice tolerized with the nonspreading encephalitogenic PLP 104-117 (±SE). However, no differences were apparent between both groups of peptide-tolerized mice in their splenocyte proliferative responses to the priming PLP 139-151 immunogen. It is worth noting that intermolecular spreading to MBP 87-99 resulted in a level of autoreactivity on a par with that directed against the priming p139-151 determinant as indicated by the ability to mediate passive transfer of EAE (Table 3) and DTH (Fig. 3 B). Moreover, splenocyte proliferative responses to MBP 87-99 were actually greater than those directed against p139-151.

## Discussion

This study shows that determinant spreading during the development of autoimmune disease is an ordered physiologic process in which defined self-determinants are recognized in a sequential predictable manner. Moreover, the mediation of passively transferred EAE with spreading determinants, the invariant relationship between spreading and chronicity, and the therapeutic effect on disease progression after determinant-specific inhibition of spreading estab-

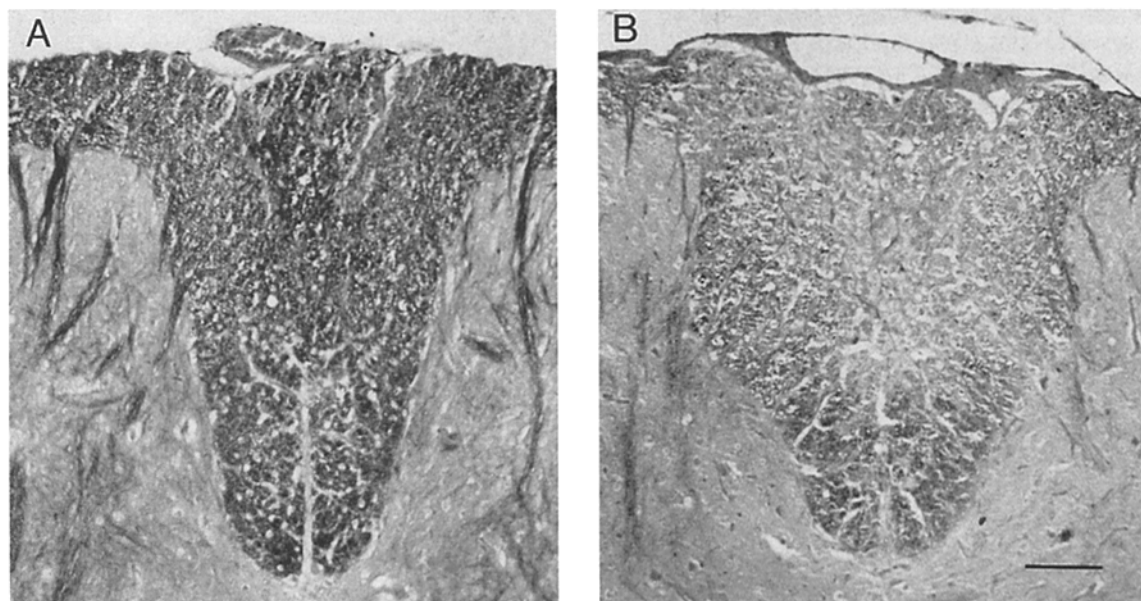
lishes an essential pathogenic role of determinant spreading in the progression of autoimmunity and provides a foundation for therapeutic intervention of ongoing disease.

Although the detailed mechanisms responsible for exacerbation of autoimmune demyelinating disease are presently unclear, numerous studies have implicated bursts of T cell-inflammatory cytokine activity before or during early stages of relapse. Increased IFN-γ and TNF-α activity (30) and increased TNF-α and TNF-β mRNA levels (31) occur in PBL of MS patients before both clinical relapse and active magnetic resonance scans (31). Relapse in MS has also been associated with decreased expression of the anti-inflammatory cytokines TGF-β and IL-10 in MS (31). Similarly, inflammatory cytokines have been demonstrated in the CNS during active EAE (32–34), whereas recovery has been associated with concomitant decreased CNS expression of IFN-γ, IL-2, IL-4, and IL-6 and increased expression of IL-10 mRNA (35). Furthermore, bursts of mRNA expression for inflammatory chemokines occur immediately before the initiation of both EAE onset (36, 37) and relapse (38) in SJL/J mice, whereas abrupt decreased expression often occurs during remission. However, perhaps the most convincing evidence to date for immune mediation of relapse are the results obtained from an IFN-γ clinical trial that produced a high enough rate of MS relapse to terminate treatment (39).

The results of this study indicate that the inflammatory cytokine and chemokine activity associated with relapse and chronicity of autoimmune demyelination are due at least in part to spreading of Th1 recognition to new myelin self-determinants. However, numerous reports have associated failure to relapse with innate immunoregulatory mechanisms that appear to target immunogen-primed autoreactive T cells. Apoptosis (40, 41), CD4<sup>+</sup> anti-TCR inhibitory cells (42–44), and perhaps CD8<sup>+</sup> cells (45, 46) have been implicated in downregulation of the response to the priming immunogen and may explain the nonrelapsing nature of EAE in Lewis rats and in PL/J and B10.PL mice.

Conversely, such inhibitory mechanisms may be deficient in relapsing and chronic progressive EAE models, thus predisposing to a sustained pathogenic response to the priming immunogen with subsequent transfer of autoreactivity to spreading determinants. Furthermore, it is conceivable that patients with single demyelinating episodes (e.g., optic neuritis) have intact immunoregulatory mechanisms that result in aborted autoreactivity, whereas patients that develop MS fail to down-regulate their initial self-reactivity and progress by spreading the response to new self-determinants. From this perspective, chronic progression of autoimmune demyelinating disease would appear to require that a threshold inflammatory response to the priming immunogen be maintained long enough to allow the transfer of autoimmune targeting to new determinants. Autoreactivity would be perpetuated by serial transfer of recognition through a determinant-spreading cascade. In this context, it is worth noting that spreading to the nondominant p249-273 occurs by day 28, coinciding with or immediately following EAE onset. This suggests that p249-273 may repre-





**Figure 5.** Histopathology of (SWR×SJL) $F_1$  mice tolerized after EAE onset with spreading versus nonspreading determinants. Photomicrographs ( $\times 300$ ; luxol fast blue-cresyl violet) of the dorsal columns of cervical spinal cords from mice tolerized intraperitoneally with peptide/IFA emulsion 7 d after EAE onset. Mice tolerized with MBP 87-99 consistently showed less severe inflammation and demyelination than mice tolerized with PLP 104-117. (A) Representative mouse tolerized with the spreading determinant MBP 87-99. Demyelination is not apparent 32 d after EAE onset. (B) In contrast, EAE mouse tolerized with the nonspreading PLP 104-117 determinant shows an extensive area of demyelination involving most of the dorsal columns 33 d after EAE onset. Bar, 50  $\mu\text{m}$ .

sent an “enhancer” determinant, incapable of mediating EAE induction by itself but capable of amplifying and sustaining the early inflammatory response directed initially against the priming p139-151 immunogen. This type of determinant-mediated inflammatory enhancement may thereby assist in spreading the response to the immunodominant MBP 87-99, a determinant completely capable of mediating and maintaining pathogenicity.

The order in which determinants are recognized during spreading does not follow the traditional immunodominant→nondominant hierarchy, nor does it favor in any sense an intramolecular bias. Spreading to PLP 249-273, a nondominant determinant according to traditional criteria, occurs before broadening of the response to the immunodominant peptides MBP 87-99 and PLP 173-198. Thus, the dominant recognition of p249-273 during the flow of autoimmune determinant spreading stands in direct conflict with its nondominant character as traditionally defined. However, traditional criteria for defining dominant/nondominant antigenic peptides are based on relative responses to exogenous proteins such as hen egg-white lysozyme (47-49). It is not surprising, therefore, that self-determinants having native antigen depots are not recognized during the development of autoimmune disease according to criteria obtained from exogenous immunizations. In essence, spreading flows from one determinant to another based on internal recognition patterns that appear to reflect factors not involved in the recognition of non-self proteins, such as processing and determinant availability within the target organ. Thus, the order in which determinants are

recognized during the development of autoimmunity reflects an innate spreading dominance based on an immunologic bias that appears to be independent of traditional dominant/nondominant criteria.

Responses to the epitope-mapping PLP peptide series reveals an extraordinary plasticity inherent in immune recognition of autoreactive proteins. At least four components constitute the observed plasticity of self-recognition: (a) recognition of broad protein regions incorporating core and flanking sequences of encephalitogenic determinants; this pattern is typically characteristic of early self-recognition events initiated by either exogenous priming or endogenous spreading (Table 1); (b) focusing of recognition to core sequences within each protein region; (c) shifting of epitope responses within each region; and (d) spreading of the response to new regions incorporating new encephalitogenic core determinants. In addition, the development of ephemeral aborted responses (e.g., the response to PLP 47-60 in one mouse on day 56 only) further adds to the inherent subtle features of self-recognition.

It appears that immunization with a discrete peptide determinant is sufficient to consistently induce a response to a sequence adjacent to the priming determinant in the native protein but not involved in the initial immunization. This implies that immune recognition of adjacent self-determinants is cognate and that an innate relationship exists between recognition of core determinants and recognition of their adjacent flanking sequences. The nature of such relationships may reflect protein sequence motifs requiring structural or functional complementary ligands, as has been

suggested (50–52). The ephemeral and shifting nature of flanking region recognition is also of interest and may represent aborted autoimmunity that fails to develop pathogenic consequences. Nevertheless, the plasticity of the observed recognition patterns indicates that autoimmune disease involves self-recognition events characterized by a supple dynamic developmental immune agenda. This dynamic paradigm provides a rationale for therapeutic intervention at each predictable stage in the development of autoimmunity.

Recent clinical improvements have enabled the identification of patients with single episodes of idiopathic monosymptomatic demyelinating syndromes at high risk for progressing to definite MS (53). Our preliminary results indicate that such patients have a high rate of self-recognition plas-

ticity and show patterns of reactivity remarkably similar to those described in this study. Such shifting patterns of self-recognition may be responsible for the observed changes in T cell repertoire restriction occurring in patients with optic neuritis who ultimately develop MS (54). The coaccumulation of neurologic deficit and promiscuous self-determinant recognition implies that early treatment of autoimmune demyelinating disease would be most desirable. To this end, the predictable and sequential nature of self-recognition plasticity may serve both to target and to monitor the effectiveness of therapeutic strategies used for early treatment of MS and related CNS inflammatory abnormalities.

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