Spontaneous Regression of Primary Autoreactivity during Chronic Progression of Experimental Autoimmune Encephalomyelitis and Multiple Sclerosis

By Vincent K. Tuohy,*‡ Min Yu,*, Ling Yin,*, Julie A. Kawczak,* and R. Philip Kinkel‡

From the *Department of Immunology, Lerner Research Institute, and the ‡Mellen Center for Multiple Sclerosis Research and Treatment, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

Summary

Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model for multiple sclerosis (MS). EAE is typically initiated by CD4+ T helper cell type 1 (Th1) autoreactivity directed against a single priming immunodominant myelin peptide determinant. Recent studies have shown that clinical progression of EAE involves the accumulation of neo-autoreactivity, commonly referred to as epitope spreading, directed against peptide determinants not involved in the priming process. This study directly addresses the relative roles of primary autoreactivity and secondary epitope spreading in the progression of both EAE and MS. To this end we serially evaluated the development of several epitope-spreading cascades in SWXJ mice primed with distinctly different encephalitogenic determinants of myelin proteolipid protein. In a series of analogous experiments, we examined the development of epitope spreading in patients with isolated monosymptomatic demyelinating syndrome as their disease progressed to clinically definite MS. Our results indicate that in both EAE and MS, primary proliferative autoreactivity associated with onset of clinical disease invariably regresses with time and is often undetectable during periods of disease progression. In contrast, the emergence of sustained secondary autoreactivity to spreading determinants is consistently associated with disease progression in both EAE and MS. Our results indicate that chronic progression of EAE and MS involves a shifting of autoreactivity from primary initiating self-determinants to defined cascades of secondary determinants that sustain the self-recognition process during disease progression.

Key words: multiple sclerosis • autoimmunity • epitope spreading • demyelination • myelin proteolipid protein

Multiple sclerosis (MS) and its related animal model, experimental autoimmune encephalomyelitis (EAE), are often characterized by a relapsing–remitting disease course with chronic–progressive disability (1). Typically, EAE is initiated by CD4+ Th1 autoreactivity directed against a single immunodominant myelin protein determinant (2, 3). However, the basis for disease progression is less clear. In the case of EAE, progression of disease may be attributed either to sustained autoreactivity directed against an immunodominant priming determinant or to acquired autoreactivity directed against determinants not involved in the initiation of disease. This acquisition of neo-autoreactivity, commonly referred to as epitope spreading, presumably results from endogenous priming with new self-antigens generated from damaged tissue over the course of disease (4–7). Similarly, in the case of MS, disease progression may be due to either a sustained primary autoreactivity or to a secondary acquisition of neo-autoreactivity as a result of epitope spreading.

Our study was designed to define the roles of primary autoreactivity and epitope spreading on the progression of autoimmune demyelinating disease. To this end, EAE was induced in SWXJ mice by immunization with a panel of distinct priming determinants of myelin proteolipid protein (PLP), and autoreactivity was assessed over time to known encephalitogenic determinants of PLP, myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG).
In complementary experiments, autoreactivity to an overlapping PLP peptide series was evaluated over a 3-yr period in patients with isolated monosymptomatic demyelinating syndrome (IMDS), a group of related neurologic disorders with variable rates of progression to clinically definite MS (CDMS; 8–10).

Our results indicate that during progression of EAE, proliferative responses to priming determinants invariably decline with time and frequently disappear in both the periphery and in the central nervous system (CNS). The regression of primary autoreactivity is succeeded by a sequential accumulation of neo-autoreactivity to defined spreading determinants. Similarly, autoreactivity associated with onset of neurologic symptoms in IMDS patients consistently wanes with time and becomes undetectable as disease progresses to CDMS. The spontaneous waning and frequent disappearance of self-recognition involved in the initiation of autoimmunity indicates that the natural history of autoimmune disease involves a shifting of responses from primary initiating determinants to defined cascades of secondary determinants that sustain the self-recognition process during disease progression.

Materials and Methods

Mice. Female SWXJ (H-2k) mice were bred at the Biological Resources Facility of the Lerner Research Institute by mating SWR/J (H-2b) females with SJL/J (H-2b) males purchased from The Jackson Laboratory. Mice were immunized at 7–12 wk of age. All protocols for animal research met with prior approval of the Institutional Animal Care and Use Committee (IACUC) 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 HSLGKWLGHPDKF, and 178–191 NTWTTCQSIAFPSK, as well as 139–151 HSLGKWLGHPDKF (serine for cysteine at residue 140), and 178–191 NTWTTCQSIAFPSK, as well as the protein core facility of the Lerner Research Institute with standard solid phase methodology using 9-fluorenylmethoxycarbonyl (FMOC) side chains. Peptides were purified sequentially into individual wells of 96-well flat-bottomed microtiter Falcon plates (Becton Dickinson). The plates were stored at −20°C until ready for use.

Induction of EAE. EAE was induced as previously described (14). SWXJ mice were immunized by subcutaneous injection in the abdominal flanks on day 0 with 100 nmol of PLP peptides p139–151 (154 μg), p178–191 (158 μg), or p104–117 (138 μg), and 400 μg Mycobacterium tuberculosis H37RA (Difco Labs.) in 200 μl of an emulsion of equal volumes of water and IFA (Difco Labs.). On days 0 and 3 each mouse also received intravenously 6 × 10^8 Borteldia pertussis bacilli (Michigan Department of Public Health, Lansing, MI). In the study presented here, all mice developed clinical EAE within 24 d of immunization.

Clinical Evaluation of EAE. All mice were weighed and examined daily for neurologic signs as previously described (14) according to the following criteria 0, no disease; I, 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 (≥7%) weight loss.

Histologic and Immunocytochemical Evaluation of EAE. Brains and spinal cords were fixed in 10% phosphate-buffered formalin, and paraffin-embedded tissue sections were cut (10 mm each) for immunostaining as previously described (15, 16). Sections were pretreated with 0.04% O2O and 1% H2O2 in 10% Triton (Electron Microscopy Sciences) and blocked with 5% normal goat serum (Vector Labs.) and 5% nonfat dehydrated milk for 60 min. Sections were treated sequentially with PLP monoclonal IgG to antibody (Harlan) at a 1:200 dilution for 14 h at 4°C, biotinylated goat anti-mouse IgG (Southern Biotechnology Associates) at a 1:500 dilution for 30 min at 22°C, and avidin-peroxidase complex (Vector Labs.) for 1 h at 1:1,000 dilution. Sections were then treated with diaminobenzidine and 0.01% H2O2 for 8 min, 0.04% O2O for 30 s, and washed in PBS. Images were digitized using the Alphamager 2000 System (Alpha Innotech) at 640 × 480 pixel resolution. Images were captured at 10 × magnification with the black level scale set at 0, white level scale at 255, and gamma level scale at 1.0. All images were normalized by adjusting background gray matter stain to the same mean intensity value using Adobe Photoshop (Adobe Systems). The presence of demyelination in CNS meninges and parenchyma was determined visually as well as by digitized image analysis using NIH image software (version 1.57; National Institutes of Health, Bethesda, MD).

Evaluation of Epitope Spreading during EAE. At wk 2, 4, 8, 12, and in some cases 16 after immunization of SWXJ mice with a PLP determinant, splenocytes were tested for proliferative responses to PLP determinants p104–117 (14, 17), p139–151 (18), and p178–191 (19, as well as M p 87–99 V HFFKNI VTPRP and M O G 92–106 DEGYTCC FFR D H YQ , were either purchased commercially (Bio-Synthesis or synthesized at the Protein Core Facility of the Lerner Research Institute with standard solid phase methodology using 9-fluorenylmethoxy carbonyl (FMOC) side chains. Peptides were purified >90% by reverse phase HPLC using a 22 × 250 mm C-18 column (Vydac Separations Group). The identity of each purified peptide was confirmed by mass spectrometry.

Epitope-Mapping PLP Peptide Series. A PLP pin peptide series representing a walk-through of the entire 276-amino acid primary sequence of mouse PLP (11, 12) was purchased from Chinaron M immunotopes. A total of 265 overlapping 12-mers were synthesized on high-density polystyrene rod tips assembled into holders designed in 96-well microtiter plate format (13). Each successive peptide differed from the previous 12-mer by sequential NH2-terminal deletion and COOH-terminal addition of PLP amino acids. Upon arrival, 1 mg of each PLP pin peptide was dissolved in 500 μl of a solution of 40% acetonitrile (Aldrich Chemical) in 10 mM Hepes buffer (GIBCO BR L). Working aqueous concentrations of pin peptides were prepared at 150 μg/ml in PBS, pH 7.2, and 20 μg/ml of each working solution was distributed sequentially into individual wells of 96-well flat-bottomed microtiter Falcon plates (Becton Dickinson). The plates were stored at −20°C until ready for use.

Induction of EAE. EAE was induced as previously described (14). SWXJ mice were immunized by subcutaneous injection in the abdominal flanks on day 0 with 100 nmol of PLP peptides p139–151 (154 μg), p178–191 (158 μg), or p104–117 (138 μg), and 400 μg Mycobacterium tuberculosis H37RA (Difco Labs.) in 200 μl of an emulsion of equal volumes of water and IFA (Difco Labs.). On days 0 and 3 each mouse also received intravenously 6 × 10^8 Borteldia pertussis bacilli (Michigan Department of Public Health, Lansing, MI). In the study presented here, all mice developed clinical EAE within 24 d of immunization.
Evaluation of Autoreactivity by CNS-infiltrating Mononuclear Cells. M ononuclear cells were recovered from the CNS at various clinical stages of EAE according to the method of Ford et al. (25). In brief, SWXJ EAE mice were killed by CO₂ inhalation and perfused with 20 ml of HBSS to remove hematogenous leukocytes. Brain tissue was teased and digested with 1 mg/ml of collagenase D (Boehringer Mannheim) and 50 Kunitz U/ml of DNase (Sigma Chemical Co.) at 37°C for 60 min. After washing, cells were re-suspended in Percoll (Amersham Pharmacia Biotech) adjusted with HBSS to a specific gravity of 1.030 and layered on Percoll/ HBSS at a specific gravity of 1.095. After centrifugation for 30 min at 1,250 g, cells were removed from the interface, washed, and counted for total yield. Percentages of T cells (FITC-labeled anti-CD3 or anti-CD4; PharMingen) and microglia (FITC-labeled anti-CD11b; PharMingen) were determined by flow cytometry. Proliferation assays in response to peptides were performed as described above using 2 × 10⁵ CNS harvested cells/microtiter well.

Results

Chronic EAE Induced in SWXJ Mice with Three Different PLP Peptides. SWXJ mice develop acute EAE ∼3 wk after conventional immunization with either of the three encephalitogenic PLP determinants p104–117, p178–191, and p139–151 (Fig. 1). Affected mice typically undergo an incomplete recovery from the initial attack with residual neuroparalytic signs. Recovery is soon followed by a series of multiple relapse/remission cycles with each neuroparalytic episode leaving the mice progressively more impaired. By 8–12 wk after immunization, the incremental accumulation of neurologic deficit makes it increasingly more difficult to observe relapses. This chronic-progressive stage of EAE is characterized by sustained limb paresis or paralysis and marked CNS demyelination particularly pronounced in the spinal cord (Fig. 2).

Regression of Primary Autoreactivity during Progression of EAE. To evaluate the changes occurring in self-recognition after immunization with different encephalitogenic peptides, three groups of SWXJ mice were immunized with each of the PLP determinants (p104–117, p178–191, and p139–151), and splenocytes were tested at various times thereafter for proliferative responses to the three PLP determinants as well as to the defined encephalitogens MBP 87–99 (20–22) and MOG 92–106 (23). Three to five independent experiments were performed for each time point. We found that the kinetics of the response to the PLP priming determinants p104–117 (Fig. 3 a) and p178–191 (Fig. 3 b) were similar, with peak responses occurring at 2–4 wk, a decline in responses by 8 wk, and a virtual absence of detectable proliferative responses to the priming immunogens by 12 wk after immunization. Although the profile of reactivity to the
p139–151 priming determinant also showed a rise, peak, and decline, the kinetics of the p139–151 response were notably distinct from those of the other PLP determinants, in that proliferation took longer to peak (8 wk versus 2–4 wk) and decline (12–16 wk versus 4–8 wk) and reached the level of control responses in only two out of five mice (Fig. 3 c). Nevertheless, by 12–16 wk after immunization, proliferative responses to three distinct PLP priming determinants invariably declined and were often undetectable despite the concurrent development of chronic-progressive disease (Fig. 1).

Regression of Primary Autoreactivity Occurs in both Splenocytes and CNS-infiltrating Mononuclear Cells. Since it was possible that the observed regression of splenocyte responsiveness to priming determinants may simply reflect a selective sequestration of autoreactive T cells from the periphery into the CNS, we simultaneously compared splenic and CNS autoreactivities during the course of disease. At distinct clinical stages of EAE, mononuclear cells were isolated from the brain and tested along with splenocytes for proliferative responses to priming determinants. In SWXJ mice immunized with both PLP 104–117 (Fig. 4 a) and PLP 178–191 (Fig. 4 b), concurrent declines in responses to priming determinants were evident in both splenocytes and CNS-infiltrating cells during the course of disease. By the second relapse, complete regression of primary autoreactivity was clearly evident in both splenocytes and CNS-infiltrating cells despite the presence of similar numbers of infiltrating cells compared with onset and first relapse (Fig. 4 c). These data indicate that regression of primary autoreactivity occurs simultaneously in both the periphery and CNS.

Emergence of Epitope Spreading Cascades during Progression of EAE. During the regression of primary autoreactivity in EAE, responses invariably emerged to self-determinants not involved in the initial priming process. By 4 wk after immunization with PLP 104–117 (Fig. 5 a), proliferative responses to PLP 139–151 (SI = 2.5) and MBP 87–99 (SI = 2.3) became apparent, peaked by 8 wk (SI = 3.9 and 3.5, respectively), and remained markedly elevated at 12 wk when acquired responses to PLP 178–191 (SI = 3.6) first became evident and responses to the p104–117 priming determinant declined toward baseline (SI = 1.6). In contrast to the plasticity observed in self-recognition, responses to the priming adjuvant Mycobacteria tuberculosis H37RA showed little fluctuation throughout the testing period (SI = 6.13 ± 0.24 SEM). Thus, a cascading emergence of neo-autoreactivity accompanied the regression of primary autoreactivity during the development of chronic-progressive disease induced by immunization with PLP 104–117.

Epitope spreading also accompanied disease progression in SWXJ mice immunized with PLP 178–191 as indicated by the appearance of proliferative responses to PLP 139–151 (SI = 3.2) and MBP 87–99 (SI = 2.2) 4 wk after priming with the PLP 178–191 immunogen (Fig. 5 b). Moreover, the observed neo-autoreactivity remained elevated at 12 wk.
When proliferative responses to the priming PLP 178–191 determinant were virtually undetectable (SI = 1.4). Responses to H37RA were similar at all times tested (SI = 4.4 ± 0.21 SEM).

As described in our previous report (6), a cascading epitope spreading pattern occurred after immunization of SWXJ mice with PLP 139–151. A readily detectable response to MBP 87–99 occurred at 8 wk (SI = 2.6) and remained elevated at 12 wk (SI = 2.9) when additional neo-autoreactivity to PLP 178–191 (SI = 3.6) became clearly evident (Fig. 5 c). Responses to H37RA showed little fluctuation throughout the testing period (SI = 4.25 ± 0.13 SEM).

Regression of Primary Autoreactivity during Progression of IMDS to CDMS.

To determine whether the concurrent processes of regressing primary autoreactivity and emerging epitope spreading occurs during progression of MS, a related series of experiments was performed using monocentric monophasic IMDS patients who showed no evidence of prior subclinical disease activity as determined by T2-weighted MRI. Such patients often progress to CDMS (8–10) and have been shown in our previous report to develop sustained autoreactivity to defined PLP peptides (26). In serial evaluation of PBMC-proliferative responses to an overlapping PLP peptide series, three IMDS patients (VS, DL, and JB) showed sustained autoreactivity to PLP determinants (p210–244, p116–150, and p117–152, respectively) coinciding with or appearing soon after the diagnosis of IMDS (Fig. 6, a, b, and c, respectively). Serial testing over a 3-yr period showed that in each case the established autoimmune responses associated with the early onset stage of the disease process invariably declined with time and eventually became undetectable.

Emergence of Epitope Spreading during Progression of IMDS to CDMS.

The two IMDS patients, VS and DL, showed progression to CDMS, and their disease progression was accompanied by the emergence of PLP neo-autoreactivity (Fig. 7). At 154 wk after initial onset of neurologic symptoms, VS was diagnosed with CDMS after developing a cervical myelitis with a new enhancing MRI lesion of the cervical spinal cord that corresponded to new symptoms. The progression to CDMS was associated with the acquisition of newly acquired secondary responses directed against PLP.
50–69 (SI = 4.7) at 154 wk and both PLP 167–185 (SI = 2.4) and PLP 258–271 (SI = 3.4) at 170 wk (Fig. 7 a). At 154 wk and after, there were no detectable proliferative responses to the primary PLP 210–244 region associated with the early disease stage.

Patient DL showed progression from IMDS to CDMS 60 wk after initial onset of symptoms after developing symptoms consistent with a brainstem syndrome. Disease progression was associated with the development of a secondary spreading response to PLP 49–62 (SI = 2.4) at 54 wk equal in magnitude to the autoreactivity directed against PLP 116–150 (SI = 2.4), a determinant that once generated a vigorous sustained autoreactivity during the onset stage of IMDS (Fig. 7 b). At 82 wk, the response to the spreading determinant PLP 167–182 (SI = 2.7) was greater than that elicited by PLP 116–150 (SI = 2.3) and was even greater by 162 wk (SI = 3.2) when the response to the initial autoreactive PLP 116–150 was undetectable (SI = 1.1).

Thus far, patient JB has not progressed to CDMS. However, at 148 wk after initial onset of symptoms, JB developed the first indications of neo-autoreactivity with responses directed against PLP 261–274 (SI = 2.4) unaccompanied by detectable responses to PLP 117–152 associated with the early onset stage of IMDS (SI = 1.1; Fig. 7 c). Throughout the experimental period, fluctuations occurring in positive control responses of VS, DL, and JB showed no correlation with changes observed in primary and spreading autoreactivities (data not shown).

Discussion

Our results indicate that progression of both EAE and MS is consistently accompanied by the spontaneous decrease and frequent disappearance of the established primary autoreactivity and the concurrent emergence of the epitope-spreading cascade. Our findings are consistent with the view that progression of autoimmune disease involves the sequential appearance and regression of responses to a cascading series of self-determinants so that at any given time the response to one of several distinct determinants may appear to be predominant. Indeed, based on the prolif-
Our study directly challenges the widely held view that EAE and MS effectively under-represents the dynamic level of the changes that actually occur. This view is proposed in light of the fact that PLP served as the sole autogenic evaluated in this study and that proliferation was the only assay used for measuring autoreactivity. The actual level of self-recognition plasticity may best be estimated by serial assessment of the autoreactive changes to overlapping peptides of several myelin proteins implicated in MS autoreactivity, such as PLP, MBP, and MOG, as well as by the incorporation of more sensitive assays such as the ELISPOT capable of providing a 10-200-fold increased sensitivity over conventional methods for detecting immunoreactivity (37, 38) and the use of soluble peptide MHC tetramers that enable frequency analysis of antigen-specific T cells by flow cytometry (39).

It seems reasonable to speculate that shifts from one epitope response pattern to another may also be accompanied by a broadening in usage of MHC class II molecules restricting the autoreactivity. Moreover, it may be possible to sustain responses to a given determinant by expanding the autoreactive repertoire with clones that utilize restriction elements not involved in the primary response. A recent report from our laboratory showed that during progression from IMDS to CDMS, a patient homozygous for DRB1*04 responded in a DPB1*0301-restricted manner to PLP 43–64 (40), a peptide region shown in other studies to be DRB1*04 restricted (33). Thus, autoimmune spreading may involve diversification of restriction elements in addition to broadening of epitopes recognized. From this perspective, both epitope spreading and “MHC spreading” may participate together to sustain autoreactivity and thereby facilitate chronic progression of autoimmune disease.

Although shifts from primary to secondary autoreactive profiles were observed in both MS and EAE, each step in the process was markedly prolonged in MS. Typically, primary autoreactivity was detectable for over 1 yr in MS compared with 12 wk for PLP 104–117 and PLP 178–191 in murine EAE. Highly developed secondary patterns of autoreactivity occurred within weeks or months of onset of clinical symptoms in murine EAE but often required years to develop during progression of IMDS to MS. However, it should be noted that the contrasting self-recognition kinetic profiles of EAE versus MS were accompanied by corresponding differences in the development and progression of clinical symptoms, i.e., progression of murine EAE occurs within a few months, whereas progression of IMDS to CDMS often develops over many months or years. Thus, it appears that the shifting autoreactivity observed in SWXJ EAE mice repre-
sents an accelerated version of the same underlying processes responsible for the development of self-recognition in MS.

The shifting patterns of self-recognition shown in this study functionally reveals the fundamental underlying instability of the autoreactive T cell repertoire in MS. This view is supported by other studies showing changes in T cell repertoire restriction in MS patients who progress to CDMS (41). The inherent plasticity of the autoreactive repertoire has implications with regard to therapeutic applications, perhaps most notably evident in clonotypic therapies targeting specific TCR genes. Such T cell vaccination approaches result in the emergence of new autoreactive repertoires using TCR genes distinct from those used by the original responding T cells (42). Thus, the inherent capacity of the immune system to provide a continual source of neo-autoreactivity may serve ultimately as a basis for undermining the effectiveness of TCR-based therapies that fail to target the secondary spreading repertoires.

Recently, it has been suggested that it may be possible to harness epitope spreading in such a manner as to facilitate the spread of immune suppression during autoimmune disease (43). Prior reports have shown that chronic progression of EAE can be inhibited by inducing intramolecular (5) or intermolecular (6) tolerance to determinants of the epitope spreading cascade. Recent studies in our laboratory indicate that adoptive Th2 immunotherapy targeting spreading determinants results in a marked long-term inhibition of EAE progression even when transfer occurs before the development of endogenous self-priming (44). Thus, stacking the T cell repertoire to favor an active Th2 response to spreading determinants may subvert the neo-autoreactive process and produce a long-lasting therapeutic outcome. In this regard, peptide-based therapies such as those incorporating the HFFK amino acid motif of the putative human immunodominant MBP determinant (45, 46) or those involving altered peptide ligand strategies (47, 48) may prove to be most effective if the repertoire capable of responding therapeutically to the selected peptide has undergone minimal spontaneous regression.

It is evident from this study that progression of both EAE and MS may occur in the absence of primary initiating autoreactivity. However, thus far the basis for regression of primary autoreactivity is unclear. Our data indicate that the disappearance of primary self-recognition is not due to sequestration of autoreactive T cells in the CNS thereby creating an apparent loss of systemic autoreactivity. Therefore, more intricate explanations are needed to account for the spontaneous disappearance of self-recognition. Chronic self-stimulation may result in T cell exhaustion and peripheral clonal deletion (42), perhaps through apoptosis (49, 50). Alternatively, autoreactive T cells may be present but unreactive as a result of T cell anergy (51–54) or suppression (55, 56). Studies designed to determine the underlying mechanism(s) responsible for the regression of primary autoreactivity and the emergence of epitope spreading are currently in progress.

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Address correspondence to Vincent K. Tuohy, The Cleveland Clinic Foundation, Department of Immunology, N338, 9500 Euclid Ave., Cleveland, OH 44195. Phone: 216-445-3684; Fax: 216-444-8372; E-mail: tuohyv@cesmtp.ccf.org

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