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Induction of Experimental Allergic Encephalomyelitis by Myelin Proteolipid-Protein-Specific T Cell Clones and Synthetic Peptides

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Abstract. Proteolipid protein (PLP) is the major protein of central nervous system (CNS) myelin. SJL(H-2^s) mice immunized with a synthetic peptide corresponding to PLP residues 139–151 (HSLGKWLGHPDKF) develop acute experimental allergic encephalomyelitis (EAE). In the present study a T cell line and 4 clones were derived from SJL/J mice following immunization with this synthetic peptide. Severe clinical and histological EAE could be induced by adoptive transfer of the peptide-specific T cell line and 3 of 4 T cell clones. The T cell line/clones all responded strongly to PLP peptide 139–151 in in vitro proliferative assays. However, two different reactivity patterns emerged when truncated PLP peptides 141–150 and 141–149 were tested, suggesting that more than 1 epitope may be present within the PLP 139–151 determinant.

To evaluate the encephalitogenic potential of the truncated peptides, we compared the ability of 2 truncated PLP peptides to induce EAE in vivo and proliferative responses in vitro. Immunization with PLP peptide 141–150 induced acute EAE in about 70% of mice tested, but PLP peptide 141–149 induced a comparatively mild form of EAE in 4 out of 9 mice tested. Lymph node cells from mice immunized with these peptides showed in vitro proliferative responses to each of the peptides, but the response to peptide 139–151 was always strongest. These combined in vivo and in vitro data further define the epitopes involved in PLP-induced EAE in SJL mice. Furthermore, the availability of multiple PLP-specific T cell clones will enable us to study the diversity of the T cell repertoire to PLP.

Introduction

In multiple sclerosis (MS), central nervous system (CNS) myelin is the target of an immune attack but the precise mechanisms that lead to myelin breakdown remain to be elucidated. The 2 major proteins of CNS myelin are proteolipid protein (PLP) and myelin basic protein (MBP), and both have been implicated in the principal animal model of MS, experimental allergic encephalomyelitis (EAE). Although a vast amount of literature exists on the role of MBP in induction of EAE [1–3] and possibly MS [4–6], PLP has been much more difficult to study and it is only relatively recently that PLP has been accepted as an encephalitogen [7–12]. EAE can be induced in mice by synthetic PLP peptides, providing unequivocal evidence that PLP is encephalitogenic [7, 8]. Furthermore, EAE can be adoptively transferred by PLP-specific T cell lines and clones which show no cross-reactivity to MBP [9–11]. However, the mechanisms of autoimmune responses to PLP have not been elucidated.

Encephalitogenic determinants have been shown to be short contiguous sequences that can activate helper T cells [13]. These determinants have been identified for MBP in several strains of mice and other animal species [14–17]. Similarly, the encephalitogenic epitopes of PLP

are now defined in SWR(H-2^q) and SJL(H-2^s) mice. PLP peptide residues 103–116 (YKTTICGKGLSATV) induces EAE in SWR [7] but not in SJL mice, whereas peptide residues 139–151 (HSLGKWLGHPDKF) induces disease in SJL but not in SWR mice [8]. In this report we characterize a panel of PLP-peptide-specific T cell clones, some of which can induce EAE in SJL mice following adoptive transfer.

Materials and Methods

Mice

Female SJL/J(H-2^s) mice were purchased from the Jackson Laboratory (Bar Harbor, Me., USA). They were obtained at 5–8 weeks of age and were immunized between 8 and 14 weeks of age.

Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School, the Institutional Use and Care of Animals Committee of the E.K. Shriver Center, and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health and Human Services Publication 85–23, revised 1985).

Antibodies

The following cell lines producing monoclonal antibodies specific for various T cell markers were obtained from the ATCC: anti-CD4 (GK-1.5), anti-CD8 (TIB105), anti-Thy-1.1 (HO.22.1) and anti-Thy-1.2 (HO.13.4). Anti-CD3 (145–2C11) and anti-TcR-α/β (H57.597) antibodies were obtained from Drs. Jeffrey Bluestone (University of Chicago) and Ralph Kubo (National Jewish Center for Immunology and Respiratory Medicine, Denver), respectively. All antibodies were used as culture supernatants.

Antigens

All peptides were prepared according to the sequence of murine PLP [18–20]. Peptides were synthesized on a Milligen model 9050 synthesizer using FMOC chemistry. Milligen PAL® resins, which produce peptides with C-terminal amides, were used as the solid support. Peptides produced by this procedure were > 90% pure. All peptides which include PLP residue 140 were synthesized with serine substituted for cysteine in the native sequence. The peptides used in the present study included murine PLP residues 139–151 (HSLGKWLGHPDKF), 141–150 (LGKWLGHPDK), 141–149 (LGKWLGHPD) and 103–116 (YKTTICGKGLSATV). Proteolipid apoprotein (PLP) was obtained from a washed total lipid extract of bovine white matter [21] and lipid was removed by chromatography on an HPLC column [22]. MBP was purified from bovine brain as described by Deibler et al. [23] and keyhole-limpet hemocyanin (KLH) was obtained from Calbiochem (La Jolla, Calif., USA).

Immunization Protocol

SJL mice were immunized subcutaneously in both abdominal flanks on day 0 with 100 μg of PLP peptide, 50 μg of *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, Mich., USA) in 200 μl of an emulsion of equal volumes of water and incomplete Freund's adjuvant (Difco). Each mouse was also injected intravenously on day 0 and on day 3 with 0.75×10^{10} *Bordetella pertussis* bacilli (pertussis vaccine, lot No. WF 262, Massachusetts Public Health Biologics Laboratories, Boston, Mass., USA).

Derivation and Maintenance of T Cell Clones

Inguinal lymph node cells from 8- to 10-week-old female SJL mice were obtained 8 days after immunization with PLP peptide 139–151. The lymph node cells were stimulated in vitro with 10–30 μg/ml of the immunizing peptide for 3 days. Live lymphoblasts were separated by Ficoll-Hypaque density gradient centrifugation and maintained in Dulbecco's minimum essential medium (DMEM) containing 10% human T cell growth factor (TCGF; Advanced Biotechnologies, Columbia, Md., USA) or murine T cell growth promoter (TCGP; American Biotechnologies, Cambridge, Mass., USA). Bulk cultures were restimulated 3 more times with the peptide in the presence of irradiated syngeneic spleen cells at approximately 10-day intervals. The line (SPL) thus obtained was then cloned at 1 cell/well. Cloned cells were gradually expanded from 96- to 24-well plates. Cultures were fed every 48–72 h with complete DMEM supplemented with 10% human TCGF or murine TCGP and 10% fetal calf serum. Clones were maintained by restimulation every 10–14 days with irradiated spleen cells and 10–30 μg/ml of 139–151 PLP peptide and fed with DMEM containing 10% human interleukin-2, every other day. Cultures were expanded when cell densities exceeded 10^6/ml.

Proliferative Assay

In order to study the antigen response of T cell clones, 5×10^4 cloned T cells were cocultured with 5×10^5 irradiated syngeneic spleen cells and the indicated concentrations of antigen. After 48 h of incubation at 37 °C, 1 μCi of [^{3}H]-thymidine was added and cultures were harvested 20 h later by using a PHD cell harvester (Cambridge Technology, Cambridge, Mass., USA) and counted with the use of standard liquid scintillation techniques in a Beckman scintillation counter (model LS5000). The data represent mean counts per minute incorporated into insoluble DNA of triplicate wells. All standard deviations were < 20%.

To assess the proliferative response of lymph node cells, inguinal lymph nodes were obtained 8 days after immunization (100 μg peptide in complete Freund's adjuvant per mouse). Lymph node cells (3×10^5 cells/well) were incubated with the indicated concentrations of antigen for 72 h, [^{3}H]-thymidine was added and incubation continued for another 16–18 h. The plates were harvested and counted using liquid scintillation techniques, as described above.

Adoptive Transfer of EAE

Three days after in vitro activation with peptide 139–151, live cells were obtained on Ficoll-Hypaque gradients, washed 3 times and injected directly into the tail vein of naive mice. In some experiments, the mice were pretreated with 390 rad irradiation and injected with 0.75×10^{10} *B. pertussis* bacilli just prior to injecting the T cell clones.

Clinical and Histological Evaluation

All animals were assessed clinically as previously described [7, 8] 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. Animals were killed within 1 week after showing clinical signs of disease. Mice showing no clinical signs were killed 70 days after immunization. For evaluating relapses,

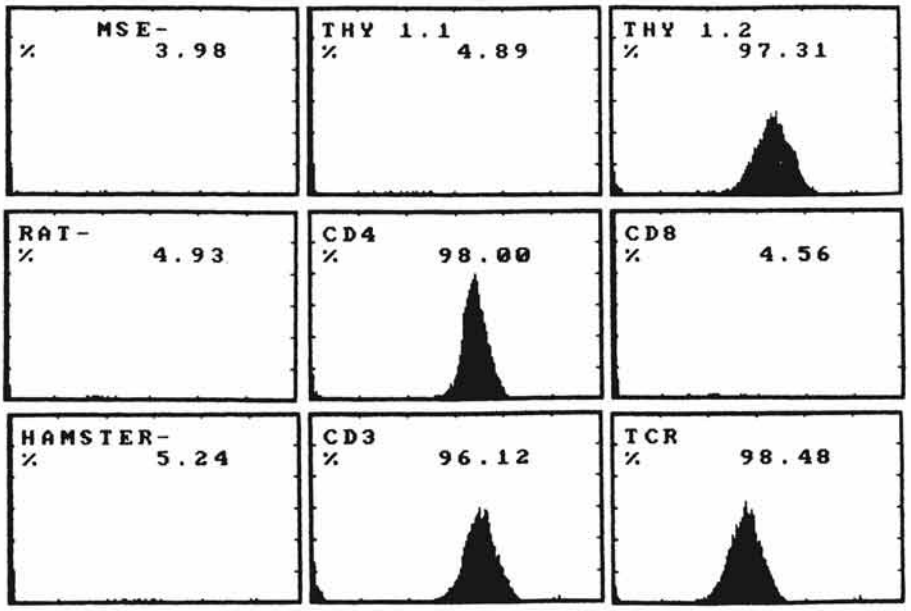

Fig. 1. Surface phenotype of PLP peptide 139–151-specific T cell line (SPL). Samples containing approximately 1 × 10^6 cells were incubated for 30 min with 50 µl of antibody-containing culture supernatant. The cells were washed and then incubated with a 1:50 dilution of an appropriate FITC-labeled second antibody. After the final incubation, cells were washed, fixed in paraformaldehyde and analyzed on a FACS II. Left-hand column gives controls for FITC-labeled anti-mouse, rat and hamster antibodies.

mice that survived an initial attack were observed regularly and followed for 135 days prior to sacrifice. Brains and spinal cords were fixed in 10% phosphate-buffered formalin, and paraffin-embedded sections were stained with luxol fast blue-hematoxylin and eosin for light microscopy. Histological disease was quantified by counting the number of inflammatory foci in meninges and parenchyma as previously described [24, 25].

Fluorescence-Activated Cell Sorter Analysis

Samples containing at least 1 × 10^6 clone or line cells were incubated on ice for 30 min with 20–50 µl of hybridoma culture supernatant and washed 2 or 3 times with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% NaN_3. These samples were then incubated for 30 min with 25 µl of the appropriate fluorescein isothiocyanate (FITC)-conjugated second antibody reagent. Second-step reagents included a 1:50 dilution of FITC-conjugated rabbit antihamster immunoglobulin, goat antirat immunoglobulin or goat antimouse immunoglobulin (Cooper Biomedicals, Organon Tecknica, Malvern, Pa., USA). After the final incubation, samples were washed twice, fixed in 1% paraformaldehyde and analyzed on a fluorescence-activated cell sorter (FACS) II or EPICS V cell sorter.

Table 1. Antigen specificity of T cell clones derived from SJL mice

Clone[a]	Stimulation index[b]					
	PLP	PLP 139–151	PLP 141–150	PLP 103–116	MBP	KLH
SPL	95.9	160.5	113.0	0.7	1.9	1.2
2E5	14.9	31.6	1.5	0.6	1.4	1.6
4E3	7.3	27.3	52.7	0.7	1.1	1.6
5B6	15.1	107.1	1.9	0.6	1.0	1.0
7A5	7.1	64.4	134.3	0.9	0.7	1.0

[a] T cell clones (5 × 10^4) were cultured with 5 × 10^5 syngeneic irradiated spleen cells and 30 µg/ml of the indicated antigen for 72 h; 1 µCi of [^{3}H]-thymidine was added for the last 16–20 h of culture.

[b] Stimulation index = Mean cpm in test wells (with antigen)/background cpm. Background counts varied between 400 and 1,200 cpm. Data are presented as stimulation index from three replicates; SD < 20%. None of the PLP peptides stimulated the 2 T cell clones specific for KLH (data not shown).

Results

Derivation of T Cell Clones

T cell clones were derived from a single line (SPL) specific for PLP peptide 139–151. Of 672 wells seeded, 8 clones arose and of these, 4 rapidly growing clones (2E5, 4E3, 5B6 and 7A5) were selected for further characterization. Phenotypic analysis of the line SPL and the 4 T cell clones showed that they express Thy-1.2, CD4, CD3 and TcR-α/β but do not express Thy-1.1 and CD8. Representative data are shown for the cell line SPL (fig. 1).

Line SPL and all of the clones showed a strong proliferative response to the whole PLP molecule and to PLP peptide 139–151 but not to PLP peptide 103–116 or other irrelevant antigens, MBP and KLH (table 1). In

Table 2. Clinical and histological EAE induction by T cell clones

Cells[a]	Number with clinical disease	Mean day of onset	Clinical grade[b]	Number of inflammatory foci[c]	Demyelination
SPL	5/7	7.8	4.00	116 (27–196)	+
2E5	3/5	11.0	4.30	ND	ND
4E3	0/5	N/A	0.00	1	–
5B6	4/7	6.5	3.75	129 (1–283)	+
7A5	3/6	6.0	4.00	122 (28–231)	+

N/A = Not applicable; ND = not determined.

[a] Cells were activated with 30 μg/ml of PLP peptide 139–151, and 3–5 × 10^6 live cells were injected intravenously into SJL mice 3 days later. The mice were either treated with 390 rad of irradiation and injected intravenously with 10^9 *B. pertussis* bacilli (for clone 7A5 only) or were left untreated.

[b] Values represent mean clinical score as described under 'Materials and Methods' for animals showing clinical disease.

[c] Mean number of total (meningeal and parenchymal) inflammatory foci in paraffin sections of CNS. Values in parentheses represent ranges.

Table 3. Comparison of the ability of truncated PLP peptides to induce EAE[a]

PLP peptide	Incidence of clinical disease		Day of onset		Clinical score[b]
	n	%	mean	range	
139–151	9/10	90	11.9	10–18	4.8
141–150	10/14	71	13.3	11–15	4.2
141–149	4/9	44	18.8	18–21	3.3
103–116	0/4	0	N/A		0.0

[a] Groups of SJL mice were immunized with 100 μg of PLP peptide in complete Freund's adjuvant and were then injected with pertussis on days 0 and 3. N/A = Not applicable.

[b] Values represent mean clinical scores as described in 'Materials and Methods' for animals showing clinical disease.

addition, the response of the line and clones to 2 truncated PLP peptides (141–150 and 141–149) was evaluated. Both truncated peptides induced similar proliferative responses in vitro, and only the data on PLP peptide 141–150 are presented. Line SPL and clones 4E3 and 7A5 reacted to the truncated peptide 141–150, whereas clones 2E5 and 5B6 failed to show significant proliferative responses. The line and clones thus showed 2 different reactivity patterns to the truncated peptides, suggesting that more than 1 epitope may be present in PLP peptide 139–151.

Disease Induction by T Cell Line and Clones

To test for encephalitogenic activity, the T cell line and clones were transferred intravenously into SJL mice 3 days after specific activation with PLP peptide 139–151. The SPL line and 3 of the T cell clones 2E5, 5B6 and 7A5, induced clinical disease in SJL mice (table 2). The mean day of onset of the disease for the different T cell clones varied between 6 and 11 days. the affected animals showed weight loss, followed by tail weakness and ascending paralysis, leading to complete hindleg paralysis. Although mice injected with clone 7A5 displayed typical signs of EAE, animals injected with clone 4E3 which has a similar fine specificity pattern (table 1) failed to induce disease when observed up to 60–70 days (table 2). The clone 7A5 induced disease only in animals that were pretreated with irradiation (390 rad) and pertussis, whereas the other cell lines (SPL, 2E5 and 5B6) did not require this type of manipulation to induce disease.

Histopathological examination of the brain and spinal cord of representative mice from each group revealed correlation of extent of inflammation with the clinical disease. EAE induced by peptide-specific T cell clones showed essentially the same pathology as observed in active disease. Inflammatory foci composed mainly of lymphocytes and macrophages were observed in meninges and parenchyma of both brain and spinal cord in the majority of mice (fig. 2a). One mouse injected with clone 5B6 with irradiation/pertussis had an acute onset of disease and showed extensive infiltration of polymorphonuclear leukocytes (fig. 2b). There was mild perivascular demyelination in the majority of clone-injected mice but more extensive plaque-like demyelination was found in SPL-line-injected mice (fig. 2c).

Disease Induction by Encephalitogenic and Truncated Peptides

To determine the encephalitogenic potential of the peptides used in this study, groups of SJL mice were injected subcutaneously with 100 μg of 4 different synthetic PLP peptides in complete Freund's adjuvant with pertussis (table 3). Peptide 139–151 induced severe clinical disease in 9/10 animals tested. The truncated pep-

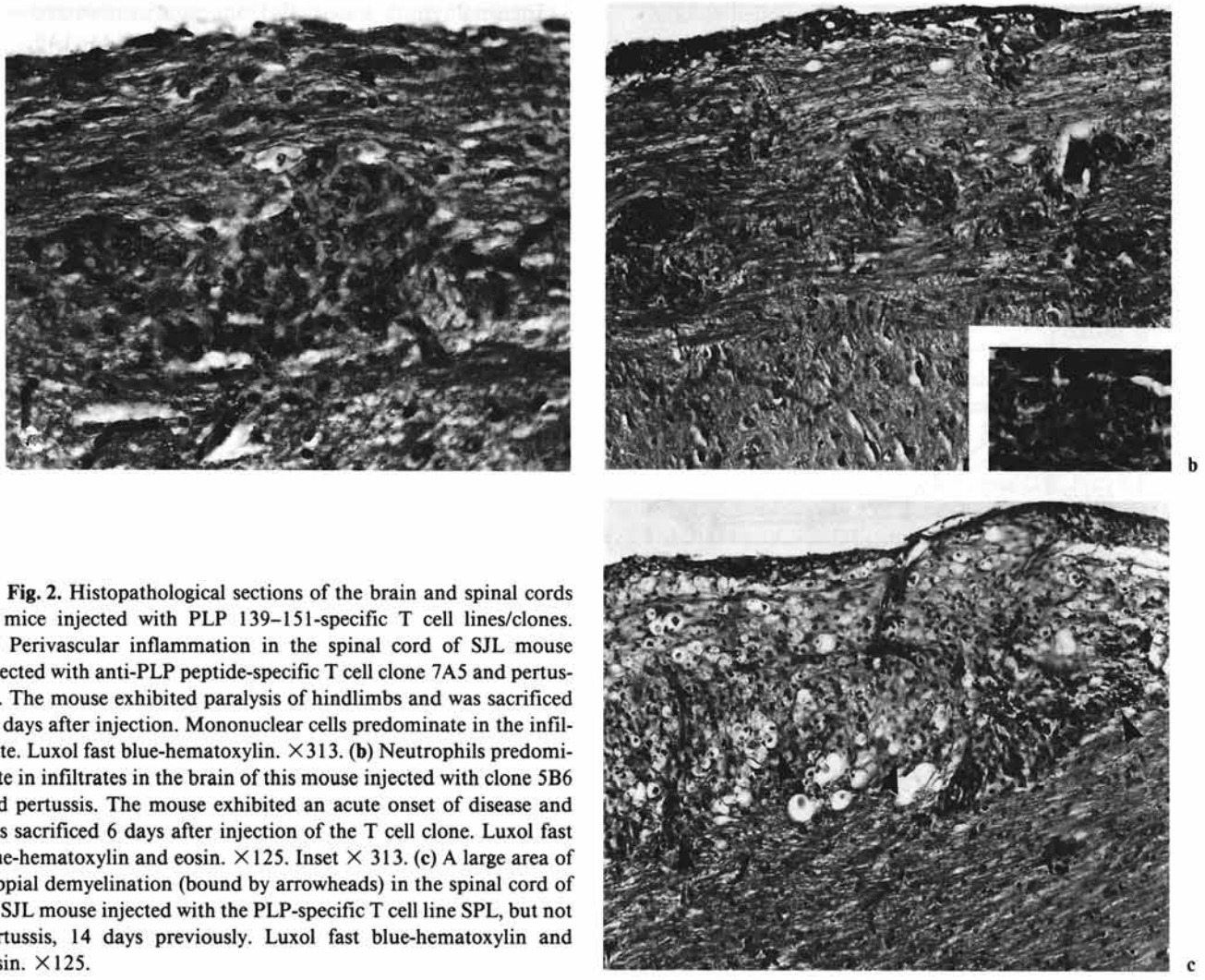

Fig. 2. Histopathological sections of the brain and spinal cords of mice injected with PLP 139–151-specific T cell lines/clones. **(a)** Perivascular inflammation in the spinal cord of SJL mouse injected with anti-PLP peptide-specific T cell clone 7A5 and pertussis. The mouse exhibited paralysis of hindlimbs and was sacrificed 14 days after injection. Mononuclear cells predominate in the infiltrate. Luxol fast blue-hematoxylin. ×313. **(b)** Neutrophils predominate in infiltrates in the brain of this mouse injected with clone 5B6 and pertussis. The mouse exhibited an acute onset of disease and was sacrificed 6 days after injection of the T cell clone. Luxol fast blue-hematoxylin and eosin. ×125. Inset × 313. **(c)** A large area of subpial demyelination (bound by arrowheads) in the spinal cord of an SJL mouse injected with the PLP-specific T cell line SPL, but not pertussis, 14 days previously. Luxol fast blue-hematoxylin and eosin. ×125.

tide 141–150 also induced clinical disease in 10 out of 14 mice tested. In one experiment, following immunization with this peptide, 4 out of the 5 mice developed clinical disease by day 11–13 and 2 of these mice died by day 14. However, 2 of the 4 mice that survived the initial acute attack were observed for 135 days and during that time these mice showed two severe episodes consistent with relapsing disease. Thus, it appears that a truncated encephalitogenic peptide of PLP is capable of inducing a relapsing disease in SJL mice that survived the initial acute attack. PLP peptide 141–149 also induced clinical disease in 4 out of 9 mice tested, and the disease induced was of milder nature. Mice were observed for more than 6 months but the sharp relapses and remissions that were observed with peptide 141–150 were not observed with this peptide. SJL mice immunized with control PLP peptide 103–116 did not show any clinical signs. The data suggest that (1) truncation of the encephalitogenic PLP peptide 139–151 may influence the incidence, onset, severity and clinical course of the disease (table 3), and (2) at least one of the encephalitogenic determinants for SJL mice lies within peptide 141–149.

Proliferative Response to Encephalitogenic and Truncated Peptides

Inguinal lymph node cells were obtained from groups of 2 SJL mice, 8 days following immunization with each of the following PLP peptides: PLP 139–151, 141–150, 141–149 and 103–116. The proliferative response of the lymph node cells to each of the peptides was ascertained.

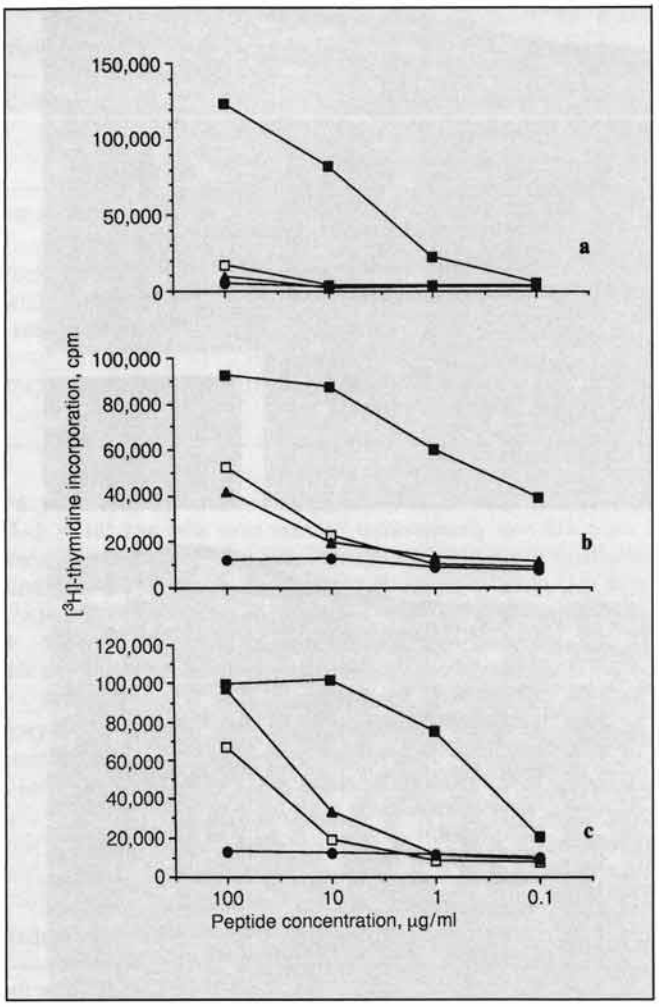

Fig. 3. In vitro proliferative response of lymph node cells to encephalitogenic and truncated peptides. Groups of 2 mice were immunized with the synthetic PLP peptide 139–151 (HSLGKWLGHPDKF) (**a**), 141–150 (LGKWLGHPDK) (**b**) or 141–149 (LGKWLGHPDK) (**c**) in complete Freund's adjuvant. Pooled lymph node cells from these mice were obtained 8 days later and then tested for their ability to proliferate to various peptides described above. Lymph node cells (3×10^5) were incubated for 72 h with various concentrations of the peptides [139–151 (■), 141–150 (□), 141–149 (▲) and 103–116 (●)], and 1 µCi of [^{3}H]-thymidine was added during the last 16–18 h of incubation. The data represent the mean counts per minute incorporated into insoluble DNA of triplicate wells. All standard deviations were < 20%.

Lymph node cells from mice immunized with PLP peptide 139–151 showed a strong response to the immunizing 139–151 peptide (>45 times above background), but little or no response to the truncated peptides (141–150 and 141–149) or to the control peptide 103–116 (fig. 3a).

Inguinal lymph node cells from mice immunized with the truncated PLP peptides 141–150 or 141–149 PLP peptides responded to these peptides with an in vitro proliferative response of 5–6 times above background. However, in both cases, the proliferative response to peptide 139–151 was heteroclitic, with 3- to 8-fold greater proliferative responses at the 1- to 10-µg/ml doses than against the immunizing peptide (fig. 3b, c). Cells from mice immunized with the truncated peptides 141–150 or 141–149 showed no cross-reactivity to the control PLP peptide 103–116, thus establishing the specificity of the response. SJL mice immunized with the control PLP peptide 103–116 did not show a significant response to this peptide or to the encephalitogenic SJL peptides described above (data not shown).

Discussion

This report describes for the first time the generation and characterization of multiple PLP-peptide-specific encephalitogenic T cell clones. Thus far, only a single previous report of a PLP-specific T cell clone has appeared [11]. That clone was generated from SJL mice immunized with whole PLP and the clone responded to the PLP molecule and PLP peptide 139–151 but other peptides were not tested. The phenotype and disease induction were similar to the clones we have obtained. Our data suggest that T cell clones of multiple specificities can be obtained from SJL mice immunized with the encephalitogenic PLP peptide 139–151. Some but not all of the clones were encephalitogenic. Thus, we now have a unique opportunity to study these T cell clones in depth and relate their characteristics with their encephalitogenic potential. It is noteworthy that the PLP-specific T cell line and some clones can transfer disease without secondary manipulations, such as irradiation or administration of pertussis, which seem to be required for induction of EAE by the MBP-specific T cell clones [2, 3]. This raises the possibility that, in SJL mice, the PLP-specific autoimmune response may differ from that of MBP.

PLP residues 139–151 induced characteristic EAE in 90% of the SJL mice tested in this study. We now show that truncated PLP peptides 141–150 (LGKWLGHPDK) and 141–149 (LGKWLGHPD) can also induce EAE in the immunized mice. Two mice that survived the initial attack after immunization with peptide 141–150 showed distinct relapses and remissions. If this observation can be replicated in a larger series of animals, this peptide

may provide a useful model system to study the nature of various immune components which regulate the cyclic course of relapses characteristic of MS.

Lymph node cells from mice immunized with PLP peptide 139–151 proliferated to the immunizing peptide but showed little or no response to the truncated peptides (fig. 3), suggesting a unique dominant epitope in the PLP sequence 139–151. This distinct pattern of T cell responsiveness was also reflected in the fine specificity patterns of encephalitogenic clones 2E5 and 5B6 (tables 1, 2). In contrast, mice immunized with the truncated peptide showed a significant reponse to the truncated peptide. However, the response to PLP sequence 139–151 was even stronger, i.e. it resulted in a heteroclitic response. This response may represent more efficient binding of the larger peptide to the antigen-presenting cells or a higher affinity of the T cell receptor toward this peptide-MHC complex. Two of the 4 T cell clones (7A5 and 4E3) prepared against peptide 139–151 (table 1) also displayed a similar reactivity pattern. In addition, 1 of the latter clones (7A5) was capable of inducing EAE, suggesting that at least two epitopes within peptide 139–151 may be responsible for inducing disease. Whether this is due to the presence of overlapping or multiple discrete epitopes within PLP peptide 139–151 is not known. An alternate interpretation would be that the same peptide is binding to MHC class II molecules in different ways and thus generating multiple functional epitopes [26]. A more extensive series of truncated and/or substituted peptides would by required to address this issue.

It has previously been suggested that PLP-specific antibodies may have a role in the response to PLP-induced EAE since (1) anti-PLP antibody levels peak just prior to onset of clinical disease [27] and (2) depletion of B cells from the PLP-primed lymph node populations reduces disease severity and incidence [10]. The data presented here clearly show that peptide-specific T cell clones alone are capable of rapidly inducing EAE in as little as 6 days (table 2), a time period too short for antibody development. Although anti-PLP antibodies or B cells may have a role in actively induced disease [10, 27], the results demonstrate that T cell clones alone can effectively induce acute EAE.

In summary, the demonstration that EAE can be induced with a nonapeptide of PLP (141–149) and the availability of several distinct PLP-specific T cell clones should permit a broader analyses of factors related to encephalitogenicity. These parameters are relevant to understanding the pathophysiology of MS.

Acknowledgments

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