Induction and Suppression of an Autoimmune Disease by Oligomerized T Cell Epitopes: Enhanced In Vivo Potency of Encephalitogenic Peptides

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Abstract

T cell epitope peptides derived from proteolipid protein (PLP139–151) or myelin basic protein (MBP86–100) induce experimental autoimmune encephalomyelitis (EAE) in "susceptible" strains of mice (e.g., SJL/J). In this study, we show that the encephalitogenic effect of these epitopes when injected subcutaneously in complete Freund's adjuvant was significantly enhanced if administered to the animal in a multimerized form as a T cell epitope oligomer (i.e., as multiple repeats of the peptide epitope, such as 16-mers). Oligomer-treated SJL/J mice developed EAE faster and showed a more severe progression of the disease than animals treated with peptide alone. In addition, haplotype-matched B10.S mice, "resistant" to EAE induction by peptide, on injection of 16-mers developed a severe form of EAE. Even more striking, however, was the dramatic suppression of incidence and severity of the disease, seen after single intravenous injections of only 50 μg of the PLP139–151 16-mer, administered to SJL/J mice 7 d after the induction of the disease. Although relapse occurred at about day 45, an additional injection several days before that maintained the suppression. Importantly, the specific suppressive effect of oligomer treatment was also evident if EAE was induced with spinal cord homogenate instead of the single peptide antigen. By contrast, the PLP139–151 peptide accelerated rather than retarded the progression of disease.

Key words: apoptosis • anergy • high zone tolerance • experimental autoimmune encephalomyelitis • multimer

Introduction

T cell epitope oligomers are linear polypeptide chains, which consist of multiple copies of a T cell epitope. They contain up to 32 repeats of the epitope connected to each other by a flexible spacer sequence (sp)[1] of ~13 amino acids. Previously, oligomers (16-mers) of an epitope derived from the influenza virus hemagglutinin protein (HA306–318) have been shown to induce an exceptionally strong CD4+ T cell proliferative response in vitro (at almost 1,000-fold lower concentration than the peptide) (1). To evaluate whether oligomers can also act as stronger immunogens in vivo, experimental animal models were needed.

Experimental autoimmune encephalitis (EAE) is the best studied experimental animal model of a T cell–mediated autoimmune disease. It serves as the animal model of human multiple sclerosis (2) and can be initiated by a subcutaneous injection of encephalitogenic peptides. In most cases, these peptides are "self-antigens" deriving from myelin proteins (3), such as proteolipid protein (PLP [4, 5]), myelin basic protein (MBP [6, 7]), or myelin oligodendrocyte protein (MOG [8]). These peptides, when associated to MHC class II molecules, are the target structure for autoreactive CD4+ T cells.

The extent of the pathological changes after the induction of the disease can be evaluated by scoring the clinical symptoms typical for EAE. This clinical score reflects the "encephalitogenicity" of the antigen and can be used to estimate its in vivo potency. However, not all mouse strains respond equally to a given encephalitogenic peptide antigen. Incidence rate and severity of the disease are strongly affected by the genotype of the recipient mouse. Primarily, the H2 haplotype dictates whether the strain is "permissive" or "nonpermissive" for disease induction by the particular antigen, but in addition other genetic factors play a role ultimately determining whether a permissive strain is actually also "susceptible" (9). The secondary mechanisms underlying "susceptibility" and "resistance" are not known at the present time, but they appear to affect the overall sensitivity for the induction of an immune response. As a consequence, the induction of EAE in resistant strains, such as B10.S, requires the coinjection of proinflammatory cytokines such as IL-12 (10) or might require exceptionally strong immunogens.

The use of the EAE system for the evaluation of the in vivo potency also allows testing the potential of an antigen to suppress the immune response. In addition to therapeutic approaches using broadly immunosuppressing mechanisms such as the administration of antiinflammatory cytokines (11) or the neutralization of proinflammatory cytokines (12), other more targeted approaches were introduced to "silence" specifically the autoreactive T cells. Most of these approaches aim at the induction of "high zone tolerance" (13), i.e., the induction of anergy (14) or the apoptotic elimination (15) of T cells by overstimulation after exposure to high concentrations of antigen. To achieve tolerance, the antigens have to be administered either by oral uptake (16–18), by intranasal deposition (19), or by intravenous or subcutaneous injection (14, 15, 20, 21). Using these routes of administration, several antigens have already been used successfully in the EAE system to induce tolerance. The list includes peptides (14, 15, 20) or peptide derivates modified by acylation (22) or amino acid substitution (23), but also proteins (21, 24) and protein–protein (25) and immunoglobulin–protein chimeras (26). However, these experiments were usually carried out in transgenic animals, which express only the TCR specific for the peptide antigen (15, 20). Few reports exist in which "normal" nontransgenic mice were treated successfully with encephalitogenic self-peptides after the disease was already induced (27). In these cases, however, a relatively high internal level of the antigen had to be maintained by the frequent administration of high doses of the peptide.

In this study, we tested the efficiency of multimerized forms of the encephalitogenic T cell epitopes PLP139–151 (4) and MBP86–100 (7) on both the induction and suppression of EAE. The disease induction was tested with several mouse strains, including resistant B10.S mice in which the peptides were known to have no encephalitogenic effect. The effect on the suppression of the autoimmune disease was tested with normal SJL/J mice, not biased by the transgenic expression of a particular TCR.

Materials and Methods

Antigens. For stability reasons, the residue Cys140 in the PLP139–151 epitope was substituted by Ser (a substitution that has no or little effect on the encephalitogenicty of the epitope [4]). The synthetic peptides PLP139–151(C140S) (HSLGKWLGHPDKF), PLP139–151 (HCLGKWLGHPDKF), sp-PLP139–151 (C140S)-sp (GGGPGGHSLGKWLGHPDKFGGPGGG), PLP178–191(C183S) (NTWTTSQSIAFPSK), MBP86–100 (NPVVHFFKNIVTPRT), and MBP86–101 (NPVVHFFKNIVTPRTP) were produced by using standard solid phase F-moc chemistry and purchased from the Biopolymers Laboratory at the Harvard Medical School. All peptides were purified on a C_4-HPLC column (Vydac).

Oligomerized T cell epitopes were produced in *Escherichia coli* bacteria using recombinant techniques as described previously (1). In brief, double-stranded oligonucleotide units encoding the T cell epitopes of the PLP139–151(C140S) oligomers and MBP86–100 oligomers were generated by annealing two complementary strands of synthetic oligonucleotides (PLP139–151 (C140S), + strand: 5'-TCACTCTCTGGGTAAATGGCTGGG-TCACCCGGATAAATTCGG, and − strand: 5'-GAATTT-ATCCGGGTGACCCAGCCATTTACCCAGAGAGTGACC; MBP86–100, + strand: 5'-CAACCCGGTTGTTCACTTCT-TCAAAAACATCGTAACTCCGCGTACTGG, and − strand: 5'-AGTACGCGGAGTTACGATGTTTTTGAAGAAGTGAA-CAAGCGGGTTGCC). They were linked to the NH_2-terminal side of a spacer sequence (amino acid sequence of the S3 spacer: GGPGGGPGGGPGG) by cloning the oligonucleotides into the BsrDI site of a modified pCITE vector (Novagen), which contained the DNA encoding the S3 spacer (the construction of full-length oligomeric DNA is described in reference 1). The same spacer was also used in MBP86–101 oligomers, which were provided by Dr. Shan Chung (Peptimmune, Inc., Cambridge, MA). The proteins were produced in TOP10 (pLysS) bacteria (Invitrogen) by using a pET22b expression vector (Novagen). The purification of the expressed protein was carried out using Ni^{2+}-NTA-agarose (Qiagen) by using a His tag located at the COOH terminus of the constructs. Endotoxin and other impurities were removed from the polypeptide oligomers by separation on a reversed-phase C_4-HPLC column (Vydac).

Spinal cords from mice were isolated, homogenized, and lyophilized as described elsewhere (28).

T Cell Lines and Clones. The T cell lines SP/2, SP/3, PLP/a, and PLP/c were generated and maintained as described previously (29). In brief, SJL mice were immunized subcutaneously with PLP139–151(C140S) peptide emulsified in CFA. Lymph node cells (LNCs) were isolated on day 8–12 after immunization and initially stimulated with 50 μg/ml PLP139–151(C140S) in the presence of 0.8–1.0% autologous mouse serum. The cultures were subsequently restimulated after 6 d and then every 2–3 wk with 10 μg/ml PLP139–151(C140S) and irradiated syngeneic splenocytes (1,200 rad). They were expanded in DMEM (supplemented with 20 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM Hepes buffer, 10% FCS [Sigma Chemical Co.]) plus 5–10% of a supernatant derived from concanavalin A–activated mouse splenocytes. The T cell line SP/178 was generated in the same way except that PLP178–191 was used instead of PLP139–151(C140S). The T cell clones SP2.2A8 and 8A1 were obtained by limiting dilution of T cell lines specific for the PLP139–151 antigen at a density of 0.5 cells/well and maintained as described for the T cell line. T cell hybridomas hPLP/1, hPLP/a9.4, and hPLP/c4 were generated by a polyethylene glycol fusion of the T cell lines PLP/a and PLP/c, respec-

tively, with TCR-α/β^- BW1100 thymoma cells, selected in the presence of histone acetyltransferase (HAT) and cloned by limiting dilution as described previously (29).

Mice. SJL/J, PL/J, SWR, B10.S, B10, BALB/c, A.SW, and AKR/J mice (6–8 wk of age) were purchased from The Jackson Laboratory and housed in the animal facility at Harvard University or at Harvard Medical School. They were maintained in accordance with the Guidelines of the Committees on Animals of Harvard University, and the Committee on Care and Use of Laboratory Animal Resources, National Research Council (Department of Health and Human Services Publication 85–23, revised 1987).

In Vitro Assays. T cell proliferation assays were performed in 96-well round-bottomed plates using 5×10^5 irradiated (1,200 rad) splenocytes and 5×10^4 T cells per well in DMEM/10% FCS. After 48 h, 5 μCi [^{3}H]thymidine was added per well and the assay was harvested after 72 h and counted in a microbeta-plate reader (Wallac). The in vitro proliferation assays of primary LNC cultures were performed in the same way except that 5×10^5 nonirradiated LNCs were plated per well, [^{3}H]thymidine was added after 72 h, and the assay was harvested after 96 h. The response of T cell hybridomas (5×10^5 cells/well) was tested with 5×10^5 splenocytes as target cells by collecting 30 μl of supernatant after 24 h for the determination of IL-2 release in a proliferation assay with CTLL.

Induction of EAE. Mice were injected in the base of the tail and the nape of the neck with indicated amounts of peptide, T cell epitope oligomer, or spinal cord homogenate together with 400 μg *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) in an emulsion consisting of equal parts of PBS and CFA (Sigma Chemical Co.). Each mouse was also injected intravenously with 200 ng of *Pertussis* toxin (List Biological Laboratories) on day 1 or on days 1 and 3 after immunization. Mice were observed once or twice a day for clinical signs of EAE and scored on a scale of 1–5 according to the severity of the clinical signs as described previously (28).

Suppression of EAE by Intravenous Injections. EAE was induced in SJL/J mice by the subcutaneous application of peptide antigens or homogenized mouse spinal cord emulsified in CFA followed by the injection of *Pertussis* toxin as described above. For the intravenous administration, the antigens were dissolved in PBS at a concentration of 0.5–1.0 mg/ml and injected into the tail vein at the dosage and time (day) indicated in the text.

Cytokine Detection. Samples of the supernatant from primary LNC cultures described above were taken after 96 h. The amount of released IL-4 and IFN-γ was determined in a sandwich ELISA using pairs of specific capture and biotinylated detection antibodies (PharMingen) and streptavidin–horseradish peroxidase (Sigma Chemical Co.). The ELISA was developed with TMB (Kirkegaard & Perry Laboratories) and measured at 450 nm using an MRX ELISA reader (Dynatech). IL-2, released by T cell hybridomas, was determined by collecting 30-μl samples of the supernatant 24 h after commencement of the T cell assay by testing the samples in a secondary proliferation assay with IL-2–dependent CTLL cells. Recombinant mIL-2 (Genzyme) was used as reference.

Immunohistochemistry. For assessment of inflammation, the brain and spinal cord were fixed in formalin, embedded in paraffin, and 7-μm sections were stained with hematoxylin and eosin according to standard pathological procedures. For assessment of demyelination, animals were perfused under anesthesia through the ascending aorta with 40 ml of Trump's fixative (4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4).

Slices of the brain and spinal cord were postfixed in cold 1% osmium tetroxide for 1 h, dehydrated through a graded series of ethyl alcohol, and embedded in epoxy resin. 1-μm sections were stained with toluidine blue and examined by light microscopy in a blinded fashion.

Results

Proliferative Response of PLP139–151-specific T Cells In Vitro. In a series of proliferation assays, the biological activity of a PLP139–151(C140S) 16-mer was tested with several different T cell lines and clones (generated as described in Materials and Methods; Fig. 1 A). Compared with the peptide, all T cell lines specific for the PLP139–151 epitope responded better (lines SP/2, SP/3), or at least equally well, to the stimulation with the oligomer (lines PLP/a, PLP/c). No proliferation was induced by the PLP139–151(C140S) 16-mer in the control line, SP/178, which is specific for PLP178–191. As had been established previously for another epitope (HA306–318; reference 1), the gain in sensitivity detected for some of these lines was significant. The T cell line SP2, for example, required ~100-fold lower concentrations of the 16-mer to induce a proliferation equivalent to that of the peptide. A more detailed picture of the proliferative response to the PLP139–151(C140S) 16-mer and peptide was obtained by analyzing single T cell clones. In particular, the response of low-avidity T cell clones improved to the 16-mer. As shown, some of these clones recognized 100-fold less 16-mer than peptide similar to the SP/2 line (SP2/2A8, hPLP/1), and for some clones (e.g., hPLP/a9.4) the specificity for 16-mer appeared even almost absolute. In contrast, other T cell clones (e.g., hPLP/c4) failed to efficiently recognize the 16-mer. For these clones, however, the failure to respond to the oligomer was found to be due to the extension of the actual T cell epitope by the spacer sequence, which presumably caused steric hindrance of the TCR–MHC–antigen interaction (data not shown).

In the HA306–318 system, it has been shown that both the spacer length and the length of the oligomer (i.e., number of repetitive epitope units) have a profound effect on the outcome of the T cell response. Testing the SP/2 line with a set of PLP oligomers revealed that also in the PLP system the oligomer length had a similar effect (Fig. 1 B, left). The strongest response was triggered with a 16-mer, whereas the 4-mer, the shortest oligomer tested in this experiment, required 5–10 times higher concentrations. Similar results were also obtained with the T cell clone 8A1 (Fig. 1 B, right). To determine the influence of the spacer on the T cell recognition, this clone was also tested with a synthetic peptide, in which the actual core epitope was extended by parts of the spacer (sp-peptide-sp, see Materials and Methods; Fig.1 B, right). Although the multimerization of the epitope resulted in a significant increase in antigenicity (>1 log for the 4-mer and >2 logs for the 16-mer), the extension of the epitope by the spacer sequence caused only a slight improvement in the proliferative response. The influence of oligomer length was not evident
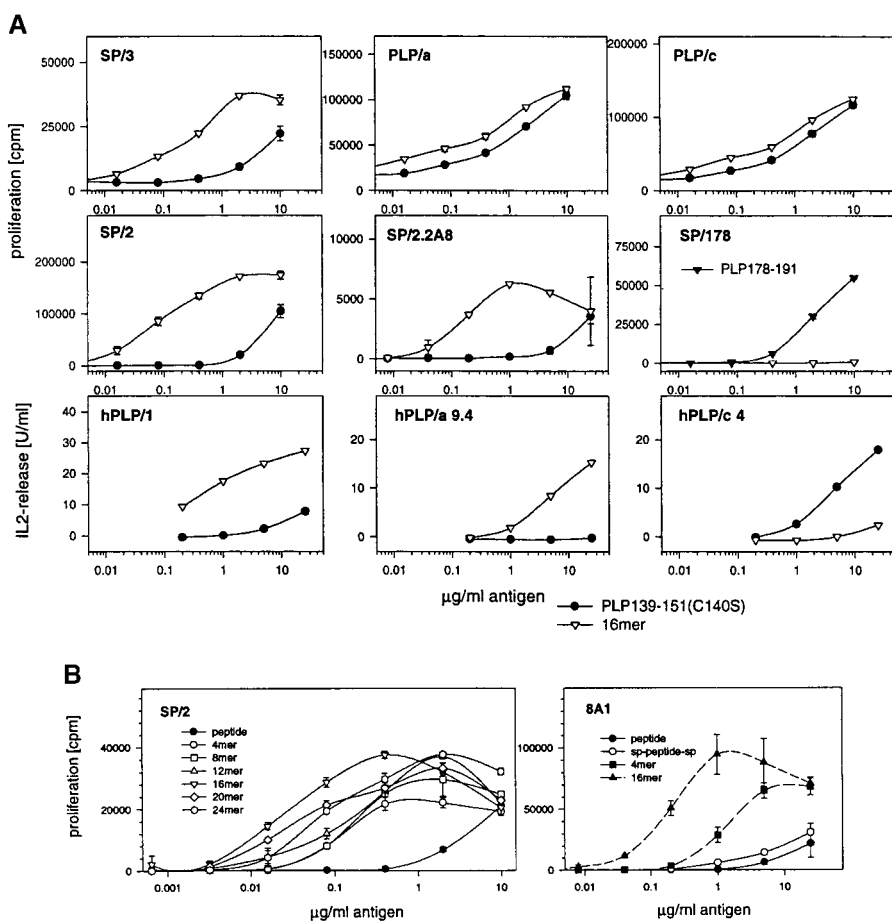
Figure 1. In vitro experiments with PLP139–151-specific T cells. (A) T cells raised against the PLP139–151(C140S) peptide were challenged with titrated amounts of the PLP139–151(C140S) peptide or the PLP139–151(C140S) 16-mer. The specific response of the T cell lines SP/3, PLP/a, PLP/c, and SP/2, and of the T cell clone SP/2.2A8 was measured in a proliferation assay. The response of the T cell hybridomas hPLP/1, hPLP/a9.4, and hPLP/c4 was determined by their IL-2 release. Also shown is the proliferative response of the line SP/178, specific for PLP178–191. SJL/J splenocytes were used as target cells. (B) Dose-response of PLP139–151-specific T cells to oligomers with an increasing number of repetitive epitope units. SP/2 T cells (left) were tested as described above with the PLP139–151(C140S) peptide and a set of PLP139–151(C140S) oligomers. The right panel shows the proliferative response of the clone 8A1. In addition to the the PLP139–151(C140S) peptide, the 4-mer, and the 16-mer, this clone was also tested with a PLP139–151(C140S) peptide containing the NH₂- and COOH-terminal extensions of the spacer sequence (sp-peptide-sp: GGG-PGG-PLP139–151[C140S]-GGPGGG).

in lines that did not show an improved response to the oligomers, and the trend was even reversed in clones that recognized oligomers only weakly (e.g., hPLP/c4, data not shown).

T cells that responded most effectively to the stimulation with the 16-mer often showed a reduced proliferation after exposure to the highest 16-mer concentrations (10 or 50 μg/ml). For instance, a maximal proliferative response for the SP/2 (Fig. 1 B, left) and SP/3 lines (Fig. 1 A, top left) or the clone 8A1 (Fig. 1 B, right) or SP/2.2A8 (Fig. 1 A, middle) was triggered at a concentration of ∼0.5–1.0 μg/ml 16-mer. The effect resembled high zone suppression and was not seen after incubation with the peptide.

Ex Vivo Response of Primary T Cell Cultures. To determine whether the improved antigenicity, observed for some of the T cells in vitro, would translate into an increased immunogenicity, mice were immunized with the PLP139–151(C140S) peptide, with the 16-mer or, as a control, with adjuvant only (Fig. 2). Primary cell cultures, established from the draining LNs of the primed mice, were then challenged in vitro with titrated amounts of the PLP139–151(C140S) peptide or the 16-mer. PLP139–151 is restricted by I-A^s, and mice from three different strains were used: SJL/J, which are permissive (I-A^s) and EAE susceptible; B10.S, which are permissive (I-A^s) but EAE resis-

tant (30), and BALB/c, which, in principle, are EAE susceptible but are not permissive for this particular epitope (I-A^d).

A specific proliferative response was only observed in the primary cultures derived from SJL/J and B10.S mice treated with antigens (Fig. 2 A, panels 2, 3, 5, and 6). No proliferation was detected in cultures established from BALB/c mice (panels 7–9) and in the control cultures derived from mice primed with adjuvant only (panels 1, 4, and 7). The strength of the T cell response was more vigorous in most cases if the primary culture was stimulated with the 16-mer. This was particularly evident in cultures generated from oligomer-primed mice. Here, often <100-fold lower concentrations of 16-mer than of the peptide were effective (panels 3 and 6). The relative enhancement was more variable in the cultures derived from peptide-primed mice, and frequently these cultures responded equally well to the stimulation by peptide or 16-mer (e.g., Fig. 2 A, panel 2). Cultures derived from mice primed with the 16-mer usually responded strongly to the 16-mer, but in most cases only relatively weakly to the stimulation with the monomeric peptide. The weak response against the peptide might be explained by the predominant stimulation by the 16-mer of low-affinity clones (as suggested by the previous in vitro experiments), whereas the monomer is recognized
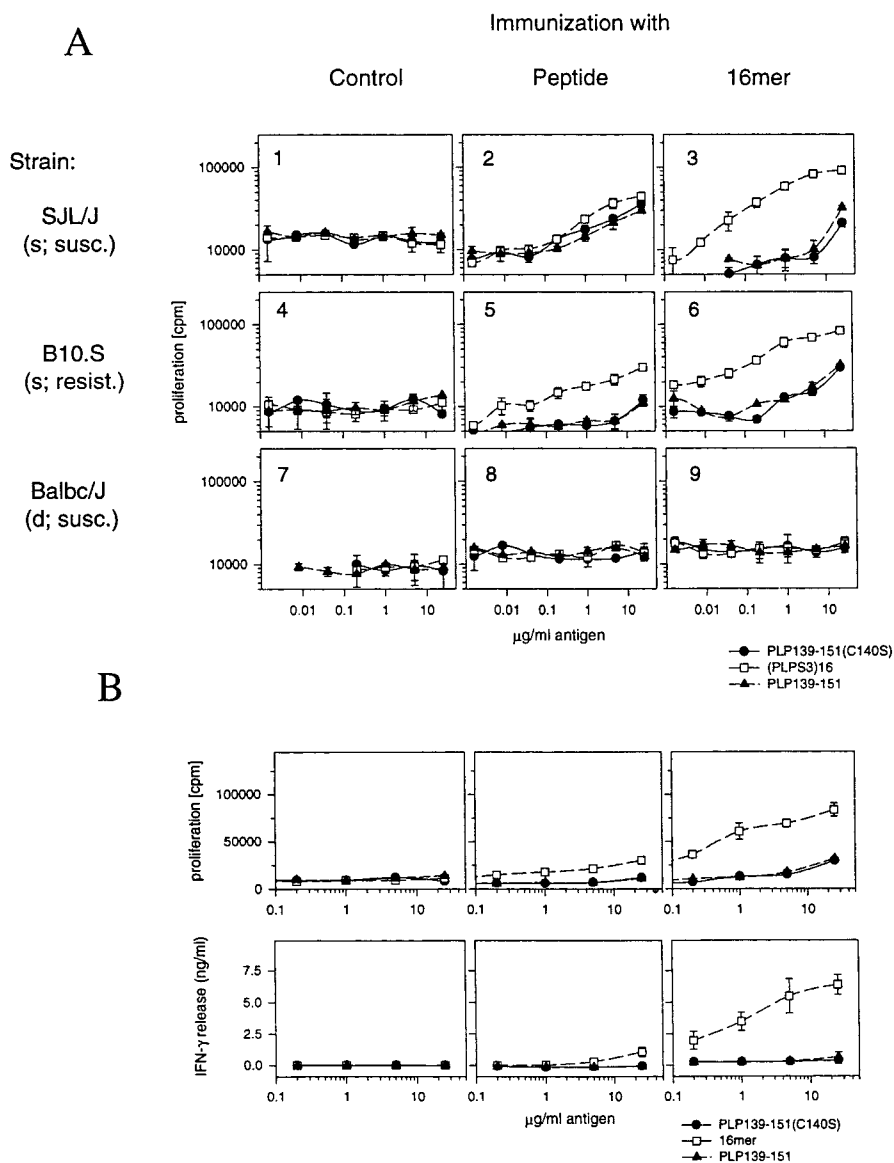
Figure 2. Ex vivo response of primary LN cultures. (A) Proliferative response of primary LN cultures of mice previously immunized with 50 μg of either the PLP139–151(C140S) peptide (panels 2, 5, and 8) or the 16-mer (panels 3, 6, and 9). Primary LN cultures were prepared from SJL/J mice (panels 1–3), B10.S mice (panels 4–6), and BALB/c mice (panels 7–9). The H2 haplotype and the EAE susceptibility (susc.)/resistance (resist.) of the strains are indicated. The LNCs were isolated 7–12 d after the immunization and challenged in vitro by adding titrated amounts of the unsubstituted PLP139–151 peptide, the PLP139–151 (C140S) peptide, or the PLP139–151(C140S) 16-mer. As control, the response of cultures derived from mice treated with adjuvant only is shown (panels 1, 4, and 7). (B) Proliferative response (top) and the cytokine release (bottom) of primary LN cultures derived from B10.S mice. The amount of IFN-γ was determined in supernatants taken 96 h after the start of the experiment.

only by high-affinity clones. In any case, in susceptible SJL mice as well as in resistant B10.S mice, a strong and specific T cell response could be generated by the immunization with the oligomer (panels 3 and 6). The priming with peptide, in particular in B10.S mice (panel 5), usually resulted in weakly responding primary cultures.

No IL-4 was detectable in any of these cultures (not shown). On the other hand, IFN-γ was found in the supernatants of all the primary cell cultures derived from antigen-primed mice, in particular after the challenge with the 16-mer. As an example, the IFN-γ release of the cultures derived from B10.S mice is shown in Fig. 2 B. Although only relatively small amounts (~1 ng/ml) were detected in cultures generated from peptide-primed mice, >6 ng/ml of IFN-γ was measured in the supernatants derived from cultures of 16-mer–primed mice. The relatively high levels of IFN-γ might be indicative of a preferred expansion of Th1 T cells after the immunization with the PLP 16-mer.

In Vivo Effect of PLP139–151(C140S) Oligomers. To determine whether the increased immunogenicity of the oligomer also results in an enhanced encephalitogenicity, a series of in vivo experiments was performed (Fig. 3). After the subcutaneous immunization of SJL/J mice with titrated amounts of either PLP139–151(C140S) peptide or the 16-mer, the mice were observed for the appearance of the typical clinical signs of EAE (Fig. 3 A). The score of the clinical symptoms revealed that SJL/J mice were more sensitive to EAE induction with the oligomer than with peptide. Compared with mice immunized with the peptide, they showed an earlier onset and a much more severe progression of the disease. At a dosage of 50 μg, the peptide-primed mice developed EAE on day 13 and had a maximal clinical score of 3.75 (incidence: 3/4, mortality: 75%). In contrast, the group primed with the 16-mer showed the first signs of the disease almost 4 d earlier (day 9) and reached the maximal clinical score of 5 (i.e., incidence: 4/4;
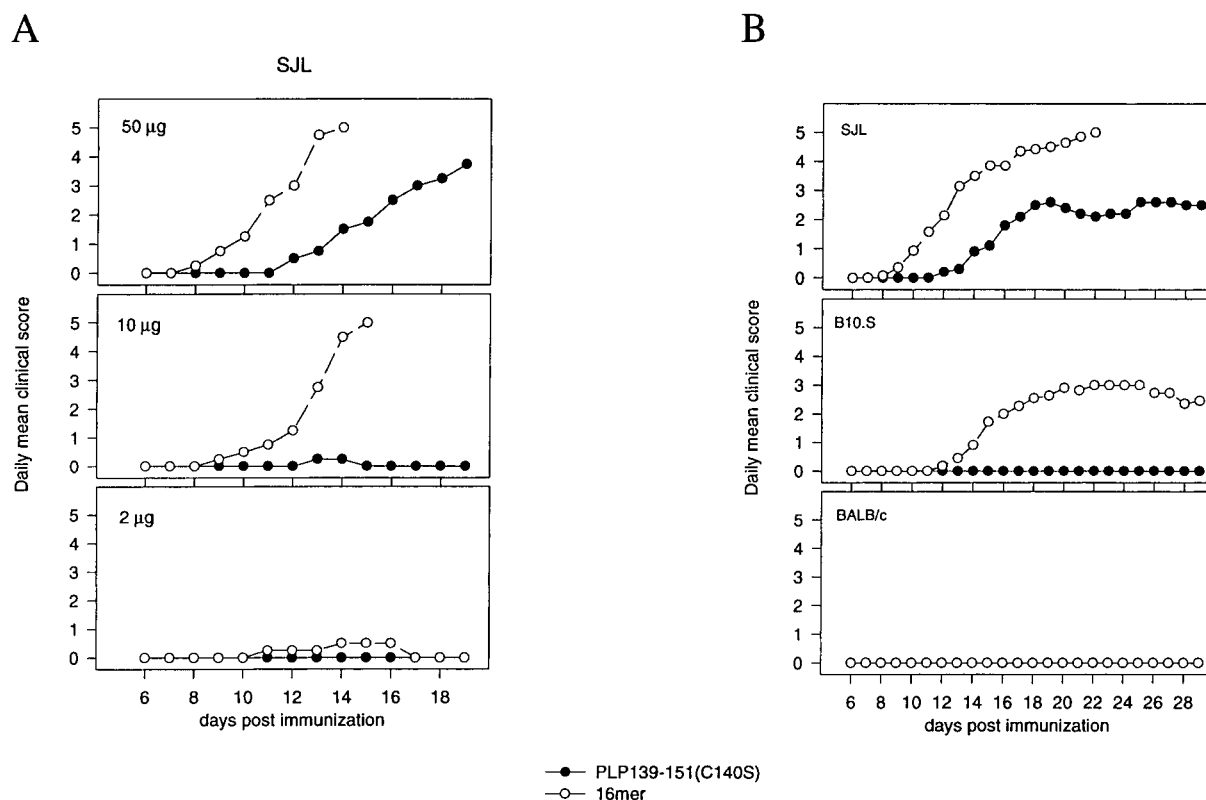
Figure 3. Induction of EAE with PLP139–151(C140S) oligomer. (A) Dose–response curves of peptide and 16-mer in susceptible SJL/J mice. EAE was induced in groups of four mice by subcutaneous injection of either 50 μg (top), 10 μg (middle), or 2 μg (bottom) of the PLP139–151(C140S) peptide or the 16-mer. The progression of the disease was determined on a daily basis by monitoring the mice for the appearance of clinical symptoms. The severity of the EAE is expressed in the mean score of these symptoms. Four mice per group were used. (B) EAE induction in susceptible and resistant strains. The encephalitogenic effect of 16-mer and peptide was tested with SJL/J mice (permissive/susceptible, top), B10.S mice (permissive/resistant, middle), or BALB/c mice (nonpermissive/susceptible, bottom). The mice were immunized subcutaneously with 50 μg of either the PLP139–151(C140S) peptide or the 16-mer. Filled circles of the peptide-treated BALB/c group are covered by the open symbol of the 16-mer–treated group. The clinical scores represent a compilation of several experiments using the following numbers of mice: SJL/J, 10 (peptide) and 14 (16-mer); B10.S, 9 (peptide) and 11 (16-mer); BALB/c, 6 (peptide) and 8 (16-mer).

mortality: 100%). The trend also continued at lower dosages of the antigens. Although at the dosage of 10 μg almost no clinical signs of disease were observed in the group primed with the peptide (incidence: 1/4; mean maximum score: 0.25), 16-mer–primed SJL mice developed a severe disease (incidence: 4/4; onset: day 11.5; mean maximum score: 5; mortality: 100%). Even at the lowest dosage tested (2 μg), a mild form of EAE was still evident in the group treated with the 16-mer (incidence: 2/4; onset: day 13; mean maximum score: 0.5; mortality: 0%). At this dosage, no effect was seen in the peptide group.

In the second part of this experiment, we addressed the question as to whether the oligomer could also induce EAE in resistant B10.S mice (Fig. 3 B). In accordance with previous reports (31), no clinical signs of the disease were observed in the B10.S group primed with the peptide. The 16-mer, in contrast, was also encephalitogenic in this strain (no effect with the peptide or the 16-mer was observed in the BALB/c control group). The EAE was only slightly less severe than in SJL/J mice and, at a dosage of 50 μg, induced the disease with an incidence rate of 100%. Compared with SJL/J mice, in B10.S mice the onset of the dis-

ease was delayed by ∼3 d and resulted in a mean maximum score of 3.0. CNS immunohistochemistry performed on brain and spinal cord samples of the 16-mer–treated B10.S mice (Fig. 4) revealed an extensive submeningeal, perivascular, and parenchymal infiltration (Fig.4, panel 2), as well as demyelination (panel 4). In contrast, only some minor submeningeal infiltration (panel 1) and no demyelination (panel 3) was detectable in the samples from peptide-primed B10.S mice.

To demonstrate that induction of EAE was limited only to permissive strains, mouse strains with various H2 haplotypes were primed with either the peptide or the 16-mer (Table I). In all tested I-A^s–expressing mouse strains, the treatment with the oligomer resulted in the induction of EAE. A.SW, for example, showed symptoms comparable to the ones described for SJL/J. No clinical signs of the disease were observed in mice of the b, d, k, q, and u haplotypes.

In Vivo Effect of MBP86–100 Oligomers.   To extend the study to other encephalitogenic T cell epitopes, oligomers were tested in which the PLP139–151(C140S) epitope was replaced by a T cell epitope derived from the MBP protein
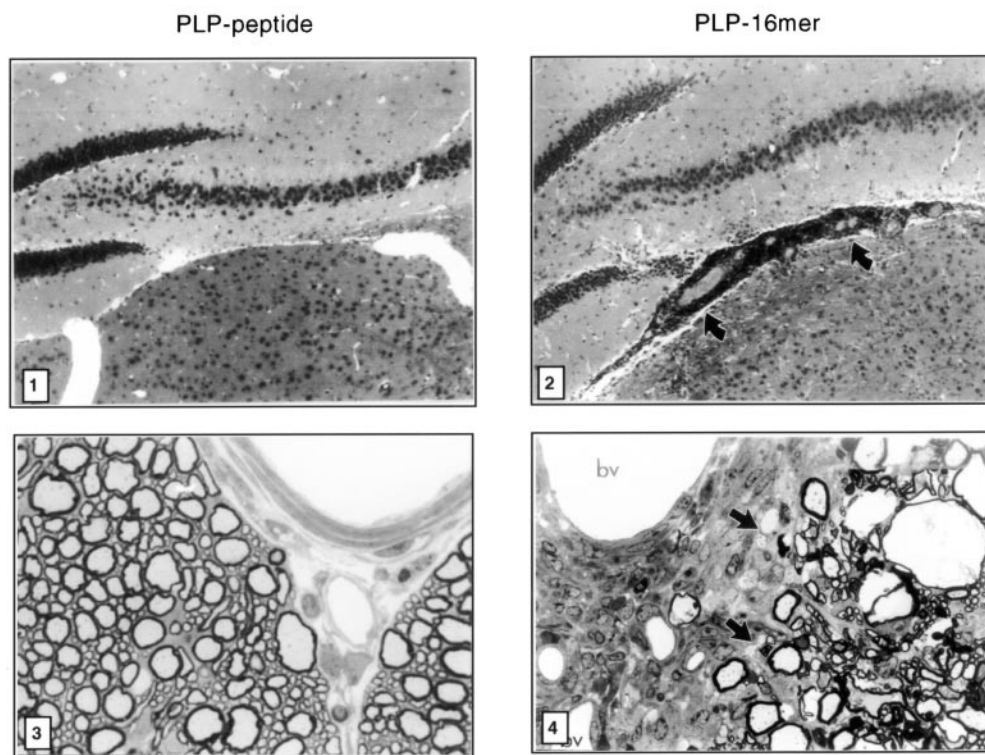
PLP-peptide

PLP-16mer

Figure 4. Pathological analysis of resistant B10.S mice after EAE induction with PLP139–151(C140S) oligomers. B10.S mice were sensitized subcutaneously with 50 μg of either the PLP139–151(C140S) peptide (panels 1 and 3) or the 16-mer (panels 2 and 4). 14 d after disease induction, the animals were perfused with fixative. Paraffin-embedded sections of the brain were stained with hematoxylin and eosin to detect inflammatory infiltrates (panels 1 and 2, original magnifications: ×350), and plastic-embedded sections of the lumbar spinal cord were stained with toluidine blue to detect demyelination (panels 3 and 4, original magnifications: ×750). In animals sensitized with PLP139–151(C140S) peptide, no evidence of inflammation was detected in the brain (panel 1), and no inflammation or demyelination was noted within the spinal chord parenchyma (panel 3). In contrast, in the brains of animals sensitized with the 16-mer, dense accumulations of perivascular cells were observed around vessels overlying the anterior thalamus (arrows, panel 2). In the spinal cord, inflammatory cells were noted around blood vessels (bv), and inflammation, primary demyelination (arrows), and intramyelinic edema were detected within the anterior columns.

(MBP86–100). In vitro experiments indicated that, at least in the I-A^s system, MBP86–100 has a significantly lower antigenicity than PLP139–151. T cells, which show an improved response to the oligomer, seem to be less frequent in SJL mice and the ex vivo experiments revealed less impressive results compared with the PLP139–151 system (data not shown). However, to test if MBP86–100 oligomers showed some enhanced encephalitogenicity, the same series of in vivo experiments was performed as described previously for the PLP oligomers (Fig. 5).

Table I. *Clinical EAE in Various Strains of Mice*

Mice		Incidence of EAE*		Percent disease[‡]		Percent mortality[§]		Maximum mean score[‖]		Mean day of onset[‖]	
Strain	H2 haplotype	Peptide	16-mer	Peptide	16-mer	Peptide	16-mer	Peptide	16-mer	Peptide	16-mer
SJL/J	s	17/20	14/14	85	100	40	100	3.1 ± 2.0	5.0 ± 0.0	14.0 ± 1.5	10.5 ± 1.2
A.SW	s	5/6	6/6	83	100	66	100	3.5 ± 2.3	5.0 ± 0.0	13.8 ± 2.0	9.8 ± 0.7
B10.S	s	0/9	15/15	0	100	0	33	0	3.1 ± 1.5	–	13.3 ± 3.0
BALB/c	d	0/6	0/8	0	0	0	0	0	0	–	–
B10	b	0/4	0/4	0	0	0	0	0	0	–	–
AKR/J	k	0/4	0/4	0	0	0	0	0	0	–	–
SWR	q	0/4	0/4	0	0	0	0	0	0	–	–
PL/J	u	0/4	0/4	0	0	0	0	0	0	–	–

EAE induction with PLP139–151(C140S) oligomers in different strains of mice. Mice from various strains were immunized with 50 μg of either the PLP139–151(C140S) 16-mer or the peptide as described in Materials and Methods.
*Values represent the number of mice with clinical signs of EAE as a fraction of the total number of immunized mice.
[‡]Values represent the percentage of immunized mice with clinical signs of disease.
[§]Values representing the percentage of mortality refer to the total number of immunized mice.
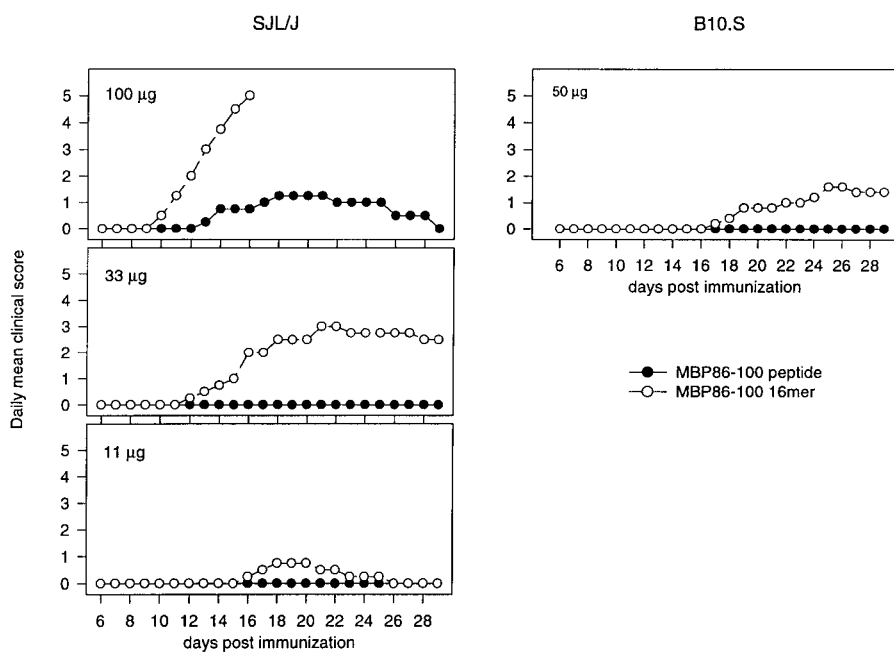[‖]Values representing mean day of onset and maximum mean clinical grade were scored as described in reference 28.

SJL/J

B10.S

● MBP86-100 peptide
○ MBP86-100 16mer

Figure 5. Induction of EAE with MBP86–100 oligomers. Clinical scores of mice treated subcutaneously with MBP86–100 peptide or MBP86–100 16-mer. The experiment was carried out as described in the legend to Fig. 3. The SJL/J and B10.S mice received the amounts of antigens indicated in the figure. Four mice per group were used in these experiments.

The MBP86–100 16-mer was found to be a fairly potent inducer of EAE. Compared with the effect of the peptide, the application of the 16-mer resulted in an earlier onset and more severe progression of the disease. In a dose–response study with SJL/J mice (Fig. 5, left), disease induction with the peptide was only observed after an injection of 100 μg, the highest dosage used in this experiment (incidence: 2/4, onset: 13.5, maximal mean score: 1.7, mortality: 0%). One third of this amount, 33 μg, was insufficient to trigger EAE. In contrast, the effect of the MBP86–100 16-mer was significantly stronger. At a dosage of 100 μg, all of the SJL mice treated with the 16-mer developed a fatal disease (incidence: 4/4, onset: 10.7, maximal mean score: 5.0, mortality: 100%). The encephalitogenic effect decreased at 33 μg (incidence: 3/4, onset: 13.6, maximal

mean score: 2.75, mortality: 50%) but was still evident at a dosage of 11 μg where some cases of mild forms of EAE were observed (incidence: 2/4, onset: day 16.5, maximal mean score: 0.75, mortality: 0%). Importantly, it was also possible to induce EAE in B10.S mice (Fig. 5, right). Compared with the PLP139–151 system, the effect of the MBP86–100 16mer was weaker but 50 μg of the antigen was still sufficient to induce the disease in the majority of the tested animals (incidence: 3/5; onset: day 18; maximal mean score: 1.6; mortality: 20%). The same treatment with the peptide did not cause any effect.

Effect of Intravenous Administration of Oligomers on the Progression of EAE. To examine whether the oligomers could have a potential therapeutic impact in the suppression of EAE, mice were treated by intravenous injections
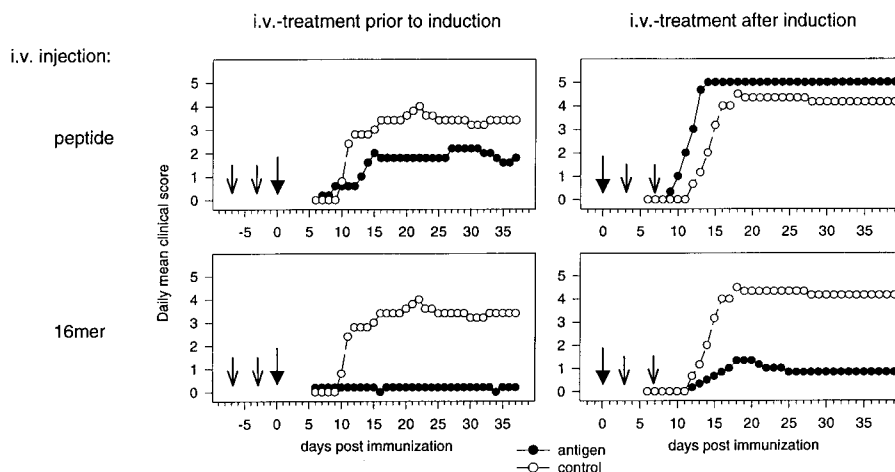

Figure 6. Suppression of EAE by intravenous injections of PLP139–151(C140S) oligomers. SJL/J mice were treated with intravenous injections of either PLP139–151(C140S) peptide (top) or the 16-mer (bottom) several days before (left) or after (right) disease induction. EAE was induced by a subcutaneous injection of 50 μg PLP139–151(C140S) peptide emulsified in CFA (filled arrowhead). The mice received the intravenous injections of peptide or 16-mer on the day indicated by small open arrows. The daily mean clinical scores of the mice treated intravenously with antigens are shown in comparison to the score of control groups, which received mock intravenous injections of PBS instead of antigen.

Groups of five or six mice were used for the experiments. The incidence rates were determined as 5/5 (control), 5/5 (peptide), and 2/5 (16-mer) for the groups treated before the disease induction and as 6/6 (control), 6/6 (peptide), and 4/6 (16-mer) for the groups treated after the disease induction.
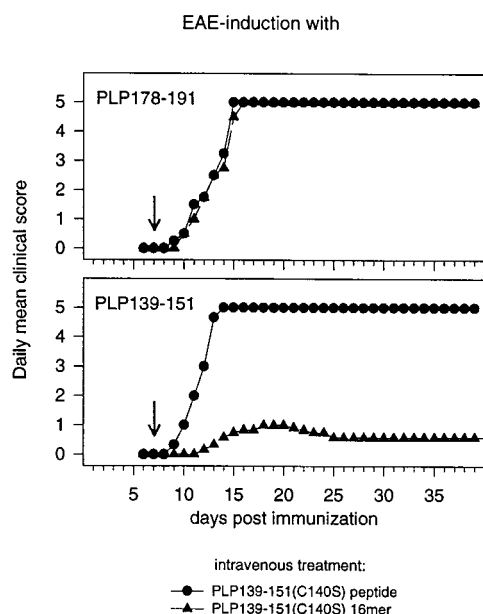
Figure 7. Specificity of EAE suppression by intravenous injections of PLP139–151(C140S) 16-mers. SJL/J mice received intravenous injections of 50 μg PLP139–151(C140S) peptide or PLP139–151(C140S) 16-mer 7 d after the induction of the disease by the subcutaneous injection of 50 μg PLP178–191(C183S) (top) or PLP139–151(C140S) peptide (bottom). Groups of six mice were used for the experiment. The incidence rates were determined as 6/6 (peptide) and 6/6 (16-mer) for the groups immunized with the PLP178–191(C183S) peptide and as 6/6 (peptide) and 4/6 (16-mer) for the groups immunized with the PLP139–151(C140S) peptide.

Figure 8. Suppression of EAE by intravenous injections of oligomers containing the MBP86–100 epitope. SJL/J mice were treated with intravenous injections of MBP86–100 (top) or MBP86–101 (bottom). EAE was induced on day 0 by the subcutaneous injection of 100 μg MBP86–100 or 200 μg MBP86–101 peptide, respectively. The mice were treated intravenously with peptide, 16-mer, or PBS at the indicated time points (arrows) and received dosages of 100 μg of the MBP86–100 or 200 μg of the MBP86–101 antigens. Groups of 5–10 mice were used for the experiment. The incidence rates were determined as 5/6 (control), 6/6 (peptide), and 4/6 (16-mer) for the groups primed and treated with the MBP86–100 epitope and as 5/5 (control), 9/10 (peptide), and 4/10 (16-mer) for the groups primed and treated with the MBP86–101 epitope.

with PBS solutions of the PLP139–151(C140S) antigens (Fig. 6). The experiment was carried out in SJL/J mice in which the disease was induced by the subcutaneous administration of 50 μg PLP139–151(C140S) peptide in CFA. The mice were treated either before (days −7 and −3; Fig. 6, left) or after the disease induction (days 3 and 7; Fig. 6, right). The animals received the intravenous injections of peptide (top) or 16-mer (bottom) at a dosage of 50 μg, and control groups received a mock treatment with PBS.

The intravenous treatment with the 16-mer almost completely prevented the development of the disease (Fig. 6, bottom). With the treatment before the disease induction (bottom left), four of the five mice did not show any clinical signs, and only one mouse developed a very mild form of EAE (score of 1, resulting in a mean score of 0.2). Importantly, the effect of the 16-mer was also evident if the treatment was done several days after the induction of the disease (bottom right). Two injections of 50 μg were sufficient to reduce the maximal mean clinical score to a value of 1.3 compared with a score of 4.5 in the control group. After the initial attack, which peaked at day 19, most of the mice returned within 6 d to a condition virtually free of any clinical symptoms. In contrast to the treatment with the 16-mer, the effect of the peptide was far less dramatic. Although peptide treatment before the disease induction (Fig. 6, top left) resulted at least in some reduction of the severity of the EAE progression (maximal mean score of
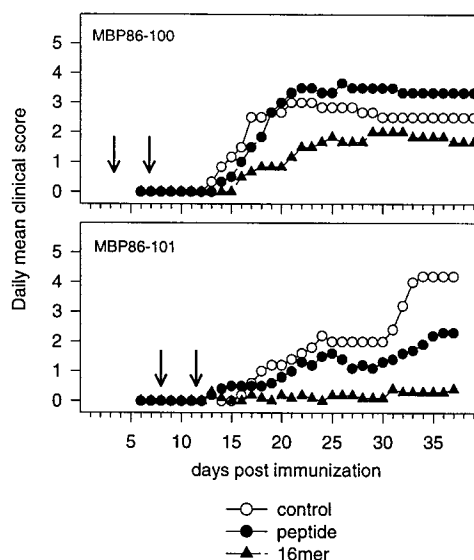
2.2), the treatment after disease induction (top right) resulted in acceleration of disease progression. The onset shifted by almost 2 d, and a maximal mean score of 5 (i.e., 100% mortality) was reached earlier, by day 14.

The effect of peptide treatment on enhancing instead of suppressing the disease progression could not be overcome by an increase in dosage. Even when 250 μg was administered to the animals, no reverse in the trend was apparent (data not shown). On the other hand, with the 16-mer a single injection of 50 μg on day 7 was found to be sufficient to produce a strong suppression (Fig. 7, bottom). At this time point, only very little submeningeal, perivascular infiltration was evident in brain sections taken from SJL/J mice immunized with the peptide (data not shown). However, the effect of the 16-mer was found to be highly specific, and no suppression was evident if the disease was induced with the PLP178–191 peptide (Fig. 7, top) instead of the PLP139–151 peptide (Fig. 7, bottom). Similar results were also obtained with MBP antigens containing the epitopes MBP86–100 and MBP86–101 (Fig. 8). Only a relatively modest suppression was observed after an injection of 100 μg of MBP86–100 16-mer on days 3 and 7 (triangles, top). The effect was greatly enhanced after using a 16-mer containing the more antigenic epitope MBP86–101 at an increased dosage (200 μg) on days 8 and 12. Also in the MBP system, the intravenous treatment with peptides had little or no effect (filled circles).
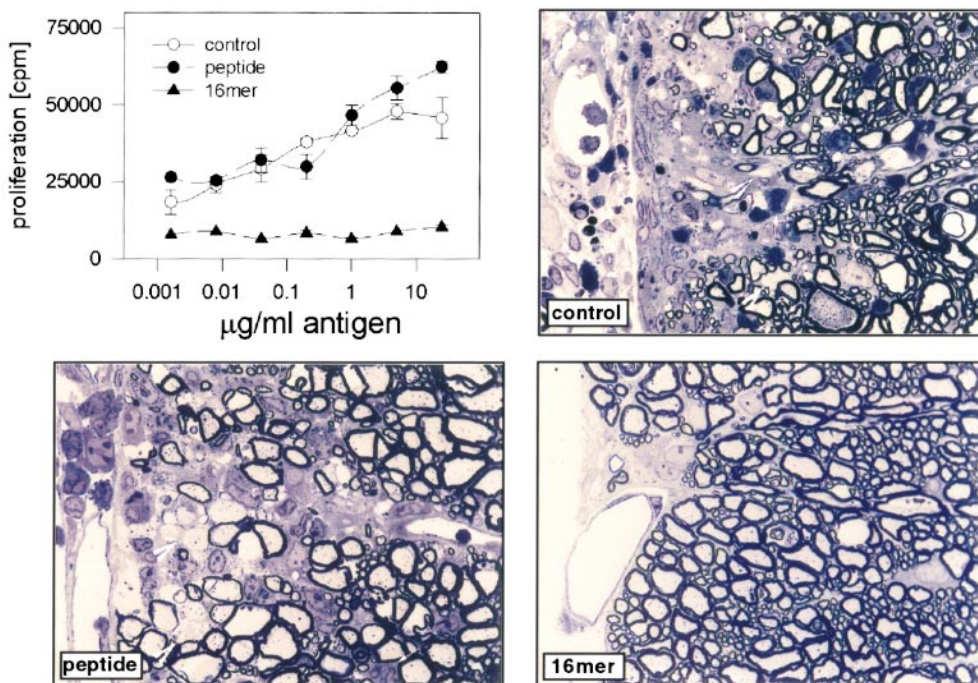
Figure 9. Pathological analysis and lymphocyte proliferative response after intravenous treatment with PLP139–151(C140S) 16-mer. EAE was induced in SJL/J mice with PLP139–151 (C140S) peptide. 7 d later, the mice received intravenous injections of either 50 μg PLP139–151 (C140S) peptide, PLP139–151 (C140S) 16-mer, or mock injections of PBS. 7 d later (day 14), LNs were removed to test the ex vivo response, and the mice were perfused with Trump's fixative before the removal of the spinal cord for the histological analysis of disease. The ex vivo response of primary LNCs of peptide-, 16-mer–, and mock-treated mice is shown in the top left panel. LNCs were challenged in a proliferation assay with titrated amounts of PLP139–151(C140S) 16-mer (top left). The proliferation assay was performed essentially as described in the legend to Fig. 2. The other panels show the histochemical image of 1-μm-thick plastic-embedded tissue stained with toluidine blue. The samples derived from the mock-treated control animal (top right) and from the peptide-treated mouse (bottom left) show submeningeal lesions typical of EAE (meninges located on the left of the panels). The meningeal vessels were inflamed, and numerous inflammatory cells were detected within the cord parenchyma (arrows). In tissues from control animals, large numbers of polymorphonuclear cells were evident (small arrows). Demyelinated axons (arrowheads) were present within the inflamed area of the cord. In contrast, in the sample derived from the 16-mer–treated mouse (bottom right), the meningeal vessels were not inflamed and the cord parenchyma showed no evidence of inflammation or demyelination. Original magnifications: ×500.

Ex vivo experiments with primary LN cultures (LNCs) further indicated that in mice treated with the PLP139–151(C140S) 16-mer, the specific response against this antigen was either reduced, or, as shown in the top left panel of Fig. 9 (triangles), completely absent. In contrast, the antigen-specific response of LNCs from peptide-treated mice (filled circles) was similar to that of the control group (open circles). This silencing of the T cell response, presumably due to apoptosis or anergy induction, was found to be antigen specific, since the T cell proliferation triggered by staphylococcal enterotoxin A or B was unaffected (not shown). The histological analysis of the tissues taken from the mice revealed interesting differences in the pathology between the three groups. In the mock-treated animals (Fig. 9, top right), the lesions showed prominent demyelination (arrowheads) in the presence of an inflammatory infiltrate that contained numerous polymorphonuclear cells (small arrows). In the peptide-treated animals (bottom left), inflammation and demyelination were also evident and the lesions contained mostly mononuclear cells. Importantly, however, in cord tissue from animals tolerized with the 16-mer no inflammation or demyelination was noted (bottom right), although a low level of inflammation was still present in the brains of these animals (data not shown).

Long-Term Effects, Control of Relapse, and Induction with Spinal Cord. Without the 16-mer treatment, in most cases the mice did not recover from the initial attack, whereas with the 16-mer treatment the disease was suppressed for at least 40 d. Long-term studies revealed that after that period the mice started to relapse (Fig. 10 A, top). The time frame between the acute episode and the relapse is consistent with the appearance of a new wave of autoreactive PLP139–151-specific T cells from the thymus. Although during the relapsing phase the T cell response reportedly becomes more heterogeneous due to epitope spreading (32–34), an additional intravenous injection with the PLP139–151(C140S) 16-mer on day 40 was still effective in further preventing the appearance of clinical symptoms (Fig. 10 A, bottom). A similar suppression was also achieved when the disease was triggered by broadly stimulating spinal cord homogenate instead of a single epitope (Fig. 10 B). Compared with mice induced with the PLP139–151 peptide, the treatment was slightly less effective, but a strong suppression was also evident after induction with spinal cord (Fig. 10 B, top). Previous reports indicated that PLP139–151 is the dominant epitope in SJL/J mice after immunization with spinal cord homogenate (35). By using a 16-mer with this immunodominant epitope the maximal daily mean score of the initial attack was reduced to only 1.7 compared with 4.5 for the control group, and a complete recovery of the 16-mer–treated mice was observed by day 34 before the mice started to relapse. A second dose, administered on day 12, greatly enhanced the suppressive effect (Fig. 10 B, bottom). In this experiment, the mice treated twice with the 16-mer remained free of clinical symptoms for >60 d.

A

Control of relapse

B

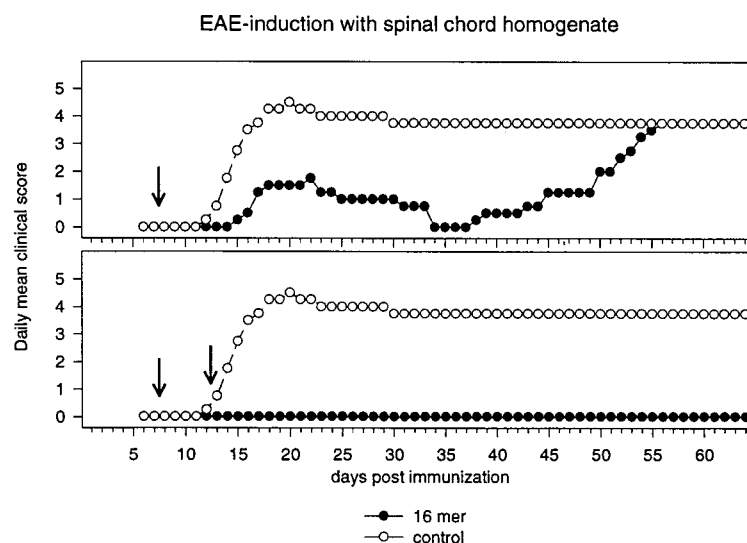**EAE-induction with spinal chord homogenate**
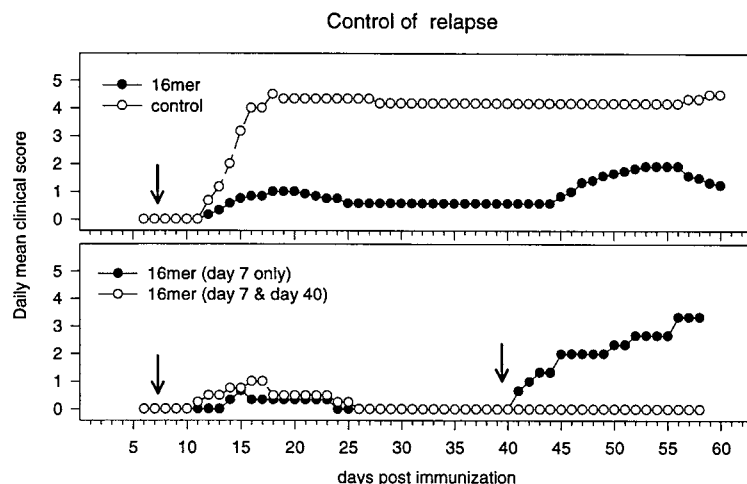
Figure 10. Control of relapse and effect of intravenous treatment with PLP139–151(C140S) 16-mer after EAE induction with spinal cord homogenate. (A) Long-term effect of the treatment with PLP 16-mer and prevention of relapse. The top panel shows an experiment in which SJL/J mice received only a single intravenous dosage of 50 µg PLP139–151 (C140S) 16-mer on day 7. The clinical score is plotted in comparison to the score of a control group treated with PBS only. In the experiment shown in the bottom panel, mice either received only the single dosage of 50 µg 16-mer on day 7 or were treated again the same way on day 40. Groups of four mice were used for the experiments, and the disease was induced by subcutaneous administration of PLP139–151(C140S) peptide. (B) Treatment of EAE with PLP139–151(C140S) 16-mer after induction with spinal cord homogenate. The experiment was carried out as described above except that 4 mg of spinal cord homogenate instead of the PLP peptide was used for the disease induction. The SJL/J mice received intravenous injections of 50 µg 16-mer either on day 8 (top) or on days 8 and 12 (bottom). The control group was treated with mock injections of PBS. Groups of five mice were used for this experiment.

Discussion

The trimolecular interaction of TCR–peptide–MHC represents the structural basis of the immune response. Several attempts have already been made to modulate this interaction with the intent to enhance the response to a vaccine (36–38), and multimerization or oligomerization is another approach in this direction. Soluble multivalent peptide–MHC complexes were previously shown to simultaneously engage multiple TCRs resulting in complexes, which are significantly more stable than the respective monomeric complex (39, 40). Furthermore, the TCR cross-linking promoted by this interaction initiates signal transduction and triggers the activation cascade of the T cell, which significantly lowers the threshold for the activation (41). In addition, the use of multimerized T cell epitopes can produce similar effects. The multimerized antigens, which were designed to form multivalent arrays of peptide–MHC complexes on the surface of the APCs, can show, in some cases, great enhancement in the antigenicity of the epitope.

The oligomerized encephalitogenic antigens presented in this study were not only more effective in the stimulation of antigen-specific T cells in vitro, but also induced stronger primary T cell responses in vivo. The priming of mice with these antigens resulted in the manifestation of very severe forms of EAE, evident even in resistant strains. Earlier studies suggested that the failure of resistant B10.S mice to develop EAE is caused by a paucity of high-affinity autoreactive T cells (42, 43). Since the oligomerized antigens were particularly effective in the stimulation of low-avidity T cells, the increased encephalitogenicity might be attributable to the induction of a broader T cell response by the recruitment of a larger number of T cell clones. Part of this might be due to a potentially reduced need for costimulatory signals. Studies with tetrameric peptide–MHC com-

plexes indicated that at least for these tetramers the influence of CD4 on the binding is diminished (40). In addition, the activation of the APCs by MHC cross-linking (1, 44) and the "proteinization" of the epitope could contribute to the effect. An increased stability of the epitope, altered routes of antigen presentation, and a more effective T cell stimulation by an activated APC might be relevant particularly for the increase in antigenicity in vivo. Another factor might be the strong enhancement of a Th1-type T cell response. Autoreactive CD4$^+$ T cells of the Th1 type are the driving force in T cell–mediated autoimmune diseases, and a preferred activation of this T cell subset would certainly promote the progression of EAE. In fact, a recent study (45) indicated that the resistance of B10.S mice might be due to a failure of antigen-specific CD4$^+$ T cells to upregulate CD40 ligand. This failure abrogates the release of IL-12 and subsequently affects the expression level of the β2 subunit of IL-12R, a receptor crucial in controlling Th1 lineage commitment. At this point, however, it is not clear whether the enhancement of encephalitogenicity is actually caused by a selective activation of Th1 T cells.

Maybe even more important than the improved stimulation is the effect of oligomerized antigens if administered intravenously on the suppression of the autoimmune disease. High zone tolerance as a mechanism of T cell tolerization has been known for some time (13), but its practicality has been limited by the requirement of extremely high antigen (peptide, protein) levels. The enhanced antigenicity of multimerized antigens might make this approach more feasible. A drastic decrease in incidence and severity of EAE was achieved after a single intravenous treatment with a relatively low dosage of 50 μg per mouse. In vitro experiments with HA306–318 oligomers and human influenza virus–specific T cells indicated that the underlying mechanism is most likely the apoptotic elimination of the "overstimulated" autoreactive T cell (data not shown). The reduction in the antigen-specific ex vivo response of mice treated with encephalitogenic oligomers and the long period of protection after treatment are consistent and suggest that this mechanism is also responsible for the specific suppression in the EAE system. Importantly, the suppression was also observed at the onset of the relapsing phase and after disease induction with spinal cord homogenate. The effectiveness of the oligomers in these situations together with the observation that a fraction of the autoreactive T cell population actually fails to respond to the multimerized antigen suggests that, in addition to the antigen-specific suicidal T cell elimination, "bystander" effects contribute to the high zone suppression.

Thus, oligomerized antigens can be effective in the induction and suppression of the immune response. In addition to their use as vaccines for the stimulation of preventive pathogen-specific T cell responses, they might be particularly valuable for the immunotherapy of tumors. Most chronic autoimmune diseases are mediated by CD4$^+$ T cells, and the recruitment of autoreactive CD4$^+$ T cells specific for tumor antigens (46) might permit the develop-

ment of a similar situation, with the immune response directed towards the specific destruction of the transformed tissue. Furthermore, the striking effects of multimeric T cell epitopes in high zone applications might provide a new stimulus for the use of autoantigens in therapies aimed at the specific suppression of acute or chronic autoimmune states.

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