Soluble Mannosylated Myelin Peptide Inhibits the Encephalitogenicity of Autoreactive T Cells during Experimental Autoimmune Encephalomyelitis

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We have previously shown that immunization with a mannosylated myelin peptide in complete adjuvant induces tolerance instead of disease in experimental autoimmune encephalomyelitis (EAE), a rodent model for multiple sclerosis. In this report we demonstrate that treatment with a soluble mannosylated epitope of proteolipid protein (M-PLP₁₃₉₋₁₅₁) significantly inhibits disease mediated by autoreactive myelin-specific T cells during EAE. Treatment with M-PLP₁₃₉₋₁₅₁, applied in different EAE models, significantly reduced the incidence of disease and the severity of clinical symptoms. Delayed-type hypersensitivity responses were abolished after peptide treatment, emphasizing the impact on peripheral T-cell reactivity. Histological analysis of spinal cord tissue from mice treated with M-PLP₁₃₉₋₁₅₁ revealed the presence of only few macrophages and T cells. Moreover, little expression of interferon- γ , interleukin-23, or major histocompatibility complex class II antigen was detected. Immune modulation by M-PLP₁₃₉₋₁₅₁ was primarily antigen-specific because an irrelevant mannosylated peptide showed no significant effect on delayed-type hypersensitivity responses or on the course of EAE. Therefore, mannosylated antigens may represent a novel therapeutic approach for antigen-specific modulation of autoreactive T cells *in vivo*. (Am J Pathol 2007; 170:272–280; DOI: 10.2353/ajpath.2007.060335)

Experimental autoimmune encephalomyelitis (EAE) serves as an experimental model for human multiple sclerosis (MS), reproducing clinical aspects such as inflammation of central nervous system (CNS) tissue.^{1–3} It

is depicted as a prototypic CD4⁺ Th1-mediated autoimmune disease^{4,5} that depends on autoreactive Th1 cells that traffic from the periphery into the CNS.⁶ EAE can be induced in rodents by immunization with myelin fragments in adjuvant or by transfer of encephalitogenic T cells. Recently, we have shown that immunization with a mannosylated peptide of proteolipid protein (M-PLP₁₃₉₋₁₅₁) is a powerful tool to induce tolerance to EAE in mice, even in the presence of complete adjuvant. Although PLP₁₃₉₋₁₅₁-specific T cells were present early after tolerance induction, their effector functions were abolished, as evident from inhibition of delayed-type hypersensitivity (DTH) responses and the absence of EAE.⁷

C-type lectins have been described as pathogen-recognition receptors that recognize sugar residues and play a role in the induction of immunity by discriminating between self and nonself.⁸ However, recent data indicate that certain C-type lectin family members are also involved in inhibition of immune responses, especially DC-SIGN, DEC-205, and the mannose receptor, which are studied to this respect. Binding of several pathogens to DC-SIGN was shown to inhibit dendritic cell maturation and to promote pathogen survival in the host.^{9–12} Likewise, targeting of antigens to DEC-205 expressed by immature dendritic cells was shown to result in tolerance induction. Ovalbumin (OVA) peptide coupled to a DEC-205 antibody was found to induce rapid early cell division and subsequent deletion of peptide-specific CD8⁺ T cells.¹³ Furthermore, activation of CD4⁺ T cells with an OVA peptide coupled to DEC-205 antibody induced transient T-cell responses, lacking interferon (IFN)- γ production, which is a key feature of Th1 cells. A systemic challenge with OVA peptide in complete Freund's adjuvant revealed that antigen-specific unresponsiveness was induced *in vivo*.^{13,14} Moreover, treatment of EAE induced with MOG₃₅₋₅₅ in complete Freund's adjuvant

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with a myelin peptide coupled to a DEC-205 antibody induced tolerance.¹⁵

In MS an ongoing autoimmune response against myelin contributes to chronic CNS inflammation, resulting in tissue damage and clinical symptoms.¹⁶ Current treatment of MS patients is based only on nonspecific immune suppression. Drugs, such as IFN- β and glatiramer acetate, are often used for prolonged periods of time with considerable side effects.^{17,18} Therefore, the development of compounds that specifically interfere with autoimmune processes in MS may be a valuable contribution to therapy. In this report, we provide evidence that treatment with mannosylated myelin peptide, under conditions in which fully activated autoreactive T cells are present, significantly ameliorates EAE symptoms. This suggests that antigen-specific interference with T-cell effector functions of autoreactive T cells may have therapeutic potential in autoimmune disease.

Materials and Methods

Animals

Female SJL mice were purchased from Harlan (Horst, The Netherlands) and Janvier (Le Genest, France). Animals were housed under standard conditions with constant temperature, controlled lightning, and free access to food and water. All experimental procedures were approved by the Animal Welfare Committee.

Peptide Synthesis

PLP₁₃₉₋₁₅₁ (HSLGKWLGHDPKF), its mannosylated form (M-PLP₁₃₉₋₁₅₁), and mannosylated OVA₃₇₁₋₃₈₆ (M-OVA₃₇₁₋₃₈₆) (HIATNAVLFFGRSVS)^{19,20} were synthesized as described elsewhere.^{21,22} In short, the peptides were prepared using solid-phase synthesis. Mannosylation was accomplished by N-terminal elongation of the peptide with a lysine coupled to two tetra-acetyl-protected mannose groups. The PLP₁₃₉₋₁₅₁ peptide was elongated with *bis*-acetyl lysine only. The acetyl-protecting groups on the mannose moieties were removed using Tesser's base. All peptides were analyzed with matrix-assisted laser desorption ionization/time of flight mass spectrometry and showed the expected masses.

Active EAE Induction

Mice (8 to 10 weeks old) were immunized subcutaneously with 50 or 75 μ g of PLP₁₃₉₋₁₅₁ peptide dissolved in phosphate-buffered saline (PBS) and emulsified in an equal volume of complete adjuvant supplemented with 1 mg/ml *Mycobacterium tuberculosis* (H37RA; Difco Laboratories, Detroit, MI). The animals were weighed daily and monitored for EAE development. Clinical EAE was graded in 5 scores: 0, no symptoms; 0.5, partial loss of tail tonus; 1, complete loss of tail tonus or partial limb weakness; 1.5, limb weakness and partial tail paralysis; 2, limb weakness and complete tail paralysis; 2.5, partial paresis; 3, complete paralysis of hind limbs; 3.5, com-

plete paralysis from diaphragm and hind limbs; 4, moribund; and 5, death attributable to EAE.

Adoptive Transfer EAE

For adoptive transfer, donor mice were immunized with 75 μ g of PLP₁₃₉₋₁₅₁ in complete adjuvant supplemented with 1 mg/ml *Mycobacterium tuberculosis*; 1×10^9 heat-killed *Bordetella pertussis* bacteria were injected intravenously on days 1 and 3. After 2 weeks, the animals were sacrificed, and the draining lymph nodes were collected. Lymph node cells were cultured in RPMI 1640 medium (Cambrex, Walkersville, MD) supplemented with 5% fetal calf serum (Life Technologies, Inc., Gaithersburg, MD), 30 μ g/ml PLP₁₃₉₋₁₅₁, and 50 U/ml interleukin (IL)-2. After 3 days, cells were harvested and washed extensively with PBS, and mice were subsequently injected intraperitoneally into naïve recipient mice (3×10^6 T-cell blasts per mouse).

Peptide Treatment Protocols

Mice were treated with 50 μ g of PLP₁₃₉₋₁₅₁, M-PLP₁₃₉₋₁₅₁, or M-OVA₃₇₁₋₃₈₆, dissolved in 200 μ l of PBS. In active EAE experiments, one single dosage of peptide was injected intravenously 8, 17, or 24 days after immunization of the animals. In adoptive transfer EAE studies, recipient mice were treated subcutaneously. Together with the transfer of encephalitogenic cells, peptide was administered 1 day before the injection of cells and 1 and 3 days afterward. Peptide treatment after disease onset occurred twice a week. The control mice received PBS only.

DTH Measurement

The DTH response was evaluated by injecting 25 μ g of PLP₁₃₉₋₁₅₁ dissolved in 10 μ l of saline into the dorsal side of the right ear of mice, using a Hamilton syringe fitted with a 30-gauge needle. As a control for nonspecific ear swelling, 10 μ l of saline was injected into the left ear. Ear thickness was measured before and at 24 or 48 hours after intradermal injection using a Mitutoyo micrometer (Mitutoyo, Veenendaal, The Netherlands). Results are expressed as the percentage of specific ear swelling, obtained by subtracting the percentage nonspecific ear swelling.

Histological Analysis

Brains and spinal cords were collected and frozen in liquid nitrogen for histological analysis. Step serial tissue sections (8 μ m) were stained with hematoxylin to localize inflammatory regions. Cellular composition of infiltrates was analyzed with the following antibodies: rat-anti-human CD3 (polyclonal antibody; DAKO, Glostrup, Denmark), rat-anti-mouse IFN- γ (XMG1.2), rat-anti-mouse CD11b-biotin (M1/70; Pharmingen, San Diego, CA), mouse-anti-rat MHCII (RT1B; Pharmingen), and goat-

Figure 1. Soluble M-PLP₁₃₉₋₁₅₁ inhibits EAE after active immunization. **A–C:** Mice were immunized with 50 to 75 μ g of PLP₁₃₉₋₁₅₁ in complete adjuvant supplemented with 1 mg/ml *M. tuberculosis* and treated intravenously with PBS (**A**), 50 μ g of PLP₁₃₉₋₁₅₁ (**B**), or M-PLP₁₃₉₋₁₅₁ (**C**) 8 days later. EAE score and body weight were assessed daily. Combined data from two experiments are shown, and statistics are provided in Table 1. **D:** In one experiment, DTH responses were measured after injection of 25 μ g of PLP₁₃₉₋₁₅₁ into the ear at day 30. Ear thickness was measured 48 hours later, and means are indicated in the graph. **E:** Treatment with M-PLP₁₃₉₋₁₅₁ resulted in a significantly decreased DTH response ($P < 0.05$). EAE was induced in mice that were treated on day 17 or 24. The cumulative EAE score of individual mice before and 4 weeks after treatment are connected to visualize disease progression. Treatment with M-PLP₁₃₉₋₁₅₁ resulted in decreased disease progression compared with controls ($P = 0.11$).

anti-mouse IL-23 (R&D Systems, Minneapolis, MN). Rabbit anti-rat-IgG biotin (Vector Laboratories, Burlingame, CA) was applied as a secondary biotinylated antibody where needed. Subsequently, streptavidin complex (DAKO) was used for detection of biotin and this reaction was visualized with NovaRed (Vector Laboratories).

Splenocyte Cultures

Spleens were isolated and single cell suspensions were prepared through a 40- μ m filter (BD Falcon, Bedford, MA). To study *in vitro* proliferation, the cells were resuspended in RPMI 1640 containing 5% fetal calf serum (Cambrex), 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mmol/L ultraglutamine, and 50 μ mol/L β -mercaptoethanol and were seeded in 96-well flat-bottom plates (Costar, Cambridge, MA) at a density of 2×10^5 cells per well. Cells were stimulated with 30 μ g/ml PLP₁₃₉₋₁₅₁ for 4 days. Proliferation was assessed by the addition of 0.5 μ Ci of 3 H per well for 6 hours.

Statistics

Statistical analysis of data were performed with a χ^2 test or Mann-Whitney test.

Results

Treatment with Soluble M-PLP₁₃₉₋₁₅₁ Inhibits EAE after Active Immunization

To study immune modulation by soluble mannosylated myelin peptide after induction of EAE with PLP₁₃₉₋₁₅₁ in complete adjuvant containing *M. tuberculosis* H37RA, a single intravenous treatment was administered on day 8 after immunization. Mice were treated with 50 μ g of soluble PLP₁₃₉₋₁₅₁ or M-PLP₁₃₉₋₁₅₁ or with PBS as a control. All control mice developed clinical symptoms at approximately day 14 (Figure 1A and Table 1). Treatment with soluble PLP₁₃₉₋₁₅₁ on day 8 resulted in 80% EAE incidence (Figure 1B and Table 1), whereas administration of M-PLP₁₃₉₋₁₅₁ resulted in less than 40% incidence (Figure 1C and Table 1; $P < 0.05$ compared with controls). The animals that did develop EAE showed a significantly delayed disease onset compared with controls after treatment with either peptide ($P < 0.01$). However, only injection of M-PLP₁₃₉₋₁₅₁ resulted in a significantly decreased maximal and cumulative EAE score compared to controls ($P < 0.01$).

Peripheral T-cell reactivity was evaluated by measuring DTH responses 30 days after immunization. Single treatment with M-PLP₁₃₉₋₁₅₁ on day 8 significantly re-

Table 1. Soluble M-PLP₁₃₉₋₁₅₁ Inhibits EAE Induced by Active Immunization

Treatment*	EAE incidence	Day of onset†	Maximal EAE score	Cumulative EAE score
PBS	11 of 11	13.6 ± 3.1	2.5 ± 1	35.4 ± 17.9
PLP ₁₃₉₋₁₅₁	9 of 11	27.4 ± 11 [§]	2.0 ± 1.0	29.5 ± 18.3
M-PLP ₁₃₉₋₁₅₁	4 of 11 [‡]	27 ± 3.5 [§]	0.8 ± 1.1 [¶]	7.5 ± 11.7

Combined data of two experiments are presented.

*Mice were treated intravenously on day 8 with one single dosage of PBS or 50 µg of peptide.

†Only mice with EAE.

‡ $P < 0.05$ compared with controls and $P = 0.08$ compared with PLP₁₃₉₋₁₅₁-treated animals.

§ $P < 0.01$ compared with controls.

¶ $P < 0.01$ compared with controls and $P < 0.05$ compared with PLP₁₃₉₋₁₅₁-treated animals.

|| $P < 0.01$ compared with controls and PLP₁₃₉₋₁₅₁-treated animals.

duced the peptide-specific DTH response at day 30 compared with control mice (Figure 1D; $P < 0.05$). Such an effect was not observed with nonmannosylated peptide. This suggests that treatment with M-PLP₁₃₉₋₁₅₁ interferes with effector functions of peptide-specific T cells in the periphery.

To study the efficacy of treatment on established disease, a single peptide dose was administered to mice that showed clinical symptoms for at least 2 days. The mice were monitored for 4 weeks after treatment and as depicted in Figure 1E, treatment with M-PLP₁₃₉₋₁₅₁ resulted in limited disease progression in five of seven mice. However, because of two nonresponding animals, this observation did not reach significance ($P = 0.11$ compared to controls). Collectively, these data indicate that treatment with soluble M-PLP₁₃₉₋₁₅₁ can mediate long-lasting modulation of an autoimmune response, well after priming of self-reactive T cells.

M-PLP₁₃₉₋₁₅₁ Treatment Inhibits Disease in an Adoptive Transfer Model of EAE

To exclude that treatment with M-PLP₁₃₉₋₁₅₁ only interfered with the expansion of antigen-specific T cells after active immunization, we studied the effect of peptide treatment in an adoptive transfer model of EAE. For this purpose encephalitogenic T cells were induced in donor mice and transferred into naive recipient mice. Three adoptive transfer experiments were performed in which triple subcutaneous treatment was applied together with

the transfer of encephalitogenic T cells (combined in Table 2). Because of severity of clinical symptoms in this model, prolonged monitoring until day 28 was feasible in only one experiment, and these data were used to evaluate the effect of peptide treatment on the cumulative EAE score. Transfer of encephalitogenic cells into recipients that were treated with PBS only resulted in development of clinical symptoms at approximately day 7 in all animals (Figure 2A and Table 2). Treatment with M-PLP₁₃₉₋₁₅₁ resulted in a significantly reduced EAE incidence compared with control mice, whereas treatment with PLP₁₃₉₋₁₅₁ did not (Table 2; $P < 0.05$).

Analysis of the disease course in affected mice revealed that treatment with PLP₁₃₉₋₁₅₁ delayed the onset of EAE ($P < 0.01$) but only slightly reduced severity of clinical symptoms (Figure 2B and Table 2). In contrast, mice treated with soluble M-PLP₁₃₉₋₁₅₁ showed both a significant delay in disease onset ($P < 0.001$) and a significantly reduced maximal EAE score compared with both control and PLP₁₃₉₋₁₅₁-treated animals (Figure 2C and Table 2; $P < 0.001$). Accordingly, the cumulative EAE score was significantly diminished compared with both groups ($P < 0.01$). To study the antigen specificity of mannosylated peptide treatment in this EAE model, a nonrelevant mannosylated OVA peptide (M-OVA₃₇₁₋₃₈₆) was included. Triple injection of M-OVA₃₇₁₋₃₈₆ into recipient mice showed no significant effect on the course of EAE (Table 2), although two animals included in the long-term experiment presented in Figure 2D did not develop EAE.

Table 2. M-PLP₁₃₉₋₁₅₁ Treatment Ameliorates EAE in an Adoptive Transfer Model

Treatment*	EAE incidence	Mean day of onset†	Mean maximal EAE score	Mean cumulative score‡
PBS	13 of 13	7.0 ± 1.2	3.7 ± 1.0	56.3 ± 25.2
PLP ₁₃₉₋₁₅₁	12 of 14	8.6 ± 1.2 [¶]	2.8 ± 1.3	38.9 ± 3.4
M-PLP ₁₃₉₋₁₅₁	10 of 15 [§]	11.7 ± 1.9 ^{**}	1.0 ± 0.9 ^{††}	13.7 ± 9.6 ^{‡‡}
M-OVA ₃₇₁₋₃₈₆	6 of 8	7.0 ± 0.9	4.2 ± 0.9	30.0 ± 30.2

Combined data of three experiments are presented.

*Mice were treated subcutaneously on days -1, 1, and 3 with PBS or 50 µg of peptide.

†Only mice with EAE.

‡Data from one experiment, $n = 5$ mice per group.

§ $P = 0.05$ compared with controls.

¶ $P < 0.01$ compared with controls and M-OVA₃₇₁₋₃₈₆-treated animals.

|| $P < 0.09$ compared with controls.

** $P < 0.001$ compared with all other groups.

†† $P < 0.001$ compared with controls and PLP₁₃₉₋₁₅₁-treated mice and $P < 0.01$ compared with M-OVA₃₇₁₋₃₈₆-treated animals.

‡‡ $P < 0.01$ compared with controls and PLP₁₃₉₋₁₅₁-treated mice.

Figure 2. M-PLP₁₃₉₋₁₅₁ treatment ameliorates EAE in an adoptive transfer model. **A–D:** Lymph node cells obtained from donor mice were cultured for 3 days and 3×10^6 T-cell blasts were injected into naïve recipient mice. These animals were treated subcutaneously with PBS (**A**), 50 μ g of PLP₁₃₉₋₁₅₁ (**B**), 50 μ g of M-PLP₁₃₉₋₁₅₁ (**C**), or 50 μ g of M-OVA₃₇₁₋₃₈₆ (**D**) on days –1, 1, and 3. EAE score and body weight were assessed daily. This experiment was performed three times, and mean data from a study monitored for a period of 28 days are presented. See Table 2 for combined data and statistical analysis.

Frequently, IL-12 is included in T-cell cultures for EAE transfer, to enhance the encephalitogenic potential of Th1 cells.^{23,24} In our hands culturing of T cells in the presence of IL-12 indeed resulted in severe EAE on transfer, resulting in 40% mortality in control recipients. Even under these conditions a single dose of M-PLP₁₃₉₋₁₅₁ injected before disease onset resulted in ameliorated disease, whereas no such effect was induced by nonmannosylated PLP₁₃₉₋₁₅₁ (unpublished data).

In addition, peptide was injected twice a week into recipient mice that showed clinical symptoms for at least 2 days. The animals were monitored for 10 days, and as depicted in Figure 3A, only treatment with M-PLP₁₃₉₋₁₅₁ ameliorated the course of disease compared with controls ($P = 0.055$). Mice were sacrificed after this treatment period, and splenocytes were cultured for 4 days in the presence of PLP₁₃₉₋₁₅₁. In all treatment groups, comparable peptide-specific proliferation was detected (Figure 3B).

M-PLP₁₃₉₋₁₅₁ Treatment Is Associated with Reduced CNS Inflammation

The effect of peptide treatment together with the transfer of autoreactive T cells on CNS inflammation was evaluated by immunohistochemistry. Hematoxylin staining revealed that on day 16 after transfer inflammatory regions were located mainly in the spinal cord (Figure 4) and to some extent in the cerebellum. In control recipients (Figure 4, left), these infiltrates comprised large numbers of CD3⁺ T cells and CD11b⁺ cells, representing infiltrating macrophages and resident microglia. Moreover, abundant expression of MHC class II and IFN- γ was observed. The majority of infiltrates also contained IL-23-positive cells. Treatment of recipient mice with PLP₁₃₉₋₁₅₁ did not influence the number and composition of these infiltrates (Figure 4, middle).

Figure 3. Treatment of established disease with soluble M-PLP₁₃₉₋₁₅₁. Recipient mice of encephalitogenic T cells with apparent clinical symptoms were randomized (cumulative EAE scores ranged between 1.5 and 9.5). Subcutaneous treatment was applied twice a week with PBS, 50 μ g of PLP₁₃₉₋₁₅₁, 50 μ g of M-PLP₁₃₉₋₁₅₁, or 50 μ g of M-OVA₃₇₁₋₃₈₆, and EAE score and body weight were assessed daily. **A:** Disease progression is expressed by the cumulative EAE score during the treatment period and medians are indicated. Only treatment with M-PLP₁₃₉₋₁₅₁ resulted in a trend toward decreased disease progression compared with controls ($P = 0.055$). Splenocytes were isolated after sacrifice and cultured with PLP₁₃₉₋₁₅₁ for 4 days. **B:** Proliferation revealed no differences between the groups.

In line with the decreased clinical scores, inflammation of the CNS was less severe in mice that were treated with M-PLP₁₃₉₋₁₅₁. This was substantiated by lower numbers of CD11b⁺ cells and less MHC class II expression. CD3 staining revealed decreased numbers of T cells and only little IFN- γ and IL-23 expression (Figure 4, right). Besides inflammation, demyelination is a well-known neuropathological feature in MS and in several EAE models. However, in the EAE models we used no detectable demyelination was observed. These data indicate that treatment with M-PLP₁₃₉₋₁₅₁ reduced inflammation in the CNS.

Treatment with M-PLP₁₃₉₋₁₅₁ Interferes with Peripheral T-Cell Reactivity

To study whether peptide treatment together with the transfer of autoreactive T cells interfered with T-cell effector functions in the periphery, we analyzed DTH responses 3 to 4 days after transfer of PLP₁₃₉₋₁₅₁-specific T cells. Intradermal injection of 25 μ g of PLP₁₃₉₋₁₅₁ into the ear of control recipients resulted in a mean antigen-specific ear swelling of $56 \pm 27\%$ (Figure 5). Treatment with M-OVA₃₇₁₋₃₈₆ resulted in similar DTH responses as those of control mice ($58 \pm 33\%$). Although not significant, PLP₁₃₉₋₁₅₁-treated recipients showed a slightly decreased DTH response ($41 \pm 19\%$). In contrast, a significantly decreased DTH response was detected ($13 \pm$

Figure 4. M-PLP₁₃₉₋₁₅₁ treatment is associated with reduced CNS inflammation. Recipient mice were treated with PBS (left), PLP₁₃₉₋₁₅₁ (middle), or M-PLP₁₃₉₋₁₅₁ (right) on days -1, 1, and 3 and they were sacrificed on day 16. Step serial, longitudinal sections from the cervical to lumbar part of the spinal cord were collected for immunohistochemistry. Antibodies specific for CD3, IFN- γ , CD11b, MHC II, and IL-23 were used. The presence of infiltrates was not limited to particular spinal cord regions. Instead, infiltrates were scattered throughout the whole tissue, predominantly located in white matter tissue. Representative regions are depicted. Original magnifications, $\times 200$.

11%) after treatment with M-PLP₁₃₉₋₁₅₁. Therefore, it can be concluded that treatment with M-PLP₁₃₉₋₁₅₁-affected T-cell effector functions in the periphery in an antigen-specific way.

In addition, PLP₁₃₉₋₁₅₁-specific antibody titers were determined in sera of recipient mice as described elsewhere.⁷ IgG levels in controls and M-PLP₁₃₉₋₁₅₁-treated mice were similar. Treatment with nonmannosylated pep-

Figure 5. Abolished DTH responses after treatment with M-PLP₁₃₉₋₁₅₁. Three to 4 days after transfer of encephalitogenic T cells, 25 μ g of PLP₁₃₉₋₁₅₁ was injected into the right ears of recipient mice; as a control saline was injected into the left ears. Peptide-specific ear swelling was determined 24 hours after challenge. Combined data of three different experiments are shown.

tide induced increased total IgG titers compared with controls, involving increased levels of IgG1 and IgG2b. In all adoptive transfer recipients, IgG2a levels were below the detection limit (unpublished observations).

Discussion

In this report we describe that the encephalitogenic potential of autoreactive T cells during EAE in SJL mice can be inhibited by treatment with soluble mannosylated myelin peptide. To demonstrate this, both active immunization and adoptive transfer EAE models were used. The fact that peptide treatment can be studied in the absence of complete adjuvant is an additional advantage of EAE transfer models. Significant reduction of EAE incidence and clinical symptoms was obtained when mannosylated peptide was administered before the onset of clinical symptoms. Treatment of established disease induced less pronounced effects, suggesting that peptide treatment is particularly effective during disease stadia involving (re)activation of autoreactive T cells. Both intravenous injection, a common way to induce peptide-specific tolerance in mice, and subcutaneous injection, more suitable for clinical application, were applied. The therapeutic effect of mannosylated myelin peptide was associated with reduced inflammation in CNS tissue and impaired T-cell effector functions in the periphery as determined by DTH responses. Irrelevant mannosylated peptide did not affect DTH responses against PLP₁₃₉₋₁₅₁ and only slightly modulated the course of EAE. We have infrequently observed such modest nonspecific effects also during previous studies,⁷ and therefore we conclude that treatment effects of M-PLP₁₃₉₋₁₅₁ were primarily antigen-specific.

Additional adjuvantia, such as pertussis toxin, are indispensable for EAE induction in most mouse strains.²⁵ These adjuvantia break tolerance induced by mannosylated peptides, and therefore our EAE studies have been limited to SJL mice, in which pertussis toxin is not required for EAE induction. Besides EAE development, DTH responses are also affected by treatment with mannosylated peptide. We have used this DTH model to demonstrate that immune modulation was achieved in other

mouse strains using a range of mannosylated antigens (JM Kel, ED de Geus, MJ van Stipdonk, JW Drijfhout, F Koning, L Nagelkerken, manuscript in preparation), implicating that a broad application of mannosylated peptides for immune modulation is feasible.

Treatment with nonmannosylated PLP₁₃₉₋₁₅₁ showed no significant effect on DTH responses in both EAE models. In addition, the development of disease in the adoptive transfer model was not affected. After active immunization, injection of nonmannosylated peptide significantly delayed disease onset but did not decrease severity of clinical symptoms. Intravenous injection of soluble antigens has been described as a mechanism for tolerance induction, via clonal deletion of antigen-specific T cells.²⁶ Although we detected slight effects, the applied dosage of PLP₁₃₉₋₁₅₁ was too low to mediate substantial tolerance. Therefore, we consider the tolerizing effect of mannosylated peptide dependent on a mechanism mediated by mannose-binding structures.

Th1 effector cells play a major role in both DTH responses and inflammation during EAE.²⁷ Because our studies show that M-PLP₁₃₉₋₁₅₁ modulates both inflammatory processes under conditions in which autoreactive T cells are already present, it is highly likely that peptide treatment interferes with the effector functions of these Th1 cells. Several candidate mechanisms involved in tolerance induction after injection of soluble antigens, have been described in literature, such as clonal deletion, anergy, or induction of regulatory T cells.²⁸⁻³⁰

Antigen-specific proliferation of splenocytes isolated from adoptive transfer recipient mice that were treated with mannosylated peptide was not affected. Previous studies also showed normal *in vitro* proliferation of T cells with a Th1 phenotype after immunization with mannosylated myelin peptide⁷ and preliminary data using a TCR transgenic mouse model indicate that mannosylated peptide induces normal expansion of antigen-specific T cells *in vivo* (JM Kel, ED de Geus, MJ van Stipdonk, JW Drijfhout, F Koning, L Nagelkerken, manuscript in preparation). Together, these data are not in favor of T-cell deletion or anergy induction after treatment with M-PLP₁₃₉₋₁₅₁. Additional analysis of PLP₁₃₉₋₁₅₁-specific IgG antibody levels in sera of recipient mice revealed that mannosylated myelin peptide induced no alterations in IgG antibody production. This may be considered an additional indication that mannosylated antigens do not induce a Th2 response or a regulatory response.

Both DTH responses and EAE are highly dependent on migration of effector T cells to local tissue.^{6,31} Because only few T cells and little IFN- γ was detected in spinal cord tissue of recipient mice, it cannot be excluded that treatment with mannosylated M-PLP₁₃₉₋₁₅₁ interferes with migration of effector cells, resulting in decreased local inflammation in tissues. Studies to elucidate the effect of mannosylated peptide on the migration capacities of T cells are ongoing.

C-type lectin family-members that recognize mannose structures, such as the mannose receptor and DEC-205, have been described to be expressed by APC in the periphery.^{32,33} Therefore, the routes of administration applied in our studies may result in loading of M-PLP₁₃₉₋₁₅₁

on peripheral APC. Flugel and colleagues³⁴ reported that encephalitogenic T cells transferred into rats reside in peripheral lymphoid organs before they migrate toward the CNS. Therefore, it is very likely that injected PLP₁₃₉₋₁₅₁-specific T cells encounter peripheral APC loaded with mannosylated PLP₁₃₉₋₁₅₁. The impaired DTH response in mice treated with mannosylated peptide likely reflects a tolerizing mechanism mediated by peripheral APC.

Besides peripheral effects, mannosylated peptides may target APC in the CNS and mediate local effects that may also contribute to diminished EAE pathology. Several studies showed that local reactivation of myelin-specific T cells by microglia in the CNS, for example by production of IL-23, is pivotal for initiation of EAE.^{24,35-37} Mannose receptor expression on CNS APC has been described in the literature.^{38,39} Preliminary data indicate that the mannose receptor (CD206) was indeed expressed in the CNS tissue of recipient mice during our studies, but initial experiments aimed to monitor biodistribution of peptides did not reveal selective targeting or accumulation of mannosylated myelin peptide toward the CNS or other organs.

At present, it is unclear how interactions between APC and T cells are modulated via binding of mannosylated peptides to C-type lectins. Downstream signaling of C-type lectins is still primarily a black box, although a couple of clarifying studies have been published. Chieppa and colleagues⁴⁰ showed that cross-linking of the mannose receptor on human dendritic cells *in vitro* induces an anti-inflammatory program, including down-regulation of IL-12 production. This idea is further supported by Pathak and colleagues⁴¹ who described that triggering of the mannose receptor inhibits nuclear factor- κ B-driven inflammatory signaling pathways.

In conclusion, we have shown that mannosylated PLP₁₃₉₋₁₅₁ is a valuable tool to inhibit the encephalitogenic potential of T cells during EAE, most likely by modulation of autoreactive T cells in the periphery. The observation that DTH responses remained suppressed for more than 4 weeks after treatment demonstrates the long-lasting effect of mannosylated peptide treatment. The ongoing search for (auto)antigens involved in (auto-)immune diseases, will enhance the feasibility of selective antigen-specific treatment of patients. Mixtures of mannosylated peptides might be effective in more complex diseases, such as MS, and may open new avenues for treatment in the future.

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