Oral Administration of Myelin Basic Protein Is Superior to Myelin in Suppressing Established Relapsing Experimental Autoimmune Encephalomyelitis


*J Immunol* 1999; 162:6247-6254;
http://www.jimmunol.org/content/162/10/6247
Oral Administration of Myelin Basic Protein Is Superior to Myelin in Suppressing Established Relapsing Experimental Autoimmune Encephalomyelitis

Jacqueline M. Benson,* Scott S. Stuckman,* Karen L. Cox,* Richard M. Wardrop,* Ingrid E. Gienapp,* Anne H. Cross,† John L. Trotter,† and Caroline C. Whitacre‡

Oral administration of a myelin component, myelin basic protein (MBP), induces immunological unresponsiveness to CNS Ags and ameliorates murine relapsing experimental autoimmune encephalomyelitis (REAE). However, a recent clinical trial in which multiple sclerosis patients were treated with repeated doses of oral myelin was unsuccessful in reducing disease exacerbations. Therefore, we directly compared the tolerizing capacity of myelin vs MBP during REAE in B10.PL mice. Oral administration of high doses of myelin, either before disease induction or during REAE, did not provide protection from disease or decrease in vitro T cell responses. In contrast, repeated oral administration of high doses of MBP suppressed established disease and MBP-specific T cell proliferation and cytokine responses. The frequency of IL-2, IFN-γ, and IL-5-secreting MBP-specific T cells declined with MBP feeding, implicating anergy and/or deletion as the mechanism(s) of oral tolerance after high Ag doses. We have previously shown that the dosage and timing of Ag administration are critical parameters in oral tolerance induction. Studies presented here demonstrate that Ag homogeneity is also important, i.e., homogenous Ag (MBP) is more effective at inducing oral tolerance than heterogeneous Ag (myelin). The Journal of Immunology, 1999, 162: 6247–6254.

Multiple sclerosis (MS) is an inflammatory disease of the human CNS thought to be mediated by autoreactive T cells. Striking clinical, histopathological, and immunological similarities between MS and an experimentally induced disease, relapsing experimental autoimmune encephalomyelitis (REAE), allow REAE to be used as a model for testing therapeutic approaches to MS. REAE is a CD4+ T cell-mediated disease that follows immunization of susceptible mouse strains (SJL, PL/J, and B10.PL) with myelin Ags. Mice exhibit an initial episode of acute paralysis followed by remission and relapses of varying severity. Histologically, REAE is characterized by CNS mononuclear cell infiltrates coupled with demyelination.

One therapeutic strategy under investigation for autoimmune disorders is oral tolerance. This form of tolerance is defined as specific immunological unresponsiveness following the oral administration of Ag. Several experimentally induced autoimmune diseases, such as, REAE, adjuvant or collagen-induced arthritis, uveoretinitis, insulin-dependent diabetes, myasthenia gravis, thyroiditis, and allograft transplantation, have been suppressed by the oral administration of myelin Ags (1, 2), type II collagen (3, 4), S Ag (5), insulin (6), acetylcholine receptor (7), thyroglobulin (8), and alloantigen (9), respectively. Currently, there are several proposed mechanisms for oral tolerance. Low doses of orally administered Ag are suggested to induce suppressive cytokine (IL-4, IL-10, TGF-β) production from regulatory T cell populations (10, 11). Oral administration of high Ag doses results in clonal anergy or deletion of Ag-specific CD4+ T cells (12–17). In addition to Ag dose, the timing of Ag administration influences oral tolerance induction. A single high dose of myelin basic protein (MBP), orally administered before disease induction or on the first day of clinical signs, protects B10.PL mice from REAE. However, repeated high doses of MBP are required to ameliorate REAE once disease is established (18). Therefore, we and others have identified Ag dose and timing of Ag administration as important factors in oral tolerance induction.

In experimental studies, a single component of myelin, MBP, suppressed ongoing REAE when orally administered in repeated high doses (18). Oral tolerance has been tested as a therapeutic strategy in MS using the oral administration of myelin. A phase I double-blind study of 30 relapsing-remitting MS patients suggested a reduction in the number of exacerbations in male DR2-negative patients receiving bovine myelin daily for 1 yr (19). Issues of small sample size, steroid usage, and lack of gender or DR2 matching precluded drawing definitive conclusions from this study. More recently, a multicenter trial controlled for patient gender and steroid treatment was conducted in which myelin was administered orally to over 500 early remitting-relapsing MS patients. Individuals received either 300 mg of bovine myelin or casein daily and were monitored for exacerbation, expanded disability status scale score, and magnetic resonance imaging. Contrary to studies in laboratory animals that demonstrated protection from EAE after oral Ag administration (1, 2, 12, 18, 20, 21), daily

---

*Address correspondence and reprint requests to Dr. Caroline Whitacre, Department of Medical Microbiology and Immunology, Ohio State University College of Medicine and Public Health, 333 West Tenth Ave., Columbus, OH 43210. E-mail address: Whitacre.3@osu.edu

†Address correspondence and reprint requests to Dr. Caroline Whitacre, Department of Medical Microbiology and Immunology, Ohio State University College of Medicine and Public Health, 333 West Tenth Ave., Columbus, OH 43210. E-mail address: Whitacre.3@osu.edu

‡Address correspondence and reprint requests to Dr. Caroline Whitacre, Department of Medical Microbiology and Immunology, Ohio State University College of Medicine and Public Health, 333 West Tenth Ave., Columbus, OH 43210. E-mail address: Whitacre.3@osu.edu

1 Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; REAE, relapsing EAE; MBP, myelin basic protein; GP, guinea pig; NAc, N-acetylated; ELISPOT, enzyme-linked immunospot; Tg, transgenic; EAMS, experimental autoimmune myasthenia gravis; EAN, experimental autoimmune neuritis; PLP, proteolipid protein.
administration of bovine myelin did not significantly improve disease in MS patients (H. L. Weiner, personal communication). Therefore, we undertook a direct comparison of myelin vs MBP administered during ongoing experimentally induced disease. Mice recovering from the acute episode of REAE were fed myelin, MBP, or vehicle, and treatment was continued for 7 wk. Analyses showed that repeated oral administration of MBP, but not myelin, suppressed REAE, proliferation responses, and T cell cytokine production. Myelin was not tolerogenic when orally administered before disease onset or during REAE. Therefore, studies presented here show that in addition to dose and timing of administration, Ag complexity also influences the induction of oral tolerance.

Materials and Methods

Animals

Female B10.PL mice (6–8 wk old) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed at Ohio State University (Columbus, OH).

Neuroantigens

MBP was extracted from guinea pig (GP) spinal cords (Harlan Sprague-Dawley, Indianapolis, IN) using the method of Deibler et al. (22) or Swanborg et al. (23). For REAE immunization, MBP was further purified on a Sephadex G-50 column eluted with 0.1% N HCl. Individual fractions were analyzed by SDS-PAGE, and fractions containing a single band of the appropriate m.w. were pooled. The purified protein was dialyzed against water and lyophilized. Myelin was prepared from GP spinal cords at Washington University (St. Louis, MO) (24). SDS-PAGE revealed all the major myelin proteins to be present, including MBP, MBP peptides NAc_{1-11} (Ac-A-S-Q-K-R-P-S-Q-R-H-COOH; m.w., 1293.5) and NAc_{43-67} (NH2-F-G-F-G-S-D-R-A-P-K-R-G-S-G-K-D-S-H-H-A-A-R-T-T-H-COOH; m.w., 2695.5) were synthesized by the Ohio State University peptide facility and puriﬁed by HPLC.

Induction of REAE

For MBP immunization, mice were injected s.c. over four sites on the flank with 100 μl containing 200 μg of GP-MBP combined with CFA (containing 200 μg of Mycobacterium tuberculosis, Jamaica strain). Mice also received i.p. injections of 200 ng of pertussis toxin (List Biological, Campbell, CA) (two sites on the flank with 100 μl containing 650 μg of GP-myelin combined with CFA, and 200 ng of pertussis toxin was administrated at the time of immunization and 2 and 7 days later (25). Animals were observed daily for clinical signs and 200 ng of pertussis toxin was administered at the time of immunization. Control mice were untreated before myelin immunization.

Induction of oral tolerance

Animals were deprived of food, but not water, for 4–6 h before oral administration of Ag. GP-MBP or myelin was suspended in 0.5 ml of PBS and administered by gastric intubation to ether-anesthetized mice. For feeding before myelin immunization, mice were fed a single dose of 120 mg of myelin 7 days before disease induction, mice were fed a single dose of 120 mg of myelin 7 days before active EAE induction with myelin immunization. Control mice were untreated before myelin immunization.

Proliferation analysis

Single-cell suspensions were prepared from spleens, peripheral lymph nodes (inguinal, axillary, brachial, cervical, deep cervical, popliteal, periaortiic), and mesenteric lymph nodes and were cultured in RPMI 1640 containing 10% FBS, 25 mM HEPES, 2 mM l-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5 x 10^{-5} M 2-ME in round-bottom 96-well plates (5 x 10^5 cells/well). Cells were cultured with MBP (40 μg/ml), MBP NAc_{1-11} (10 μg/ml), MBP_{3-50} (10 μg/ml), or medium alone in triplicate for 72 h, including a final 18-h pulse with [3H]thymidine. Cultures were harvested onto glass-fiber filter mats using a Skatron harvester (Skatron, Sterling, VA) and were counted by liquid scintillation with an LKB Betaplate (LKB, Rockville, MD). The means of triplicate wells were determined, and results are expressed as the stimulation index (mean counts per minute of cultures with Ag/mean counts per minute of cultures with medium alone) ± SEM for all animals in the group.

ELISPOT cytokine analysis

ELISPOT analysis was performed as previously described (26). ELISPOT plates (Polybiotinics, Rockland, MA) were coated with specific capture Ag. For MBP immunization, mice were injected s.c. over four sites on the flank with 100 μl containing 650 μg of GP-MBP, or cultured in X-Vivo serum-free medium alone (BioWhittaker). Chicken anti-TGF-β (2.5 μg/ml; R&D Systems, Minneapolis, MN) was incubated in 96-well Immulon II ELISA plates (Dynatech, Chantilly, VA) at 4°C overnight. After washing, the plates were blocked with 0.25% casein or 2% BSA and incubated with 40 μg/ml anti-IL-2-biotin (JES6-5H4), 75 μg/ml anti-IL-4-biotin (BVD4-24G2), 4 μg/ml anti-IL-5-biotin (TRFK4), and 2 μg/ml anti-IFN-γ-biotin (XMG1.2, PharMingen). After overnight incubation, plates were washed and incubated with alkaline phosphatase-conjugated goat anti-biotin IgG (Vector, Burlingame, CA) for 2 h. Plates were washed, developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium phosphate substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), dried, and analyzed by computer-assisted image analysis using a Series I Immunospot Analyzer (Resolution Technologies, Columbus, OH). The number of cells responding to medium alone was subtracted from the number of cells responding to MBP. Frequencies are expressed as the number of MBP-responsive cells per million ± SEM for all animals in the group.

TGF-β ELISA analysis

TGF-β analysis was performed as previously described (27). Supernatants were harvested at 72 h from 24-well plate cultures of spleen cells (4 x 10^6 cells/ml) or 48 h (for IL-4 and IL-5). Plates were washed with PBS, pH 7.1, with and without Tween-20, then cytokine-specific secondary Abs were added: 2 μg/ml anti-IL-2-biotin (JES6-5H4), 75 μg/ml anti-IL-4-biotin (BVD4-24G2), 4 μg/ml anti-IL-5-biotin (TRFK4), and 2 μg/ml anti-IFN-γ-biotin (XMG1.2, PharMingen). After overnight incubation, plates were washed and incubated with alkaline phosphatase-conjugated goat anti-biotin IgG (Vector, Burlingame, CA) for 2 h. Plates were washed, developed with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium phosphate substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), dried, and analyzed by computer-assisted image analysis using a Series I Immunospot Analyzer (Resolution Technologies, Columbus, OH). The number of cells responding to medium alone was subtracted from the number of cells responding to MBP. Frequencies are expressed as the number of MBP-responsive cells per million ± SEM for all animals in the group.

Ab ELISA analysis

For Ab determinations, Immulon II plates (Dynatech) were coated overnight at 4°C with 5 μg of GP-MBP. Wells were washed then blocked for 30 min at 25°C with 1% BSA (Sigma). After washing, 200 μl of serum dilutions (in 1% BSA) were added and incubated for 2 h at 37°C. Plates were washed, and then anti-Ig Abs were added: 1/3000 goat anti-mouse IgA, 1/1000 goat anti-mouse IgG1, and 1/1000 goat anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL). Plates were incubated for 2 h at 25°C, then wells were developed with phosphatase substrate in diethanolamine buffer (Sigma) and read at 405 nm on a Bio-Rad ELISA reader.

Statistical analysis

In experiments with two groups (Table II and Fig. 3), two-tailed Student’s t test was used to determine statistical significance between experimental and control values. In experiments with more than two groups (Figs. 1, 2, 6, 7).
FIGURE 1. Clinical course of REAE in individual mice fed vehicle, myelin, or MBP multiple times during established REAE. Female B10.PL mice were immunized for REAE, monitored daily for clinical signs, then divided into treatment groups after recovering from the acute phase of disease. Mice were fed a single dose of 50 mg of myelin or 20 mg of MBP upon recovery from initial paralysis, then fed 20 mg of myelin or 10 mg of MBP two times per week for 7 wk. Control mice were fed vehicle (PBS) concurrent with treatment groups. The REAE clinical course for two representative mice per group is shown with arrows indicating days of feeding. The area under the curve was measured in square microns by computer image analysis and is shown for each animal.

Results
Repeated oral doses of MBP, but not myelin, suppress established REAE

REAE was established in female B10.PL mice, then animals were divided into treatment groups after recovering from the acute disease episode so that the mean highest clinical score (2.6–2.9), the mean cumulative clinical score (14.5–18.2), and the mean clinical score per day (0.7–0.9) were comparable between groups. Mice were fed an initial loading dose of MBP (20 mg) or myelin (50 mg) on the day they were judged to have recovered from acute disease. Subsequently, mice were fed maintenance doses of MBP (10 mg) or myelin (20 mg) twice a week for 7 wk. A third group was fed vehicle (PBS) concurrently with treatment groups to control for the stress of repeated handling. The clinical course of representative mice from each treatment group is shown in Fig. 1. Mice fed vehicle or myelin exhibited typical REAE disease courses. However, repeated high oral doses of MBP decreased the clinical signs of established REAE. Animals in each group were observed for 2 wk after cessation of treatment for relapse recurrence. Those mice showing clinical signs maintained a chronic clinical course, typical for late stage REAE. Mice without REAE on the day of the last treatment demonstrated continued protection from disease.

To determine the number of feedings required to suppress REAE, clinical signs were analyzed after 3 wk (six feeds) and 7 wk (14 feeds), corresponding to days 40 and 68 after MBP immunization, respectively. Shown in Table I are cumulative clinical scores, clinical scores per day, the number of relapses, and the highest clinical score for each feeding group after 6 or 14 feeds. Repeated oral administration of myelin did not significantly suppress any disease parameter at either time point compared with vehicle-fed control mice. Indeed, the number of relapses was highest in the myelin-fed group. After 14 oral doses of MBP, the mean cumulative clinical score, the mean clinical score per day, and the mean highest clinical score were reduced compared with those in vehicle-fed controls. The reduction in the mean highest clinical score was statistically significant (\(p = 0.02\)), and the cumulative clinical score approached significance at \(p = 0.07\). The number of relapses did not change after multiple MBP feeds despite the suppression of overall disease severity. It is important to note that suppression of REAE was greater after 14 MBP feeds than after six feeds. Therefore, protection from disease was enhanced with increasing exposure to orally administered Ag. In addition, these results show in a direct comparison that repeated high oral doses of MBP were more efficacious than myelin.

### Table I. Repeated oral doses of MBP, but not myelin, inhibit established REAE

<table>
<thead>
<tr>
<th>Feeding Group</th>
<th>No. of Feeds</th>
<th>Cumulative Clinical Score</th>
<th>Clinical Score/Day</th>
<th>No. of Relapses</th>
<th>Highest Clinical Score</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6</td>
<td>33.6 ± 2.3</td>
<td>1.7 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>2.9 ± 0.2</td>
<td>14</td>
</tr>
<tr>
<td>Myelin</td>
<td>6</td>
<td>30.4 ± 2.8</td>
<td>1.5 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>2.6 ± 0.3</td>
<td>17</td>
</tr>
<tr>
<td>MBP</td>
<td>6</td>
<td>27.7 ± 3.1</td>
<td>1.4 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>2.1 ± 0.3*</td>
<td>17</td>
</tr>
<tr>
<td>Vehicle</td>
<td>14</td>
<td>74.8 ± 6.3</td>
<td>1.7 ± 0.1</td>
<td>1.3 ± 0.4</td>
<td>2.7 ± 0.3</td>
<td>8</td>
</tr>
<tr>
<td>Myelin</td>
<td>14</td>
<td>68.7 ± 11.8</td>
<td>1.5 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>2.8 ± 0.4</td>
<td>10</td>
</tr>
<tr>
<td>MBP</td>
<td>14</td>
<td>43.1 ± 9.3*</td>
<td>1.0 ± 0.2</td>
<td>1.4 ± 0.4</td>
<td>1.5 ± 0.2†</td>
<td>7</td>
</tr>
</tbody>
</table>

* REAE was induced in B10.PL mice by immunization with 200 μg of MBP in CFA and coadministration of pertussis toxin, and animals were divided into treatment groups after recovering from initial paralysis (acute phase). Mice were fed 50 mg of myelin or 20 mg of MBP upon recovery and subsequently with 20 mg of myelin or 10 mg of MBP two times/wk for 7 wk, for a total of 14 feeds. Control mice were fed vehicle (PBS) concurrent with treatment groups. Analysis of clinical signs (cs) began the day of the first feeding through the day of the 6th or 14th feed. The mean for each group is shown ±SEM.

+ Group mean of the sum of daily clinical scores for individual animals.

- Group mean of the number of days the animal was observed. Values were different from vehicle-fed controls at \(p = 0.07\) (*).

- Group mean of the number of relapses in individual animals. Relapses were defined as a decrease in clinical score sustained for ≥2 days followed by an increase in clinical score sustained for ≥2 days.

† Group mean of the highest clinical score exhibited by individual animals between the 1st and 6th or 14th feed. Values were statistically different from vehicle fed controls at \(p ≤ 0.05\) (†).
MBP can reduce established REAE, whereas comparable doses of myelin cannot.

The possibility exists that MBP was tolerogenic because MBP was also the immunizing Ag. Therefore, the tolerizing capacity of myelin was assessed when myelin was the immunizing Ag. For these studies, the most highly reproducible feeding regimen for tolerance was chosen, i.e., feeding before challenge. Previous studies have demonstrated protection from REAE with a single high oral dose of MBP 7 days before challenge (18). Therefore, groups of mice were fed a high dose of myelin (120 mg) or were not treated, then immunized with myelin/CFA/pertussis toxin 7 days later. Table II compares EAE incidence, the day of EAE onset, cumulative clinical score, clinical score per day, and the highest clinical score of untreated vs myelin fed mice. There were no differences between myelin-fed and untreated mice in the day of EAE onset, the cumulative clinical score, or the clinical score per day. Interestingly, EAE incidence was higher and the mean highest clinical score was significantly enhanced with myelin feeding. Therefore, no protection was afforded by feeding myelin before myelin immunization; in fact, EAE was slightly worse. These results and those presented in Fig. 1 and Table I show that myelin is indeed encephalitogenic, but not tolerogenic, when administered either before disease onset or during established REAE.

Repeated oral doses of MBP, but not myelin, inhibit proliferative responses to MBP and MBP peptides

Because repeated high doses of orally administered MBP were shown to suppress disease, functional changes in MBP-specific lymphocyte populations were assessed in vitro. Mice were fed vehicle, myelin, or MBP multiple times during ongoing REAE, as described in Fig. 1, then analyzed after 3 or 7 wk of treatment (six or 14 feeds). Fig. 2 shows splenocyte proliferative responses to MBP and its immunodominant peptides, NAc1–11 and MBP43–67, after 14 oral doses of vehicle, myelin, or MBP. Repeated oral doses of myelin did not decrease proliferation in response to MBP or MBP peptides. However, there was significant suppression of the proliferative response to MBP, MBP NAc1–11, and MBP43–67 after 14 oral doses of MBP were administered. Significant decreases in proliferation in response to MBP and MBP peptides were also observed in peripheral lymph node and mesenteric lymph node cell cultures after MBP feeding, but not in cultures from mice fed myelin or vehicle (data not shown). These analyses demonstrate that feeding homogeneous Ag (MBP) during ongoing REAE can diminish T cell responses to the fed Ag and Ag peptides, whereas feeding heterogeneous Ags (myelin) cannot.

Repeated oral doses of MBP reduce IL-2 secretion

Maximum suppression of clinical signs and MBP-specific proliferative responses required 14 oral administrations of MBP. However, to optimize future oral tolerance strategies, we determined the minimum number of applications required to influence the encephalitogenic MBP-specific T cell population. Mice were fed repeated doses of MBP or vehicle after recovering from acute EAE, and after 10 days or 3 wk of treatment (three or six feeds) IL-2 secretion and those presented in Fig. 1 and Table I show that myelin is indeed encephalitogenic, but not tolerogenic, when administered either before disease onset or during established REAE.

Repeated oral doses of MBP, but not myelin, inhibit proliferative responses to MBP and MBP peptides

Because repeated high doses of orally administered MBP were shown to suppress disease, functional changes in MBP-specific lymphocyte populations were assessed in vitro. Mice were fed vehicle, myelin, or MBP multiple times during ongoing REAE, as described in Fig. 1, then analyzed after 3 or 7 wk of treatment (six or 14 feeds). Fig. 2 shows splenocyte proliferative responses to MBP and its immunodominant peptides, NAc1–11 and MBP43–67, after 14 oral doses of vehicle, myelin, or MBP. Repeated oral doses of myelin did not decrease proliferation in response to MBP or MBP peptides. However, there was significant suppression of the proliferative response to MBP, MBP NAc1–11, and MBP43–67 after 14 oral doses of MBP were administered. Significant decreases in proliferation in response to MBP and MBP peptides were also observed in peripheral lymph node and mesenteric lymph node cell cultures after MBP feeding, but not in cultures from mice fed myelin or vehicle (data not shown). These analyses demonstrate that feeding homogeneous Ag (MBP) during ongoing REAE can diminish T cell responses to the fed Ag and Ag peptides, whereas feeding heterogeneous Ags (myelin) cannot.

Repeated oral doses of MBP reduce IL-2 secretion

Maximum suppression of clinical signs and MBP-specific proliferative responses required 14 oral administrations of MBP. However, to optimize future oral tolerance strategies, we determined the minimum number of applications required to influence the encephalitogenic MBP-specific T cell population. Mice were fed repeated doses of MBP or vehicle after recovering from acute EAE, and after 10 days or 3 wk of treatment (three or six feeds) IL-2 secretion and those presented in Fig. 1 and Table I show that myelin is indeed encephalitogenic, but not tolerogenic, when administered either before disease onset or during established REAE.

Repeated oral doses of MBP, but not myelin, inhibit proliferative responses to MBP and MBP peptides

Because repeated high doses of orally administered MBP were shown to suppress disease, functional changes in MBP-specific lymphocyte populations were assessed in vitro. Mice were fed vehicle, myelin, or MBP multiple times during ongoing REAE, as described in Fig. 1, then analyzed after 3 or 7 wk of treatment (six or 14 feeds). Fig. 2 shows splenocyte proliferative responses to MBP and its immunodominant peptides, NAc1–11 and MBP43–67, after 14 oral doses of vehicle, myelin, or MBP. Repeated oral doses of myelin did not decrease proliferation in response to MBP or MBP peptides. However, there was significant suppression of the proliferative response to MBP, MBP NAc1–11, and MBP43–67 after 14 oral doses of MBP were administered. Significant decreases in proliferation in response to MBP and MBP peptides were also observed in peripheral lymph node and mesenteric lymph node cell cultures after MBP feeding, but not in cultures from mice fed myelin or vehicle (data not shown). These analyses demonstrate that feeding homogeneous Ag (MBP) during ongoing REAE can diminish T cell responses to the fed Ag and Ag peptides, whereas feeding heterogeneous Ags (myelin) cannot.

Repeated oral doses of MBP reduce IL-2 secretion

Maximum suppression of clinical signs and MBP-specific proliferative responses required 14 oral administrations of MBP. However, to optimize future oral tolerance strategies, we determined the minimum number of applications required to influence the encephalitogenic MBP-specific T cell population. Mice were fed repeated doses of MBP or vehicle after recovering from acute EAE, and after 10 days or 3 wk of treatment (three or six feeds) IL-2 secretion and those presented in Fig. 1 and Table I show that myelin is indeed encephalitogenic, but not tolerogenic, when administered either before disease onset or during established REAE.
levels in supernatants from lymph node cells cultured with MBP were determined by ELISA (Fig. 3). Minimal changes were observed after three MBP feedings (data not shown); however, IL-2 levels were undetectable in animals fed MBP six times. Therefore, although protection from clinical REAE required 14 doses of oral Ag (Table I), reduced IL-2 production could be detected after as few as six MBP feeds (Fig. 3).

One of the proposed mechanisms for oral tolerance is Ag-specific immune deviation, i.e., a switch from an encephalitogenic Th1 response to a protective Th2 response (28). We therefore analyzed cytokine production in neuroantigen-fed vs control animals using a single-cell assay (ELISPOT) that is 10–200 times more sensitive than ELISA determinations (29). Mice were fed vehicle, myelin, or MBP as described in Fig. 1, and then were analyzed after six or 14 feeds. Peripheral lymph node cells were cultured in vitro with MBP, then assayed for cytokines by ELISPOT, and the results are shown in Fig. 4. Interestingly, the frequency of IL-4-producing cells increased only in animals fed myelin, even though these mice were not protected from disease (Table I). No changes in the frequency of IL-2-, IFN-\(\gamma\), or IL-5-producing cells were observed in myelin-fed groups compared with that in vehicle-fed controls. In contrast, the frequency of cells producing IL-2, IFN-\(\gamma\), and IL-5 was profoundly reduced in MBP-fed mice. Additionally, the frequency of IL-2- and IL-5-producing cells was reduced in Peyer’s patches only in the MBP-fed group (data not shown).

There was a reduction in IL-2 responder frequency in lymph nodes after six feeds (data not shown), but suppression of other cytokine-producing populations (IFN-\(\gamma\), IL-5) required 14 exposures to oral Ag. In summary, single-cell ELISPOT cytokine analysis revealed that 7 wk of oral MBP (14 feedings) inhibited both Th1 (IL-2, IFN-\(\gamma\)) and Th2 (IL-5) MBP-specific populations. These observations are consistent with inhibition of all MBP-specific T cell cytokine responses rather than immune deviation.

Another proposed mechanism for oral tolerance involves the production of TGF-\(\beta\) from Ag-specific suppressor cells (10, 11). Therefore, we measured the TGF-\(\beta\)-producing capacity of cells from mice fed vehicle, myelin, or MBP. Splenocytes were cultured with or without MBP in vitro for 72 h, then supernatants collected from these cultures were analyzed for the presence of TGF-\(\beta\) by ELISA. There were equivalent amounts of TGF-\(\beta\) in the presence or the absence of MBP stimulation in all feeding groups (Fig. 5). Compared with cultures from vehicle-fed control mice, TGF-\(\beta\) levels decreased with myelin feeding and increased with MBP feeding.

It has been reported that under certain conditions, oral administration of self Ag can promote autoimmunity (30) or prime B cell responses (31). Therefore, evidence of specific Ab production after repeated vehicle, myelin, or MBP during ongoing REAE as described in Fig. 1, and peripheral lymph node cells were harvested after 14 feeds for ELISPOT cytokine analysis. The number of cells producing cytokine in response to 40 \(\mu\)g/ml MBP in vitro was analyzed by computer image analysis. The number of cells responding to medium alone was subtracted from the number of cells responding to MBP. Responding cells per million were determined for replicate cultures from individual animals and the mean for each group ± SEM is shown \((n = 3–5)\). Values were statistically different from vehicle-fed controls at \(p \leq 0.05\) (*).
required to decrease MBP-specific IFN-γ production regardless of whether lymphocyte cultures were restimulated with MBP, whereas myelin feeding resulted in decreased TGF-β levels compared with those in vehicle-fed controls (Fig. 5). It is difficult to correlate changes in TGF-β levels with MBP feeding mediated disease suppression, or if protection from disease can be attributed more to decreased levels of Th1 cytokine production (Fig. 4). The data with myelin feeding of mice conflicts with observations from MS patients fed myelin daily for 2 yr, which resulted in increased serum TGF-β1 levels (34). The role of TGF-β in mediating oral tolerance has recently been called into question by the report of successful oral tolerance induction in TGF-β null mice (35).

After repeated oral administrations of MBP during REAE, we did not observe evidence for Th1 to Th2 immune deviation. Instead, protection from ongoing disease correlated with decreased IL-2, IFN-γ, and IL-5 Ag-specific responses (Fig. 4). Recently, it was shown that adoptive transfer of MBP-specific Th2 cells results in EAE characterized by polymorphonuclear cell and mast cell infiltration into the CNS (36). Therefore, it is possible that a shift to a CNS Ag-specific Th2 response could contribute to EAE rather than provide protection. Interestingly, cytokine levels varied within Th1 and Th2 subsets. The frequency of IL-2-producing cells was much greater than IFN-γ-producing cells. Likewise, the frequency of IL-5-producing cells was greater and responded differently to feeding regimens compared with that of IL-4-producing cells. These observations support the recently proposed concept that cytokine gene expression is independently regulated, yielding a type 1 to type 2 continuum rather than polarized Th1 and Th2 subsets (37). Indeed, coordinate cytokine expression is not a property of whole T cell populations and may vary even within clonal T cell populations (38). Our measurements of cytokine production represented a population analysis of peripheral lymphoid cells (Figs. 3–5). Therefore, T cell populations included new thymic emigrants primed by MBP/CFA immunization and memory T cell populations recirculating after exposure to tolerizing MBP in the gut. Therefore, it is of particular interest that we observed such marked changes in cytokine responses to MBP despite the presence of newly activated thymic emigrants. Because thymic emigrants would be less prevalent in adult MS patients, it is conceivable that changes in cytokine profiles would be even more profound after oral Ag administration.

High doses of orally administered Ag have been reported to induce clonal anergy or deletion (12, 15, 16, 39, 40). We have demonstrated decreased MBP-specific proliferative responses and reduced frequencies of MBP-responding T cells secreting IL-2, IFN-γ, and IL-5 after repeated MBP feeds. However, we cannot distinguish between anergy and deletion of MBP-specific T cells.

It has been proposed that presentation of orally administered self Ags by gut-associated lymphoid tissue may induce tolerance by altering Ag-specific cytokine responses. Suppression of proinflammatory Th1 (IL-2, IFN-γ) cytokine production has been suggested to be mediated by TGF-β derived from Ag-specific T cells (10, 11) or by immune deviation from Th1 to Th2 (IL-4, IL-10) cytokines (28). We found that oral administration of MBP slightly increased TGF-β production regardless of whether lymphocyte cultures were restimulated with MBP, whereas myelin feeding resulted in decreased TGF-β levels compared with those in vehicle-fed controls (Fig. 5). It is difficult to correlate changes in TGF-β levels with clinical disease, since it is not known whether increased levels of TGF-β after MBP feeding mediate disease suppression, or if protection from disease can be attributed more to decreased levels of Th1 cytokine production (Fig. 4). The data with myelin feeding of mice conflicts with observations from MS patients fed myelin daily for 2 yr, which resulted in increased serum TGF-β1 levels (34). The role of TGF-β in mediating oral tolerance has recently been called into question by the report of successful oral tolerance induction in TGF-β null mice (35).

FIGURE 5. TGF-β production after repeated oral administrations of MBP, myelin, or vehicle during REAE. Mice with established REAE were fed vehicle, myelin, or MBP multiple times (14 feeds) as described in Fig. 1. Splenocytes were cultured with or without 40 μg/ml MBP in vitro, and 72-h supernatants from individual cultures were assayed in duplicate by ELISA for TGF-β production. The TGF-β concentration (picograms per milliliter) was determined for cultures from individual animals from a standard curve, and the mean for each group ± SEM are shown (n = 3–5). Values were not statistically different from those in vehicle-fed controls.

Discussion

The results reported here show that Ag homogeneity is an important criterion in oral tolerance induction. A side-by-side comparison of the oral tolerogenicity of myelin membranes containing MBP and other protein and lipid components of myelin vs a more homogeneous purified Ag (MBP) demonstrated that repeated oral administrations of homogeneous Ag (MBP) is superior to inducing oral tolerance compared to heterogeneous Ag (myelin). Myelin feeding did not suppress clinical signs of REAE when administered before disease induction (Table II) or during REAE (Fig. 1 and Table I). MBP-specific proliferative and IL-2, IFN-γ, and IL-5 responses were unchanged with myelin feeding; however, the frequency of IL-4–producing cells increased, while TGF-β levels decreased (Figs. 4 and 5). In contrast, repeated oral administration of MBP decreased clinical signs of REAE (Fig. 1 and Table I) as well as proliferative responses to MBP and MBP peptides (Fig. 2). Reduced IL-2 production from MBP-specific T cells was detected after 3 wk of MBP treatment (Fig. 3), but 7 wk (14 feeds) were required to decrease MBP-specific IFN-γ and IL-5-secreting populations (Fig. 4). Therefore, we observed that myelin protein Ags are not well absorbed when orally administered together and are not as effective as purified myelin Ags in suppressing encephalitogenic T cell populations.

It has been proposed that presentation of orally administered self Ags by gut-associated lymphoid tissue may induce tolerance by altering Ag-specific cytokine responses. Suppression of proinflammatory Th1 (IL-2, IFN-γ) cytokine production has been suggested to be mediated by TGF-β derived from Ag-specific T cells (10, 11) or by immune deviation from Th1 to Th2 (IL-4, IL-10) cytokines (28). We found that oral administration of MBP slightly increased TGF-β production regardless of whether lymphocyte cultures were restimulated with MBP, whereas myelin feeding resulted in decreased TGF-β levels compared with those in vehicle-fed controls (Fig. 5). It is difficult to correlate changes in TGF-β levels with clinical disease, since it is not known whether increased levels of TGF-β after MBP feeding mediate disease suppression, or if protection from disease can be attributed more to decreased levels of Th1 cytokine production (Fig. 4). The data with myelin feeding of mice conflicts with observations from MS patients fed myelin daily for 2 yr, which resulted in increased serum TGF-β1 levels (34). The role of TGF-β in mediating oral tolerance has recently been called into question by the report of successful oral tolerance induction in TGF-β null mice (35).

After repeated oral administrations of MBP during REAE, we did not observe evidence for Th1 to Th2 immune deviation. Instead, protection from ongoing disease correlated with decreased IL-2, IFN-γ, and IL-5 Ag-specific responses (Fig. 4). Recently, it was shown that adoptive transfer of MBP-specific Th2 cells results in EAE characterized by polymorphonuclear cell and mast cell infiltration into the CNS (36). Therefore, it is possible that a shift to a CNS Ag-specific Th2 response could contribute to EAE rather than provide protection. Interestingly, cytokine levels varied within Th1 and Th2 subsets. The frequency of IL-2-producing cells was much greater than IFN-γ-producing cells. Likewise, the frequency of IL-5-producing cells was greater and responded differently to feeding regimens compared with that of IL-4-producing cells. These observations support the recently proposed concept that cytokine gene expression is independently regulated, yielding a type 1 to type 2 continuum rather than polarized Th1 and Th2 subsets (37). Indeed, coordinate cytokine expression is not a property of whole T cell populations and may vary even within clonal T cell populations (38). Our measurements of cytokine production represented a population analysis of peripheral lymphoid cells (Figs. 3–5). Therefore, T cell populations included new thymic emigrants primed by MBP/CFA immunization and memory T cell populations recirculating after exposure to tolerizing MBP in the gut. Therefore, it is of particular interest that we observed such marked changes in cytokine responses to MBP despite the presence of newly activated thymic emigrants. Because thymic emigrants would be less prevalent in adult MS patients, it is conceivable that changes in cytokine profiles would be even more profound after oral Ag administration.

High doses of orally administered Ag have been reported to induce clonal anergy or deletion (12, 15, 16, 39, 40). We have demonstrated decreased MBP-specific proliferative responses and reduced frequencies of MBP-responding T cells secreting IL-2, IFN-γ, and IL-5 after repeated MBP feeds. However, we cannot distinguish between anergy and deletion of MBP-specific T cells.

It is possible that MBP-specific T cells are present after repeated MBP feeds, but are not proliferating or producing cytokines upon in vitro Ag restimulation. Pape et al. (39) recently described a long-lived anergic CD4+ population in vivo whose function was restored once Ag was cleared from the periphery. Intravenous administration of OVA resulted in OVA/MHC class II complexes that were required to maintain the hyporesponsive state. Indeed, the persistence of Ag may be required for the maintenance of T cell tolerance. The persistence of MBP in IFA induces a tolerant state, i.e., anergy, in MBP TCR transgenic (Tg) mice and ameliorate established EAE (41). Therefore, repeated administrations of oral Ag may mediate tolerance by facilitating Ag persistence.

High oral doses of MBP could induce clonal deletion of MBP-specific cells. Repeated oral administrations of high doses of OVA induced apoptosis of OVA-specific lymphocytes within the Pey er’s patches of OVA TCR Tg mice (16). In addition, we have observed evidence for clonal deletion after orally administering high doses of MBP to MBP TCR Tg mice (17). Therefore, it is possible that the reduction in MBP-specific proliferative responses
and frequencies of IL-2, IFN-γ, and IL-5-producing cells represents deletion of MBP-specific cells as a result of MBP feeding. Multiple exposures to orally administered MBP would then be required to provide protection from newly derived encephalitogenic T lymphocytes responding to MBP/CFA immunization. Interestingly, protection from disease persisted beyond the cessation of oral Ag treatment. Therefore, oral Ag administration may provide long term amelioration of autoimmune responses.

It is possible that heterogeneous Ag yields insufficient epitope density within the gut-associated lymphoid tissue to ligate TCRs and deliver a tolerizing signal, which points to the potential role of APCs in oral tolerance. Recent work by Viney et al. (42) demonstrated enhanced oral tolerance to OVA after dendritic cell populations were expanded by Flt3 ligand. Similarly, studies in our laboratory showed enhanced tolerance to MBP and protection from REAE following Flt3 ligand administration (43). Dendritic cells have been shown to preferentially incorporate purified forms of Ag, i.e., peptides, for Ag presentation (44). Therefore, if dendritic cells play a key role in the mediation of oral tolerance, treatment strategies will prove more successful with oral administration of purified Ag. Interestingly, Ag complexity has also been reported to influence oral tolerance induction in experimental autoimmune myasthenia gravis (EAMG) (45) and experimental autoimmune neuritis (EAN) (46). Protection from EAMG was dependent on the dose and purity of orally administered acetylcholine receptor, and the immunogenicity of fed Ag (bovine peripheral myelin vs P2 protein) influenced protection from EAN.

One explanation for the lack of a therapeutic effect with oral myelin may be that the lipid content of myelin interferes with oral tolerance induction. Mazzanti et al. (47) have reported that in MS patients, lipid-bound human MBP is recognized separately from delipidated MBP. Their interpretation is that changes in T cell responsiveness may be due to T cell recognition of lipopeptidic epitopes or differences in APC requirements for presenting lipopeptides. Indeed, a nonclassical MHC class Ib molecule, CD1, is proposed to present lipid Ags. CD1 surface expression is dependent on Ag and frequency of feeding. Thus, oral administration of purified encephalitogenic proteins (PLP, MBP, myelin oligodendrocyte glycoprotein, and myelin-associated glycoprotein) may eliminate complications in tolerance induction from the lipid component of myelin and provide complete protection from REAE.

In conclusion, the studies presented here demonstrate that homogeneous Ag is superior in oral tolerance induction to heterogeneous Ag. In addition, there is evidence that anergy and/or deletion of Ag-specific T cells mediate oral tolerance after high Ag doses. Oral administration of self Ag did not promote autoimmunity or prime B cell responses, thereby providing a safe and Ag-specific therapeutic approach to autoimmune disorders.

Acknowledgments

We thank Drs. Kim Campbell and Phillip Popovich for critical review of this manuscript and Kennichi Dowdell and Dr. Fei Song for assistance with these studies.

References


