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A L Meyer; ... et. al

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Suppression of Murine Chronic Relapsing Experimental Autoimmune Encephalomyelitis by the Oral Administration of Myelin Basic Protein[1]

Abbie L. Meyer, Jacqueline M. Benson, Ingrid E. Gienapp, Karen L. Cox, and Caroline C. Whitacre[2]

Chronic relapsing experimental autoimmune encephalomyelitis (EAE), induced in mice by the injection of myelin basic protein (MBP), is a T cell-mediated autoimmune disease characterized by periods of paralysis and remission. We have shown previously that the oral administration of MBP or MBP peptides renders Lewis rats refractory to EAE. This study was undertaken to examine the conditions necessary to produce oral tolerance in a chronic relapsing model of EAE in B10.PL mice. The optimal tolerizing regimen for the mouse was found to be a single feeding of 20 mg of MBP suspended in PBS. To determine the ability to suppress chronic disease, a range of doses (0.4–100 mg) was administered orally in a single dose before challenge. Larger oral doses (20 or 100 mg) of MBP provided the best protection from EAE, while 0.4 mg exacerbated the clinical course of disease. Secretion of the proinflammatory cytokines, IL-2 and IFN-γ, were lowest in the group fed 20 mg. A single feeding of MBP before challenge or as late as the first day of clinical signs showed significant protection over the relapsing disease course. Once relapsing EAE was established, multiple oral doses of MBP were required to achieve suppression of clinical signs of disease. These findings suggest that vehicle, dosage, and timing are important considerations in the successful application of oral tolerance strategies for suppression of chronic disease processes. *The Journal of Immunology,* **1996, 157: 4230–4238.**

Experimental autoimmune encephalomyelitis (EAE)[3] is an inflammatory autoimmune disease of the central nervous system (CNS) that serves as a useful animal model for testing multiple sclerosis (MS) treatment strategies. EAE is induced in rats and mice by injection of whole nervous tissue, CNS myelin, myelin basic protein (MBP), or proteolipid protein (PLP) combined with adjuvant. EAE is mediated by MHC class II-restricted $CD4^+$ T cells specific for the injected neuroantigen. In the Lewis rat, the disease course is monophasic, and rats recovering from the acute phase are refractory to further reinduction of EAE. However, in susceptible mouse strains, e.g., SJL/J ($H\text{-}2^s$), PL/J ($H\text{-}2^u$), and B10.PL ($H\text{-}2^u$), EAE follows a protracted chronic relapsing course (1–8). Within 25 days after neuroantigen injection, mice exhibit acute paralysis of varying severity, followed by recovery and then various periods of relapse, often lasting for the remainder of the animal's life. This characteristic remitting-relapsing course facilitates the initiation of therapy at various times during the course of disease, making the mouse an ideal model for assessment of MS treatment strategies.

Although the etiology of MS is presently unknown, it is generally accepted that T cell-mediated autoimmunity plays a role in the pathogenesis of the disease. This notion is based on several lines of evidence, including the infiltration of T cells and macrophages into CNS white matter, the association of MS with specific MHC haplotypes and TCR genes, the identification of in vivo clonally expanded MBP-specific T cell populations, and the clinical effectiveness of immunosuppressive drugs (9, 10). At present the only approved therapies for the treatment of MS are adrenocorticotropic hormone (11, 12) and IFN-β (13, 14). Rather than relying on systemic immunosuppression, methods that result in unresponsiveness of specifically autoreactive cells would be a valuable addition to treatment strategies for MS.

We and others have shown that the oral administration of MBP results in protection from the clinical and histopathologic manifestations of EAE in Lewis rats (15–18). This tolerance has been shown to be specific for the fed Ag and extends to species-specific determinants on the MBP molecule (15, 19–21). Based on these studies and others, a phase 1 clinical trial examining the effects of oral myelin in MS was conducted (22). Remitting-relapsing MS patients were given bovine myelin orally, and a reduced number of exacerbations were observed in male myelin-treated patients. The preliminary nature of the trial coupled with the finding that small groups of myelin-treated males responded better than myelin-treated females point to the necessity for additional animal studies in chronic relapsing EAE models.

The majority of work to date on oral tolerance in EAE has been conducted using the Lewis rat strain, which exhibits an acute monophasic form of disease. In studying the feasibility of oral tolerization for treatment of human autoimmune diseases, it becomes critical to determine whether oral administration of autoantigens can suppress an ongoing chronic disease process. Early work along these lines in chronic relapsing models of EAE using strain 13 guinea pigs and Lewis rats indicated that the severity and

Department of Medical Microbiology and Immunology, Ohio State University College of Medicine, Columbus, OH 43210

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[2] Address correspondence and reprint requests to Dr. Caroline C. Whitacre, Department of Medical Microbiology and Immunology, Ohio State University, 2078 Graves Hall, 333 West 10th Avenue, Columbus, OH 43210.

[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; CNS, central nervous system; MBP, myelin basic protein; MS, multiple sclerosis; PLP proteolipid protein; PT, pertussis toxin; STI, soybean trypsin inhibitor.

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frequency of clinical relapses could be reduced by the oral administration of myelin Ags (23).

Because of the frequency of relapses, widespread demyelination, availability of well-characterized reagents for immunologic studies, and availability of transgenic animals, the mouse has become the model of choice for investigating chronic relapsing disease in EAE. In this study, we report the conditions necessary for demonstration of MBP-induced oral tolerance in the B10.PL ($H\text{-}2^u$) mouse. Because the dose of orally administered MBP has been reported to play a pivotal role in the induction of oral tolerance (24, 25), we examined the efficacy of a wide range of oral Ag doses. Results reported here reveal that while a single feeding of 20 mg of MBP in PBS generates optimal protection from challenge with MBP when given before challenge or as late as the first day of clinical signs, multiple feedings of MBP are required to suppress EAE after relapsing disease is established.

Materials and Methods

Animals

Male and female B10.PL mice (6–10 wk old) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed at Ohio State University.

Antigens

MBP was extracted from guinea pig spinal cords (Rockland, Inc., Gilbertsville, PA) using the method of Diebler et al. (26) or Swanborg et al. (27). MBP was further purified on a Sephadex G50 column eluted with 0.01N 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. Purified protein derivative was obtained from Parke-Davis (Morris Plains, NJ), and concanavalin A was obtained from Sigma Chemical Company (St. Louis, MO).

Induction of EAE

Mice were challenged with 200 μg of MBP and CFA containing 200 μg of *Mycobacterium tuberculosis* Jamaica strain. A total of 100 μl was injected s.c. in four sites over the flank. Pertussis toxin (PT) (List Biologic Laboratories, Inc., Campbell, CA) at 300–400 ng in 0.2 ml of PBS was given i.p. at the time of MBP challenge and 48 h later. Animals were observed for the onset of clinical disease, which was scored as follows: limp tail or waddling gait with tail tonicity, 1+; ataxia or a waddling gate with tail limpness, 2+; partial hindlimb paralysis, 3+; total hindlimb paralysis, 4+; and death, 5+. Additionally, a score of 2+ was assigned to animals whose paralysis was apparent in forelimbs only. Observations of clinical disease were made for the length of time indicated in the figure legends.

Induction of oral tolerance

Mice were deprived of food but not water for 5–10 h before oral administration of Ag. Mice then were given a total of 0.4–100 mg of MBP suspended in 0.5 ml of 0.15 M sodium bicarbonate buffer or PBS administered by gastric intubation in 1 feeding or divided among four or 10 feedings, depending on experimental design. In some experiments, the vehicle consisted of 0.15 M sodium bicarbonate buffer containing 10 mg soybean trypsin inhibitor (STI). Challenge with MBP/CFA/PT followed 7 days after the last feeding. In other experiments, MBP was administered as a single feeding on the first day of clinically apparent EAE or after animals had recovered from acute paralysis and had begun their first remission period. In multiple feeding experiments, animals fed on the first day of clinical signs were given 20 mg of MBP three times per week during acute EAE, followed by 10 mg of MBP two times per week for 60 days. Animals fed beginning at recovery were given a single oral feeding of 20 mg of MBP on the day they were judged to have recovered from acute EAE, followed by 10 mg MBP two times per week.

Cytokine determinations

Supernatants were harvested at 24, 48, and 72 h from 24-well plate cultures of spleen cells (4×10^6/ml) stimulated with medium, 40 μg/ml of MBP, or 5 μg/ml of concanavalin A cultured in serum-free medium (X-vivo 20, BioWhittaker, Walkersville, MD). Capture ELISAs for the detection of IL-2 and IFN-γ were conducted according to manufacturer's recommendations (PharMingen, San Diego, CA). Capture Abs (2 μg/ml in bicarbonate buffer) were incubated in Immulon II 96-well ELISA plates (Dynatech Laboratories, Chantilly, VA) at 4°C overnight. After washing the plates were blocked with 3% BSA (Sigma) for 1 h, then washed again. One hundred microliters of each sample or standard dilution (recombinant mouse IL-2 and IFN-γ, PharMingen, San Diego, CA) were added to wells in duplicate and incubated shaking at room temperature for 2 h. Biotinylated detection Abs (anti-IL-2 and IFN-γ) were diluted in 3% BSA-PBS to 1–2 μg/ml and added to wells for 1 h. For the detection of TGFβ, 2.5 μg/ml chicken anti-TGFβ (R & D Systems, Minneapolis, MN) was used as the capture Ab, followed by blocking and the addition of 72-h supernatants as above. Mouse anti-TGFβ 1,2,3 (1 μg/ml) (Genzyme Corp., Cambridge, MA) was added as the detection Ab, followed by 1 μg/ml of biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA). Following extensive washing of all plates, avidin-peroxidase (Sigma) was added, followed by 2,2'azino-di-[3 ethyl-benzthiazoline sulfonate (6)] diammonium salt (ABTS) substrate (Boehringer Mannheim, Indianapolis, IN). Plates were incubated in the dark for 15–30 min and then read at 405 nm on a Bio-Rad ELISA reader. Cytokine concentrations were determined by comparing the OD of samples to the appropriate standard curve.

Table I. *Vehicle is important in the induction of oral tolerance*[a]

Group Feeding Regimen	Animals with Disease	Mean Highest Clinical Score ± SEM[b] (Range)	Mean Score Per Day ± SEM[c]
STI	5/5	3.2 ± 0.67 (2–5)	2.0 ± 0.46
STI/4 mg MBP	7/7	3.3 ± 0.36 (2–4)	0.73 ± 0.13[d]
STI/20 mg MBP	6/7	3.1 ± 0.45 (0–4)	0.57 ± 0.19[d]
Bicarbonate	5/5	3.4 ± 0.40 (2–4)	2.1 ± 0.64
Bicarbonate/4 mg MBP	5/7	2.5 ± 0.68 (0–4)	1.0 ± 0.28
Bicarbonate/20 mg MBP	4/6	1.7 ± 0.57[e] (0–3.5)	0.32 ± 0.12[e]
PBS	9/10	3.1 ± 1.5 (0–5)	1.6 ± 0.38
PBS/2 mg MBP	5/9	1.3 ± 1.7[e] (0–4)	0.61 ± 0.24[f]
PBS/20 mg MBP	1/9[g]	0.11 ± 0.33[g] (0–1)	0.12 ± 0.10[f]

[a] Mice were fed vehicle alone (2, 4, or 20 mg MBP) in a single feeding in bicarbonate buffer containing STI, bicarbonate buffer, or PBS 7 days prior to challenge with MBP/CFA/PT. Mice were observed for clinical signs of EAE for up to 50 days.

[b] Mean of the highest clinical score exhibited by individual animals within a group during the entire course of EAE. The range of highest individual clinical scores is indicated.

[c] Mean of individual animal cumulative clinical scores divided by the number of days observed.

[d] $p < 0.01$ compared to vehicle alone.

[e] $p < 0.05$ compared to vehicle alone.

[f] $p = 0.002$ compared to vehicle alone.

[g] $p < 0.001$ compared to vehicle alone.

Statistical analysis

Differences between control and experimental groups for incidence of disease and incidence of relapse were determined using a χ^2 analysis. ANOVA with Tukey's post hoc analysis was utilized to determine differences in mean highest clinical score and mean score per day (Tables I and II and Fig. 2). A two-tailed Student's t test was used to determine statistical significance between two groups for the mean highest clinical score, cumulative clinical score, average number of relapses, and average score per day (Tables III and IV). Values were judged to be significantly different at $p < 0.05$.

Results

A single feeding of MBP induces tolerance

Experiments were performed to determine the optimum vehicle for oral administration of MBP in the mouse. Either 2 mg, 4 mg, or 20 mg was fed in one of three vehicles: in bicarbonate buffer with

Table II. *A single feeding of 20 mg protects mice from acute EAE*[a]

Group		Animals with Disease	Mean Highest Clinical Score ± SEM[b] (Range)
Number of Feedings	Each Feeding		
10	Vehicle	4/7	1.78 ± 0.68 (0–4)
4	Vehicle	3/6	1.91 ± 0.41 (0–4.5)
10	2 mg MBP	5/8	2.25 ± 0.71 (0–5)
4	5 mg MBP	4/8	1.87 ± 2.17 (0–5)
1	20 mg MBP	1/8	0.19 ± 0.53[c] (0–1.5)

[a] Mice were fed vehicle alone or a total of 20 mg MBP divided into 10, 4, or single doses with the last feeding 7 days prior to challenge with MBP/CFA/PT. Clinical signs of EAE were scored for the acute period of EAE, 35 days.

[b] Mean of the highest clinical score exhibited by individual animals within a group during the entire course of EAE. The range of highest individual clinical scores is indicated.

[c] $p < 0.05$ compared to 4 or 10 feedings of vehicle.

STI, in bicarbonate buffer alone, or in PBS. Table I shows that regardless of the vehicle used, feeding 20 mg of MBP gave better protection than feeding 2 or 4 mg when compared with vehicle-fed controls. When MBP was fed in combination with STI, the mean score per day was significantly decreased in the MBP-fed groups, although nearly all animals exhibited severe clinical signs of EAE. Mice receiving 20 mg of MBP in bicarbonate exhibited decreased severity throughout the course of EAE compared with bicarbonate-fed mice. The greatest overall protection was achieved when MBP was administered in PBS. With PBS, significant decreases were observed for the incidence of EAE, the mean highest clinical score, and mean score per day for both the 2 and 20 mg fed groups. The near complete inhibition of clinical disease when 20 mg of MBP in PBS was fed prompted the use of this vehicle and dose for the remainder of our studies.

To determine the optimum feeding schedule, three regimens were used: 20 mg of MBP in PBS was delivered in 10 doses of 2 mg each over 14 days, in four doses of 5 mg each over 7 days, or in one dose of 20 mg. In all cases the final feeding occurred 7 days before MBP/CFA/PT challenge. Table II shows that a single feeding of 20 mg of MBP provides the best protection from acute EAE. Oral administration of Ag in multiple smaller doses did not confer protection and even appeared to exacerbate disease in the group fed MBP 10 times. Therefore, subsequent experiments utilized a single oral administration of MBP.

Higher oral doses of MBP result in better protection over the course of chronic EAE

Because the dose of orally administered MBP has been reported to play such a pivotal role in the induction of oral tolerance (24), we examined the effect of a wide range of MBP doses on the long-term clinical course of EAE. Figure 1 illustrates the mean clinical score for animals fed 0.4–100 mg of MBP in a single feeding 7 days before challenge. The 0.4-mg dose of MBP was observed to exacerbate the course of clinical disease compared with the vehicle-fed group, while some protection was conferred by feeding 2 mg of MBP. However, oral administration of the higher doses of MBP, 20 and 100 mg, protected animals over the entire 67-day observation period. The clinically protective effect of orally administered MBP is also reflected in reduced proliferative responses to MBP in lymph node cells, spleen cells, and mesenteric lymph node cells from the group fed 20 mg of MBP (data not shown).

Cytokine levels were measured in the supernatants of spleen cell cultures derived from mice (Fig. 1). In animals fed 20 mg of MBP, there were decreased amounts of IL-2 and IFN-γ produced in response to MBP stimulation relative to all other groups (*Fig. 2*), corresponding with suppression of clinical disease. Interestingly, no reduction in IFN-γ or IL-2 was observed in the group fed 100 mg, despite the profound reduction in disease exhibited by these animals (Fig. 1). Unlike IL-2 and IFN-γ, high levels of TGFβ were observed in Ag-stimulated and unstimulated wells regardless of the feeding regimen.

A single oral administration of MBP before establishment of relapsing disease suppresses long-term clinical signs of EAE in B10.PL mice

To determine the ability of orally administered MBP to modulate an ongoing chronic disease process, we compared the disease course when MBP was administered orally before challenge, on the first day of clinical disease, and during recovery. The clinical course of EAE in B10.PL mice was characterized by chronic relapsing-remitting disease unique to individual animals. Over the course of 100 days following injection of MBP, CFA, and PT, B10.PL mice exhibited as many as seven relapses or maintained a chronic state of paralysis (Fig. 3). When 20 mg of MBP was given orally in a single feeding, 7 days before neuroantigen challenge, only 13 of 24 or 54% of the MBP-fed animals developed acute disease (Table III and Fig. 3*A*). The mean highest clinical scores and the cumulative clinical scores for the MBP-fed group were suppressed relative to controls. During the relapse phase of EAE, the number of relapses per mouse was reduced in the MBP-fed group as was the overall mean score per day. Therefore, feeding MBP before challenge reduces the severity of EAE during both the acute and relapse phases.

To determine whether the ongoing course of EAE could be altered by oral administration of MBP later in the disease course, a single 20-mg dose of MBP was fed on the day each animal first developed signs of EAE. In these studies mice developed signs of EAE 10–15 days after challenge, and the animals were followed for 100 days (Fig. 3*B*). The control groups, nonfed and vehicle-fed, developed a relapsing and variable course of EAE. The acute phase of disease in mice fed MBP on the first day of clinical signs was not significantly different from that of the control groups with respect to duration or severity. However, long-term assessment of the clinical course of EAE revealed that MBP-fed mice did differ from controls (Table III and Fig. 3*B*). The cumulative clinical score and the mean daily score were significantly reduced in the MBP-fed group.

To probe the limits of protection provided by a single dose of MBP, mice were fed after they had progressed through the acute phase of disease. At this point in the course of relapsing EAE, CNS-reactive effector T cells have infiltrated the brain and spinal cord. Thus, the tolerizing effect of oral feeding would have to prevent further amplification of MBP-specific cells and also inactivate those effector cells already within the CNS. In these experiments mice were fed a single 20-mg dose of MBP when they had recovered from the acute phase of EAE (Fig. 3*C*). The average day of onset, day 10–12, was similar in all groups. Because the course of acute EAE varies in individual animals, the feeding date varied by about 10 days. Also, the level of clinical recovery that each animal achieved at the time of feeding varied. An animal was fed if it had recovered to a score of 2+ or better by 10–15 days after the onset of clinical signs. The control group exhibited severe relapsing disease with varying numbers of relapses (Table III). There were no differences in mean highest clinical score, cumulative clinical score, or mean number of relapses between MBP-fed mice

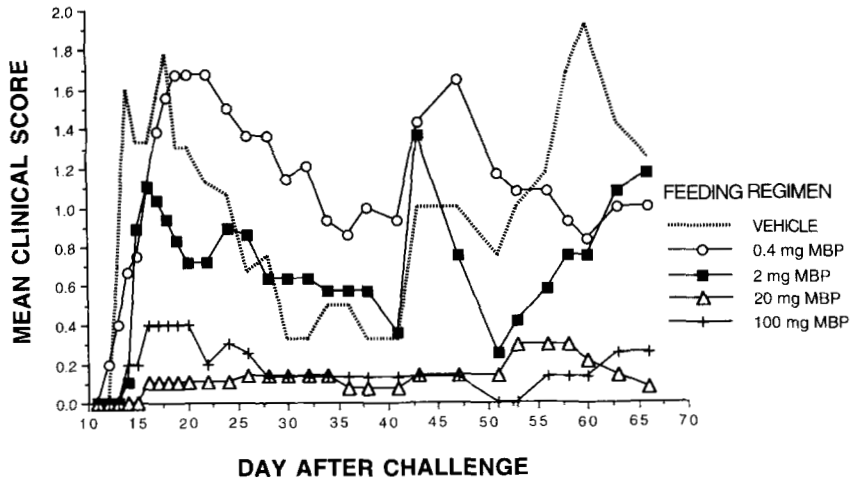

FIGURE 1. Clinical course of chronic EAE following oral administration of various concentrations of MBP. Mice (eight to nine per group) were fed increasing doses of MBP in PBS 7 days before challenge with MBP/CFA/PT. Clinical scores were followed for 67 days. Data are representative of two experiments.

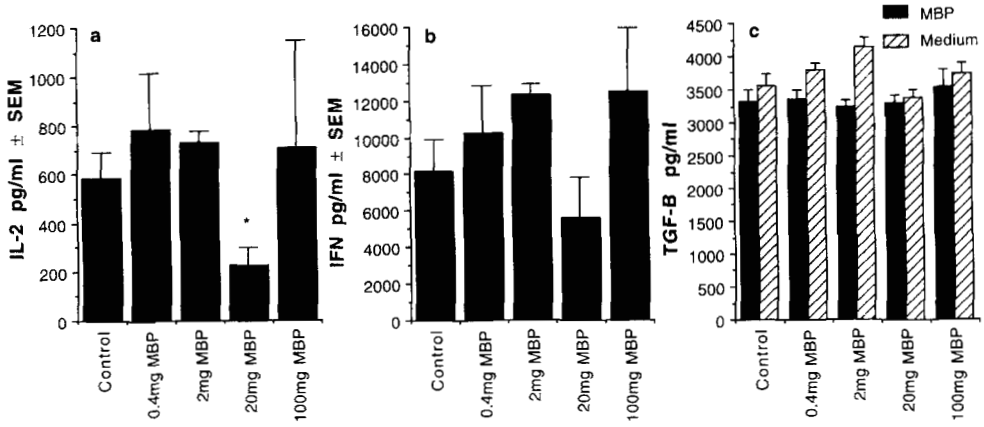

FIGURE 2. Cytokine production in supernatants of MBP-stimulated splenocytes following various oral doses of MBP. Mice were treated as described in the legend for Figure 1. Spleens were taken 10 days following challenge and cultured with MBP. Supernatants were harvested at 48 h for IL-2 and IFN-γ and at 72 h for TGFβ (n = 3–4 per group). For IL-2 and IFN-γ, data are expressed as the amount of cytokine produced in wells containing MBP minus wells with medium alone; for TGFβ, data represent wells containing MBP (■) or medium alone (▨). *Twenty milligrams of MBP is at the level of significance (p = 0.05) compared with nonfed controls and is significantly different from 2 mg of MBP ($p < 0.05$).

and controls. However, the decrease in mean score per day approached statistical significance in the MBP-fed group (p = 0.09).

Multiple oral administrations of MBP suppress relapsing disease in B10.PL mice

Although a single feeding of MBP given before challenge or as late as the first day of clinical signs protected mice from severe EAE, this treatment did not result in significant protection once the relapse phase of EAE was under way. Experiments were designed to determine whether multiple feedings of MBP have a protective effect when started either at the initiation of clinical signs or after establishment of relapsing disease. Animals treated at initiation of clinical signs were given 20 mg of MBP (three times per week) during acute EAE followed by 10 mg of MBP two times per week) during the relapse phase of disease (Fig. 4*A*). The control vehicle-fed group demonstrated severe EAE, whereas the MBP fed group exhibited markedly less disease (Table IV). The course of EAE was also significantly altered, as the cumulative clinical score and mean score per day were significantly reduced in MBP-fed mice compared with controls.

In contrast to the results observed following a single oral administration of MBP, when multiple feedings were initiated at the time mice were judged to have recovered from acute EAE, the disease course was significantly suppressed (Fig. 4*B*). Mice were fed an initial 20-mg dose of MBP when the animal was judged to have recovered from the acute phase of EAE, followed by 10 mg of MBP twice per week. A significant reduction in the cumulative clinical score and mean score per day was observed in the MBP-fed group compared with vehicle-fed controls (Table IV).

Discussion

Much of the previous work on oral tolerance in EAE has been conducted in the Lewis rat. In this model of acute disease, oral administration of MBP or MBP peptides before encephalitogenic challenge results in near complete inhibition of EAE (15–18, 21). In this study we extended the application of oral tolerance into an ongoing chronic relapsing model of EAE in the B10.PL mouse. Initially we focused on optimizing the feeding regimen for this species and strain of mouse. We observed that feeding 20 mg of MBP suspended in PBS profoundly suppressed the subsequent development of EAE clinical signs and was superior to lower doses or the same dose administered in multiple feedings (Tables I and II). Interestingly, feeding a higher dose of MBP (100 mg), although active in suppressing clinical disease, did not attenuate the production of IL-2 or IFN-γ (Fig. 2). Orally administered MBP

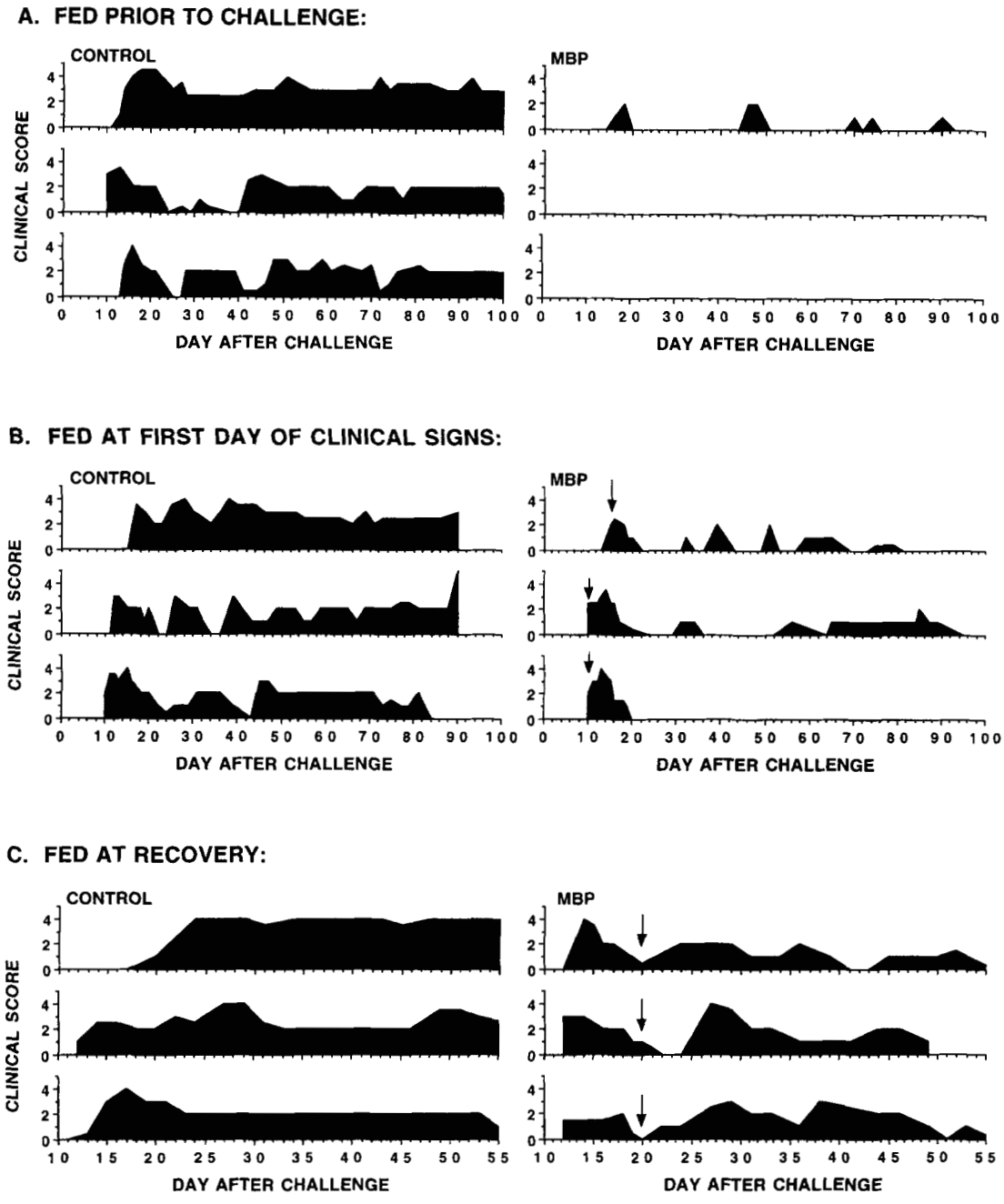

FIGURE 3. Clinical course of EAE in individual mice: feeding before challenge, at the first day of clinical signs, and during the recovery period. Mice were fed a single dose of 20 mg of MBP in PBS 7 days before challenge (*A*), on the first day of clinical disease (*B*), or when the animals were judged to be in the first remission period (*C*). Arrows (*B* and *C*) indicate the day of feeding. Interim analysis of the data in *C* and a lack of observable treatment effect led to the early termination of these animals.

proved most efficacious when given before encephalitogenic challenge, and incidence of clinical signs, mean highest clinical score, cumulative clinical score, and mean score per day were all significantly suppressed (Table III). Feeding MBP at the onset of clinical signs also resulted in a significant decrease in disease severity, whereas the effectiveness of a single dose of MBP given at the time of recovery from acute EAE was less readily apparent. Prolonged administration of MBP in multiple doses was necessary to achieve significant reduction in disease once EAE was established (Table IV).

Oral tolerance is dependent on the efficient delivery of Ag to the gut mucosal epithelium. Our results suggest that prevention of the lumenal processing of MBP by coadministration of STI does not lead to optimal induction of oral tolerance in the mouse (Table I). Saffron et al. (28) showed that oral administration of apronin, a trypsin inhibitor, enhanced the amount of protein that crosses from the gut lumen into the circulation of the mouse. Similarly, Hanson et al. (29) reported that oral administration of apronin along with OVA to mice increases the amount of immunologically active OVA found in the serum. However, apronin appeared to interfere with tolerance induction to OVA, as evidenced by increased delayed-type hypersensitivity and in vitro proliferative responses to OVA challenge in mice fed OVA with apronin compared with mice fed OVA without apronin (30). Thus, our results showing

Table III. *A single oral dose of MBP before challenge and at the onset of clinical signs protects from EAE*

	Incidence of Clinical Signs	Mean Highest Clinical Score ± SEM[a] (Range)	Cumulative Clinical Score ± SEM[b] (Range)	Mean Number of Relapses ± SEM	Mean Score Per Day ± SEM[c]
Fed before challenge[d]					
Control	43/51	3.3 ± 0.2	90.6 ± 17.1	2.4 ± 0.29	1.35 ± 0.15
	84%	(0–5)	(0–286)		
MBP	13/24[e]	1.5 ± 0.4[e]	30.7 ± 14.1[e]	1.3 ± 0.47	0.51 ± 0.18[f]
	54%	(0–5)	(0–209)		
Fed at first day of clinical signs[g]					
Control	29/29	3.41 ± 0.2	86.1 ± 17.0	2.5 ± 0.37	1.82 ± 0.15
	100%	(2–5)	(20–333)		
MBP	12/12	3.0 ± 0.3	34.8 ± 11.2[f]	1.4 ± 0.53	1.01 ± 0.29[h]
	100%	(1–5)	(2–80)		
Fed at recovery[i]					
Control	39/39	2.6 ± 0.2	55.2 ± 7.96	2.0 ± 0.22	1.39 ± 0.15
	100%	(0–5)	(0–185)		
MBP	19/19	2.4 ± 1.4	50.1 ± 10.4	2.0 ± 0.30	0.97 ± 0.20
	100%	(0–5)	(0–133)		

[a] Mean of the highest clinical score exhibited by individual animals within a group during the entire course of EAE for animals fed before challenge or during the period following feeding in the group fed on the first day of clinical signs and at recovery. The range of highest individual scores is indicated.

[b] Mean of the sum of daily scores for each animal over the observation period. The range of individual cumulative scores is indicated.

[c] Mean of individual animal cumulative clinical scores divided by the number of days observed (some animals were observed for less than the full 100 days) beginning at the onset of EAE for animals fed before challenge or beginning on the day of feeding in the other groups.

[d] A single feeding of 20 mg MBP in PBS was given 7 days before challenge. Animals were observed for 100 days (Fig. 3*A*). Data are combined from three experiments.

[e] $p < 0.01$ compared to control.

[f] $p < 0.001$ compared to control.

[g] A single feeding of 20 mg MBP in PBS or PBS alone was given on the day each animal developed clinical signs of EAE (Fig. 3*B*). Animals were observed for 100 days after challenge. Data are combined from two experiments.

[h] $p = 0.05$ compared to control.

[i] A single feeding of 20 mg MBP in PBS or PBS alone was given on the day each animal was judged to have recovered from acute EAE (Fig. 3*C*). Animals were observed for 55 days after challenge, when an interim analysis was conducted, and it was determined that there was no treatment effect. Data are combined from three experiments.

only a modest effect on clinical signs in mice following oral administration of MBP and STI are consistent with the results reported by others. This is in contrast to results obtained in the Lewis rat, in which a vehicle containing sodium bicarbonate buffer and STI resulted in maximal tolerance induction to MBP (15, 17, 21). Moreover, inclusion of STI was shown to be an absolute requirement for oral tolerance to MBP peptides in the rat (21). The differences in vehicle requirements between the mouse and rat may be explained by differences in gastrointestinal physiology. Unlike mice, rats do not have gall bladders. The gall bladder stores and delivers bile salts that emulsify fats and proteins in the lower small intestine and speed their delivery to lymphatic vessels. It is plausible that this is one of the key pathways utilized for the transport and processing of tolerizing signals in mice and is simply less efficient in the rat, requiring the MBP to be better protected from enzymatic degradation. In rats, bile salts are produced in and secreted from the liver in response to ingested fats in the diet. Given the particular feeding schedule used, in which rats are denied their normal diet for 18 h, after which they are given a small oral dose of soluble protein, this amount of protein may not induce the secretion of bile salts. Additional evidence for the importance of the gall bladder in processing of orally administered proteins was recently reported by Matsuda et al. (31), who showed that extracts from mouse liver and gall bladder activate peritoneal macrophages and enhance their interactions with T cells.

A central question relative to application of oral tolerance to human autoimmune disease is whether such an approach can be of benefit once disease is under way. Our results indicate that a single feeding of MBP before disease induction or even at the onset of clinical EAE is significantly protective. However, once EAE has progressed into the relapse phase, multiple doses of oral Ag are necessary to suppress EAE. Perhaps a single oral administration of MBP is insufficient to overcome an expanded MBP-reactive T cell population, and multiple oral administrations of MBP are required to suppress more advanced disease. In one report from a study that examined the efficacy of oral tolerization in relapsing EAE, Brod et al. (23) reported that oral administration of MBP or myelin to rats and guinea pigs, respectively, beginning at recovery from the acute phase of disease, resulted in lower clinical and histopathologic scores relative to controls. The fact that rats were fed three times per week for 5 wks and guinea pigs were fed for 13 wks agrees with our data showing that multiple oral administrations of Ag are required to suppress chronic disease.

An alternative explanation for the decreased effectiveness of a single oral dose of Ag in later stages of disease is provided by epitope spreading, which predicts that once active or passive EAE is under way, additional encephalitogenic epitopes are released from the damaged CNS and then stimulate the expansion of new encephalitogenic clones (32–37). It has been shown that animals immunized with MBP or encephalitogenic peptides of MBP also develop immunologic responses to PLP, another major protein component of myelin. These newly generated PLP-specific cells can then transfer EAE (33, 36). In experiments designed to prevent spreading to new determinants, McRae et al. (37) showed that i.v. administration of PLP 139–151-coupled splenocytes just before the onset of acute adoptive disease changed the course of relapsing EAE. Relapse was prevented, as was the spread of delayed-type hypersensitivity responses to another PLP region demonstrable in controls. Intermolecular spreading may be operative in our results, whereby late oral administration of MBP is ineffective in suppressing immune responses to PLP released as a result of MBP-induced CNS damage. Epitope spreading may explain why we did not observe complete suppression of disease in animals fed during recovery.

It is of interest to compare the efficacy of oral tolerization with other routes of neuroantigen administration in suppression of relapsing EAE. Tan et al. (38, 39) showed that i.v. administration of

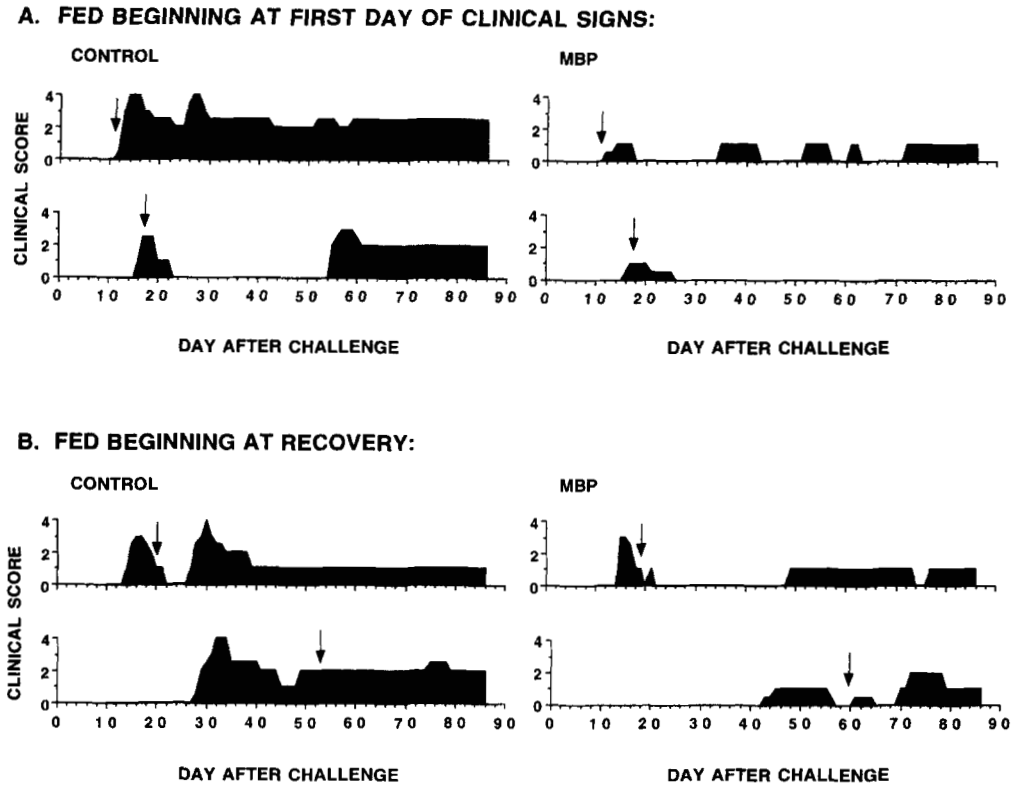

FIGURE 4. Clinical course of EAE in individual mice: multiple feedings beginning at the onset of clinical disease and during the relapse period. Mice were challenged and given multiple feedings of MBP. Arrows indicate the day on which the feeding regimen was initiated. Animals fed beginning on the first day of clinical signs received 20 mg of MBP three times per week during the acute phase of EAE. They subsequently received 10 mg of MBP two times per week during the relapse phase. Controls received PBS on the same schedule. Feeding continued for 48 days (*A*). Animals fed beginning at recovery received 20 mg of MBP when they were judged to have recovered from the acute phase of EAE. They subsequently received 10 mg of MBP twice per week during the relapse phase. Controls received PBS on the same schedule. Feeding continued for 30–50 days (*B*).

Table IV. *Multiple feedings beginning at the first day of clinical signs and at relapse protects from EAE*

	Incidence of Clinical Signs	Mean Highest Clinical Score ± SEM[a] (Range)	Cumulative Clinical Score ± SEM[b]	Mean Score Per Day ± SEM[c]
Fed beginning at first day of clinical signs[d]				
Control	5/5 100%	3.8 ± 0.2 (3–4)	99.4 ± 23	1.5 ± 0.3
MBP	4/4 100%	1.4 ± 0.4[e] (1–2.5)	29.0 ± 13[f]	0.5 ± 0.25[f]
Fed beginning at recovery[g]				
Control	8/8 100%	2.6 ± 0.46 (1–4)	50.9 ± 8.8	1.3 ± 0.13
MBP	5/5 100%	1.6 ± 0.24 (1–2)	22.8 ± 5[f]	0.9 ± 0.28[f]

[a] Mean of the highest clinical score exhibited by individual animals within a group during the period following the initiation of feeding in both groups. The range of highest individual scores is indicated.
[b] Mean of the sum of daily scores for each animal over the observation period, following the initiation of feeding.
[c] Mean of individual animal cumulative clinical scores divided by the number of days observed (animals were observed for 86 days) beginning on the day feeding was initiated.
[d] Multiple feedings of 20 mg MBP in PBS were given three times per week during acute EAE followed by 10 mg given two times per week during relapse phase of EAE. Animals were observed for 86 days (Fig. 4*A*). Data is from a single experiment.
[e] $p = 0.005$ compared to control.
[f] $p < 0.05$ compared to control.
[g] An initial feeding of 20 mg MBP in PBS followed by 10 mg given two times per week was given during relapse phase of EAE. Animals were observed for 86 days (Fig. 4*B*). Data are from a single experiment.

mouse spinal cord homogenate-coupled spleen cells after adoptive transfer of MBP-specific effector T cells in SJL/J mice, but before the onset of the relapse phase, resulted in protection from relapses of EAE. Interestingly, i.v. administration of MBP-coupled spleen cells under similar conditions inhibited the first relapse following transfer but no later ones. Furthermore, Samson and Smilek (40) showed that multiple i.v. administrations of the NAc1–11 peptide beginning at the onset of acute disease in (PL/J × SJL)F1 mice, reduced the clinical severity of relapsing EAE. Administration of the NAc1–11[4Y] peptide, which exhibits enhanced binding to

MHC, gave almost complete long-term protection when administered at the onset of paralysis. Gaur et al. (41) administered NAc1–11 and 35–47 combined i.p. in IFA at the onset of clinical signs and showed long-term suppression of disease. In contrast, when mouse spinal cord homogenate in IFA was administered s.c. to Biozzi AB/H mice later in the course of EAE, a synchronization of relapses rather than protection was observed (42). Thus, tolerance approaches have been applied to a variety of relapsing EAE models, and our results indicate that oral administration of MBP compares favorably with other routes of neuroantigen administration.

In the Lewis rat as well as the B10.PL mouse, oral administration of MBP results in decreased secretion of the proinflammatory cytokines IL-2 and IFN-γ (Fig. 2) (17). Curiously, we did not observe an increase in TGFβ in spleen cell cultures from animals fed a wide range of MBP doses. This cytokine has been reported to mediate the suppressive effects of orally administered MBP through Ag nonspecific bystander suppression (19, 20, 43, 44). Additionally, TGFβ-producing $CD4^+$ cell lines that can transfer unresponsiveness have been selected from MBP-fed SJL/J mice (43). In the OVA TCR transgenic mouse, it was observed that lower doses of OVA (0.5 and 5 mg) given orally three or more times generated an increase in TGFβ secretion, whereas higher doses (500 mg) resulted in deletion (44). Interestingly, single feeds of these same doses caused a reduction in IL-2 and IFN-γ without a concomitant increase in Th2 cytokines or TGFβ (44). In our study, using a single feeding regimen over a range of doses, we did not observe an increase in TGFβ secretion in mice fed lower doses of MBP. These data suggest that there is no effect of high or low dose associated with MBP administered in a single dose. Although there is evidence for a role for TGFβ in mediating protection following multiple oral feedings of MBP, other data suggest that the protective effects of TGFβ occur only during the induction phase of EAE (45, 46). When exogenous TGFβ was administered earlier or later than 5–7 days after challenge, the severity of EAE was greater.

It is clear from the work of many laboratories that different immune responses are operative at different stages of relapsing EAE. Therefore, a variety of tolerizing approaches may be required to completely suppress chronic disease. Clearly, our data suggest that the most complete tolerance was achieved when MBP was given orally in a single feeding before challenge or in multiple doses following the onset of disease. Additionally, substantial protection was achieved when MBP was fed at the onset of disease. Given the likely spread of the dominant immune response to new encephalitogenic epitopes of MBP or other CNS proteins or peptides over the course of EAE, multiple feeds were necessary to achieve tolerance at points later in the course of relapsing EAE. Additionally, the dose of Ag may be of critical importance. Our results indicate the existence of a linear relationship between increasing oral doses of MBP and increasing protection but also indicate that deleterious effects may be observed when an insufficient amount of Ag is fed. Therefore, our results indicate that timing, dose, and vehicle are all critical parameters in achieving optimal oral tolerance and should be carefully evaluated in each species tested.

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References

1. Olitsky, P. K., and R. H. Yager. 1949. Experimental disseminated encephalomyelitis in white mice. *J. Exp. Med. 90:213.*
2. Brown, A. M., and D. E. McFarlin. 1981. Relapsing experimental allergic encephalomyelitis in the SJL/J mouse. *Lab. Invest. 45:278.*
3. Lublin, F. D., P. H. Maurer, R. G. Berry, and D. Tippet. 1981. Delayed, relapsing experimental allergic encephalomyelitis in mice. *J. Immunol. 126:819.*
4. Fritz, R. B., C.-H. J. Chou, and D. E. McFarlin. 1983. Relapsing murine experimental allergic encephalomyelitis induced by myelin basic protein. *J. Immunol. 130:1024.*
5. Mokhtarian, F., D. E. McFarlin, and C. S. Raine. 1984. Adoptive transfer of myelin basic protein-sensitized T cells produces chronic relapsing demyelinating disease in mice. *Nature 309:356.*
6. Fritz, R. B., M. J. Skeen, C.-H. J. Chou, M. Garcia, and I. K. Egorov. 1985. Major histocompatibility complex-linked control of the murine immune response to myelin basic protein. *J. Immunol. 134:2328.*
7. Lublin, F. D. 1985. Relapsing experimental allergic encephalomyelitis: an autoimmune model of multiple sclerosis. *Springer Semin. Immunopathol. 8:197.*
8. McCarron, R., and D. E. McFarlin. 1988. Adoptively transferred experimental autoimmune encephalomyelitis in SJL/J, PL/J, and (SJL/J X PL/J)F_1 mice: Influence of I-A haplotype on encephalitogenic epitope of myelin basic protein. *J. Immunol. 141:1143.*
9. Traugott, U. 1990. Evidence for immunopathogenesis. In *Handbook of Multiple Sclerosis*, Vol. 8. S. D. Cook, ed. Marcel Dekker, New York, p. 101.
10. Khoury, S. J., H. L. Weiner, and D. A. Hafler. 1990. Immunologic basis of multiple sclerosis. In *Handbook of Multiple Sclerosis*, Vol. 8. S. D. Cook, ed. Marcel Dekker, New York, p. 129.
11. Miller, H., D. J. Newell, and A. Ridley. 1961. Multiple sclerosis: treatment of acute exacerbations with coritcotrophin (ACTH). *Lancet 2:1120.*
12. Rose, A. S., J. W. Kuzma, and J. F. Kurtzke. 1970. Cooperative study in the evaluation of therapy in multiple sclerosis: ACTH vs. placebo: final report. *Neurology 20:1.*
13. Arnason, B. G. 1993. Interferon beta in multiple sclerosis. *Neurology 43:641.*
14. Group, T. I. M. S. S. 1993. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis: clinical results of a multicenter randomized, double-blind, placebo-controlled trial. *Neurology 43:655.*
15. Bitar, D. M., and C. C. Whitacre. 1988. Suppression of experimental autoimmune encephalomyelitis by the oral administration of myelin basic protein. *Cell. Immunol. 112:364.*
16. Higgins, P. J., and H. L. Weiner. 1988. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments. *J. Immunol. 140:440.*
17. Whitacre, C. C., I. E. Gienapp, C. G. Orosz, and D. M. Bitar. 1991. Oral tolerance in experimental autoimmune encephalomyelitis. III. Evidence for clonal anergy. *J. Immunol. 147:2155.*
18. Miller, A., O. Lider, A. Al-Sabbagh, and H. L. Weiner. 1992. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. V. Hierarchy of suppression by myelin basic protein from different species. *J. Neuroimmunol. 39:243.*
19. Miller, A., O. Lider, A. B. Roberts, M. B. Sporn, and H. L. Weiner. 1992. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor β after antigen-specific triggering. *Proc. Natl. Acad. Sci. USA 89:421.*
20. Miller, A., A. Al-Sabbagh, L. M. B. Santos, M. P. Das, and H. L. Weiner. 1993. Epitopes of myelin basic protein that trigger TGF-β release after oral tolerization are distinct from encephalitogenic epitopes and mediate epitope-driven bystander suppression. *J. Immunol. 151:7307.*
21. Javed, N. H., I. E. Gienapp, K. L. Cox, and C. C. Whitacre. 1995. Exquisite peptide specificity of oral tolerance in experimental autoimmune encephalomyelitis. *J. Immunol. 155:1599.*
22. Weiner, H. L., G. A. Mackin, M. Matsui, E. J. Orav, S. J. Khoury, D. M. Dawson, and D. A. Hafler. 1993. Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. *Science 259:1321.*
23. Brod, S. A., A. Al-Sabbagh, R. A. Sobel, D. A. Hafler, and H. L. Weiner. 1991. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin antigens. IV. Suppression of chronic relapsing disease in the Lewis rat and strain 13 guinea pig. *Ann. Neurol. 29:615.*
24. Friedman, A., and H. L. Weiner. 1994. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage. *J. Immunol. 91:6688.*
25. Whitacre, C. C., I. E. Gienapp, A. Meyer, K. L. Cox, and N. Javed. 1996. Oral tolerance in experimental autoimmune encephalomyelitis. *Ann. NY Acad. Sci. 778:217.*
26. Diebler, G. E., R. E. Martensen, and M. W. Kies. 1972. Large-scale preparation of myelin basic protein from central nervous tissue of several mammalian species. *Prep. Biochem. 2:139.*
27. Swanborg, R. H., J. E. Swierkosz, and R. G. Saieg. 1974. Studies on the species variability of experimental allergic encephalomyelitis in guinea pigs and rats. *J. Immunol. 112:594.*
28. Saffron, M., R. Franco-Saenz, A. Kong, D. Pepthadjopoulos, and F. Szoka. 1979. A model for the study of oral administration of peptide hormones. *Can. J. Biochem. 57:548.*
29. Hanson, D. G., M. J. Roy, S. D. Miller, E. G. Seidman, M. J. Thomas, I. R. Sanderson, J. N. Udall, I. Ely, and G. M. Green. 1993. Endopeptidase inhibition and intestinal antigen processing in mice. *Reg. Immunol. 5:85.*
30. Hanson, D. G., M. J. Roy, G. M. Green, and S. D. Miller. 1993. Inhibition of orally-induced immune tolerance in mice by prefeeding an endopeptidase inhibitor. *Reg. Immunol. 5:76.*
31. Matsuda, H., Y. Ikeda, and M. Kubo. 1992. Pharmacological study on Naja naja kaouthia. Lesson III. Effects of 50% ethanolic extracts from liver and gall bladder

on phagocytic activity of mouse reticuloendothelial system. *Yakugaku Zasshi J. Pharmaceut. Soc. Jpn. 112:183.*
32. McCarron, R. M., R. J. Fallis, and D. E. McFarlin. 1990. Alterations in T cell antigen specificity and class II restriction during the course of chronic relapsing experimental allergic encephalomyelitis. *J. Neuroimmunol. 29:73.*
33. Perry, L. L., E. Barzaga-Gilbert, and J. Trotter. 1991. T cell sensitization to proteolipid protein in myelin basic protein-induced relapsing experimental allergic encephalomyelitis. *J. Neuroimmunol. 33:7.*
34. Lehmann, P. V., T. Forsthuber, A. Miller, and E. E. Sercarz. 1992. Spreading of T cell autoimmunity to cryptic determinants of an autoantigen. *Nature 358:155.*
35. Lehmann, P. V., E. E. Sercarz, T. Forsthuber, C. M. Dayan, and G. Gammon. 1993. Determinant spreading and the dynamics of the autoimmune T cell repertoire. *Immunol. Today 14:203.*
36. Cross, A. H., V. K. Tuohy, and C. S. Raine. 1993. Development of reactivity to new myelin antigens during chronic relapsing autoimmune demyelination. *Cell. Immunol. 146:261.*
37. McRae, B. L., C. L. Vanderlugt, M. C. DalCanto, and S. D. Miller. 1995. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. *J. Exp. Med. 182:75.*
38. Tan, L.-J., M. Kennedy, M. C. DalCanto, and S. D. Miller. 1991. Successful treatment of paralytic relapses in adoptive experimental autoimmune encephalomyelitis via neuroantigen specific tolerance. *J. Immunol. 147:1797.*
39. Tan, L.-J., M. K. Kennedy, and S. D. Miller. 1992. Regulation of the effector stages of experimental autoimmune encephalomyelitis via neuroantigen-specific tolerance induction. *J. Immunol. 148:2748.*
40. Samson, M. F., and D. E. Smilek. 1995. Reversal of acute experimental autoimmune encephalomyelitis and prevention of relapses by treatment with a myelin basic protein peptide analogue modified to form long-lived peptide-MHC complexes. *J. Immunol. 155:2737.*
41. Gaur, A., B. Wiers, A. Liu, J. Rothbard, and C. G. Fathman. 1992. Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy. *Science 258:1491.*
42. O'Neill, J. K., D. Baker, and J. L. Turk. 1992. Inhibition of chronic relapsing experimental allergic encephalomyelitis in the Biozzi AB/H mouse. *J. Neuroimmunol. 41:177.*
43. Chen, Y., V. K. Kuchroo, J.-I. Inobe, D. A. Hafler, and H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science 265:1237.*
44. Chen, Y., J.-I. Inobe, R. Marks, P. Gonnella, V. K. Kuchroo, and H. L. Weiner. 1995. Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature 376:177.*
45. Santambrogio, L., G. M. Hochwald, B. Saxena, C.-H. Leu, J. E. Martz, J. A. Carlino, N. H. Ruddle, M. A. Palladino, L. I. Gold, and G. J. Thorbecke. 1993. Studies on the mechanisms by which transforming growth factor-β (TGF-β) protects against allergic encephalomyelitis: antagonism between TGF-β and TNF. *J. Immunol. 151:1116.*
46. Santambrogio, L., G. M. Crisi, J. Leu, G. M. Hochwald, T. Ryan, and G. J. Thorbecke. 1995. Tolerogenic forms of autoantigens and cytokines in the induction of resistance to experimental allergic encephalomyelitis. *J. Neuroimmunol. 58:211.*