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Immunology

Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit

(oral tolerance)

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ABSTRACT **Oral administration of autoantigens can prevent and partially suppress autoimmune diseases in a number of experimental models. Depending on the dose of antigen fed, this approach appears to involve distinct yet reversible and short-lasting mechanisms (anergy/deletion and suppression) and usually requires repeated feeding of large (suppression) to massive (anergy/deletion) amounts of autoantigens to be effective. Most importantly, this approach is relatively less effective in animals already systemically sensitized to the fed antigen, such as in animals already harboring autoreactive T cells and, thus, presumably also in humans suffering from an autoimmune disorder. We have previously shown that feeding a single dose of minute amounts of antigens conjugated to cholera toxin B subunit (CTB) can effectively suppress delayed-type hypersensitivity reactions in systemically immune animals. We now report that feeding small amounts of myelin basic protein (MBP) conjugated to CTB either before or after disease induction protected rats from experimental autoimmune encephalomyelitis. Such treatment was as effective in suppressing interleukin 2 production and proliferative responses of lymph node cells to MBP as treatment involving repeated feeding with much larger (50- to 100-fold) doses of free MBP. Different from the latter treatment, which led to decreased production of interferon-γ in lymph nodes, low-dose oral CTB–MBP treatment was associated with increased interferon-γ production. Most importantly, low-dose oral CTB–MBP treatment greatly reduced the level of leukocyte infiltration into spinal cord tissue compared with treatment with repeated feeding of large doses of MBP. These results suggest that the protection from experimental autoimmune encephalomyelitis achieved by feeding CTB-conjugated myelin autoantigen involves immunomodulating mechanisms that are distinct from those implicated by conventional protocols of oral tolerance induction.**

Experimental allergic encephalomyelitis (EAE) is a $CD4^+$ T-cell-dependent paralytic autoimmune disease of the central nervous system (CNS) that can be induced in experimental animals by the injection of myelin-derived autoantigens and peptides (1). This experimental disease serves as a widely studied model for human autoimmune inflammatory demyelinating disorders such as multiple sclerosis (2).

Oral administration of antigens including autoantigens has long been recognized as a method to prevent peripheral T-cell responses and, in the case of autoantigens, has also been shown to prevent or delay the onset of several experimental autoimmune diseases (3–9), including EAE (5, 6). Major problems recognized with strategy is that it requires feeding large if not massive doses of autoantigens and it is generally less efficient in an immune as opposed to a naive host (10, 11). The latter problem has limited the therapeutic potential of this strategy.

We have recently shown (12) that oral administration of minute amounts of prototype particulate and soluble protein antigens conjugated to cholera toxin B subunit (CTB), the nontoxic receptor-binding moiety of cholera toxin, can readily induce tolerance in the peripheral T-cell compartment and is effective not only in naive but also in systemically sensitized animals. We now report that oral administration of minute amounts of an autoantigen, myelin basic protein (MBP), coupled to CTB can prevent EAE in Lewis rats, a particularly susceptible strain. Most importantly, the oral CTB–MBP is also effective when given after induction of the experimental EAE disease and reduces leukocyte infiltration into the CNS.

MATERIALS AND METHODS

Animals and Induction of EAE. Female Lewis rats (7 to 8 weeks old) were purchased from Harlan (Blackthorn, United Kingdom). To induce EAE, animals were injected in the hind footpads with 50 μg of guinea pig MBP (prepared as described below) emulsified in Freund's complete adjuvant (CFA) containing killed *Mycobacterium tuberculosis* H37Ra cells (Difco; 5 mg/ml).

Animals were examined daily for clinical signs of EAE. Clinical severity was scored as follows: 0, no disease; 1, limp tail; 2, tail paralysis and hind limb weakness; 3, hind limb paralysis; 4, tetraplegia; 5, death.

Antigens and Preparation of CTB–Antigen Conjugates. Highly purified CTB, free of any contaminating A subunit, was produced in a mutant strain of *Vibrio cholerae* deleted of the cholera toxin A subunit gene and transfected with a plasmid encoding CTB (13, 14). CTB was purifed from the bacterial culture supernatants by sequential precipitation and gel filtration chromatography (14). MBP was purified from guinea pig spinal cord and brain tissue by the method of Deibler *et al.* (15). Purity was confirmed by SDS/polyacrylamide gel electrophoresis, and staining with Coomassie blue showing a single protein band.

MBP was covalently conjugated to CTB by using *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) as bifunctional coupling reagent (16). Briefly, CTB and MBP were separately derivatized with SPDP at a molar ratio of 1:5. After incubation (23°C for 30 min), free SPDP was removed by gel filtration through Sephadex G-25 (Pharmacia). The SPDP-derivatized MBP was reduced with 10 mM DTT and the

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Abbreviations: CTB, cholera toxin B subunit; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; IL, interleukin; IFN, interferon; MBP, myelin basic protein; CNS, central nervous system; CFA, complete Freund's adjuvant; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; HGG, human gamma globulin; MNC, mononuclear cell.

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resulting preparation was freed of excess DTT and pyridine-2-thione by Sephadex G-25 chromatography. SPDP-derivatized CTB and MBP were then mixed at equimolar ratio and incubated for 16 hr at 23°C. The resulting CTB–MBP conjugate was purified by gel filtration through a column of Sephacryl S-300. The conjugate retained both GM1-binding activity and MBP serological reactivity, as judged by a solid-phase ELISA using GM1 as the capture system (17) and enzyme-labeled anti-MBP antibodies as the detection reagent. Quantitations of free and bound MBP and free and bound CTB were made by reference to standard curves established by assaying known amounts of unconjugated antigens (16). On average, conjugates used in this study contained less than 10% free CTB and negligible amounts of unconjugated MBP.

Human gamma globulin (HGG) was conjugated to CTB as above and the HGG–CTB conjugate was purified as described (12).

Induction of Oral Tolerance. Lewis rats were fed MBP–CTB conjugate, HGG–CTB conjugate, MBP alone, or a mixture of free CTB and MBP, diluted in an antacid buffer containing 0.35 M $NaHCO_3$, by means of gastric intubation with a baby feeding tube. Animals were fed either once or two to five times at 2-day intervals either before or after EAE induction.

Delayed-Type Hypersensitivity (DTH) to MBP. DTH reactivity was tested 18 days after EAE induction by "challenge" intracutaneous injection into the ear of 50 μg of MBP in pyrogen-free saline. Ear thickness was measured before and 24 hr after challenge, by using a micrometer caliper (Oditest, Essen, Germany). The intensity of DTH reactions after challenge was determined for each individual animal by substracting the value obtained before challenge from that obtained 24 hr after challenge. Specific ear thickness increment was calculated by substracting the mean background swelling of unprimed control animals at 24 hr after challenge from the swelling of the primed test animals to provide the net ear skin responses, expressed in units of cm $\times 10^{-3}$.

Lymphocyte Proliferative Responses. Triplicate cultures of mononuclear cell (MNC) suspensions from popliteal lymph nodes draining the sites of EAE induction with MBP in CFA were established in round-bottomed microtiter plates (Nunc) in Iscove's medium with 5% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, and antibiotics. MBP (10 μg/ml), *M. tuberculosis* purified protein derivative (10 μg/ml) (Serum Institute, Copenhaguen, Denmark), or Con A (2 μg/ml) was added at the start of the culture period and aliquots of supernatants were collected at 24 hr for interleukin 2 (IL-2) measurement (see below). Proliferation was assessed by measurement of [^{3}H]thymidine uptake during the last 16 hr of a 72-hr culture period. Results were expressed as mean stimulation indices, calculated as the ratio of radioactivity incorporated in antigen- or mitogen-exposed cultures compared with replicate cultures exposed to medium alone.

Cytokine Assays. Culture supernatants from lymph-node MNCs were assayed for IL-2 contents by using the murine IL-2-dependent CTLL-2 subclone (18). IL-2 levels were determined by measuring [^{3}H]thymidine uptake by CTLL cells exposed to test supernatants, and values were calculated in units per ml by reference to a standard curve obtained by assaying known amounts of recombinant human IL-2 (Genzyme).

Interferon-γ (IFN-γ) production was determined by reverse ELISPOT assay (19) using mouse monoclonal anti-rat IFN-γ antibody (DB-1 clone, provided by Peter van der Meide, Biomedical Primate Research Centre, Rijswijk, The Netherlands) immobilized on nitrocellulose membranes (Millipore) as solid-phase capture system and rabbit antiserum to rat IFN-γ as primary detection reagent. Briefly, popliteal and mesenteric lymph node cells were incubated for 48 hr in anti-IFN-γ-coated wells with MBP (10 μg/ml). Plates were then thoroughly washed with PBS/0.05% Tween 20, and individual wells were exposed to primary anti-IFN-γ antiserum appropriately diluted in wash buffer. After sequential incubation with biotinylated swine anti-rabbit Ig (Dakopatts), avidin-peroxidase (Sigma), and chromogen substrate, plates were thoroughly washed with running water and examined for the presence of spots. The latter, indicating the presence of IFN-γ secreted from individual cells, were enumerated under low magnification and values were adjusted to 10^6 MNCs.

Histology. Spinal cords from animals sacrificed 15 days after EAE induction were dissected, and segments from the lumbar region were processed for routine histology. Serial sections (4 μm) of formalin-fixed paraffin-embedded tissue were stained with hematoxylin and eosin. Randomly selected serial sections were examined for the presence of infiltrating leukocytes. All histological determinations were performed by a single observer who was unaware of the treatment assigned to each animal.

RESULTS

Pretreatment with Oral CTB–MBP Conjugate Protects Rats from Clinical EAE. Lewis rats were fed 25 μg of MBP conjugated to CTB given once or thrice before a systemic subcutaneous challenge with MBP in CFA. Additional groups of rats received unconjugated MBP given orally as small (25–100 μg) or large (1 mg) doses. Control animals received an irrelevant antigen, HGG, coupled to CTB or saline only. As Table 1 shows, pretreatment with a low dose (25 μg) of MBP conjugated to CTB strongly suppressed disease: 13 out of the 14 Lewis rats pretreated with a single oral dose of CTB–MBP were completely protected from EAE, and all five rats given

Table 1. Prevention of EAE by oral pretreatment with CTB-conjugated MBP

Feeding					
Compound	Amount	No. of times	Day(s)	Mean clinical score	Incidence of paralysis
CTB–MBP	25 μg	1	−4	0.3 ± 0.2**	1/14** (93)
CTB–MBP	25 μg	3	−2, −4, −6	0.2 ± 0.2*	0/5** (100)
MBP	25 μg	3	−2, −4, −6	2.5 ± 0.3	4/4 (0)
MBP	100 μg	5	−2, −4, −6, −8, −10	2.8 ± 0.2	5/5 (0)
MBP	1 mg	5	−2, −4, −6, −8, −10	0.4 ± 0.2*	1/9** (89)
CTB–HGG	100 μg	1	−4	3.0 ± 0.3	5/5 (0)
Saline			−2, −4, −6	3.4 ± 0.3	16/16 (0)

Lewis rats were fed unconjugated or CTB-conjugated MBP at the indicated times before induction of EAE by systemic injection of MBP in CFA on day 0. The mean clinical score is the mean (±SEM) maximal clinical score. Numbers in parentheses are the protective efficacy, which is the percentage of animals with a maximal clinical score of ≤1. Significant differences with saline-fed animals, determined by Wilcoxon's rank test for mean clinical score and by Fisher's exact test for incidence of paralysis are indicated (*, $P < 0.01$; **, $P < 0.001$).

three doses of CTB–MBP were also protected. In contrast, all rats fed comparable doses (25–100 μg) of unconjugated MBP, given once (data not shown) or on repeated (three to five times) occasions, developed severe EAE 2 weeks after challenge, as did all control rats fed saline alone or HGG–CTB conjugate. In keeping with earlier reports (5, 6), effective oral pretreatment with unconjugated MBP required considerably larger amounts (1 mg) of free MBP given on five consecutive occasions. These data are consistent with our earlier study with prototype antigens in that they show that targeting an antigen to the gut by chemically linking the antigen to the strong mucosa-binding and immunomodulating molecule CTB can induce a profound state of peripheral tolerance (12).

Oral CTB–MBP Conjugate Protects Rats from Already Induced EAE. Rats were injected on day 0 with MBP in CFA to induce EAE. Six days later (i.e., 4–6 days before the onset of clinical symptoms) animals were fed a single dose of 50 μg of MBP–CTB. As can be seen in Table 2, 12 of 15 rats were protected from EAE and the remaining 3 rats developed a mild form of the disease (clinical score = 2). Further, all 8 rats given the same oral MBP–CTB conjugate on three consecutive occasions were completely protected from EAE. In contrast, all animals fed the same amounts of unconjugated MBP given either once or three times developed EAE, as did all but 1 of 15 animals fed unconjugated MBP with free CTB (Table 2). Three out of 4 animals that received one oral dose of CTB–MBP previously saturated with a 100-fold molar excess of GM1 ganglioside, the natural cell-surface receptor for CTB, developed EAE (Table 2), indicating that the tolerogenic effects of CTB–MBP were dependent on receptor-specific cell surface binding in the host. Oral administrations of a single large (5 mg) dose of unconjugated MBP or of three consecutive 1-mg doses had modest effects on EAE. However, oral administration of large (1 mg) doses of unconjugated MBP repeated on five consecutive occasions effectively reversed EAE in 10 of 13 rats (Table 2).

Lymph Node Cells from Rats Orally Treated with CTB–MBP Have Impaired Proliferative Responses to MBP Associated with Decreased IL-2 Production and Increased IFN-γ Production. MNCs from the draining popliteal lymph nodes of rats injected with MBP in CFA and subsequently treated orally with unconjugated or CTB-conjugated MBP were stimulated *in vitro* with MBP. Proliferation to MBP was almost abrogated in animals treated with one or three doses of CTB-conjugated MBP (Table 3). Similar results were obtained with lymph node cells from animals repeatedly fed large doses (five 1-mg doses) of unconjugated MBP (Table 3). In contrast, animals fed a small dose (50 μg) of unconjugated MBP on three consecutive occasions after disease induction had normal proliferative responses to MBP. Irrespective of treatment, lymph-node MNCs from all animals injected with MBP in CFA mounted proliferative responses to Con A and purified protein derivative that were comparable to those of saline-fed control animals (Table 3).

Table 3. Suppression of MBP-induced T-cell proliferative responses after oral treatment with CTB-conjugated MBP

Oral tolerogen			Stimulation index		
Compound	Amount	No. of times	MBP	PPD	Con A
CTB–MBP	50 μg	1	2.5 ± 0.8*	17 ± 5.6	32 ± 11
CTB–MBP	50 μg	3	2.2 ± 0.6*	19 ± 7.3	43 ± 14
MBP	50 μg	3	8.1 ± 2.7	26 ± 9.1	58 ± 19
MBP	1 mg	5	3.1 ± 0.9*	25 ± 8.4	20 ± 6
Saline			10.3 ± 2.8	17 ± 5.1	32 ± 4.8

Rats were fed unconjugated or CTB-conjugated MBP given one (day +6), three (day +4, +6, +8), or five (day +2, +4, +6, +8, +10) times after induction of EAE by subcutaneous injection of MBP in CFA (day 0). PPD, purified protein derivative. Proliferative responses of popliteal lymph node cells were determined for four to six animals per group in cultures initiated on day 15 and are expressed as mean stimulation index (±SD). (*, $P < 0.05$; Wilcoxon rank test).

IL-2 levels in MBP-stimulated lymph-node MNC cultures from rats treated with low-dose oral CTB–MBP conjugate or repeated high dose of unconjugated MBP were markedly reduced compared with those from saline-fed control animals or from animals given a low dose of MBP admixed with free CTB (Fig. 1). Lymph-node MNC cultures from rats fed repeated large doses of unconjugated MBP had decreased numbers of IFN-γ-secreting cells compared with saline-fed control animals or animals given a low dose of MBP admixed with free CTB (Fig. 1). Unexpectedly, the frequency of IFN-γ-secreting cells in cultures of MBP-stimulated lymph node cells was markedly increased in animals treated with low-dose CTB–MBP (Fig. 1). Thus, oral treatment with low-dose CTB–MBP appears to involve a modification of the peripheral Th-1 cytokine response to MBP, which is distinct from that observed after treatment with large doses of free MBP.

Decreased DTH Reactivity to MBP in Rats Treated with Oral CTB–MBP Conjugate. Skin DTH reactivity in rats treated with a low-dose oral CTB–MBP conjugate or repeated large doses of unconjugated MBP given after EAE induction was determined after ear challenge with MBP. As shown in Fig. 2, animals treated with 50 μg of MBP conjugated to CTB and given orally on one or three occasions after EAE induction had markedly reduced DTH reactivity to MBP compared with animals fed comparable or even much larger (five 1-mg doses)

Table 2. Reversal of EAE by oral treatment with CTB–MBP conjugate

Feeding					
Compound(s)	Amount	No. of times	Day(s)	Mean clinical score	Incidence of paralysis
CTB–MBP	50 μg	1	+6	0.4 ± 0.2**	3/15** (80)
+GM1	50 μg	1	+6	2.0 ± 0.4	3/4 (25)
CTB–MBP	50 μg	2	+4, +6	0.5 ± 0.2*	1/8** (88)
	50 μg	3	+4, +6, +8	0*	0/8** (100)
MBP	50 μg	1	+6	3.3 ± 0.3	4/4 (0)
+CTB	50 μg	1	+6	2.2 ± 0.2	14/15 (7)
MBP	50 μg	3	+4, +6, +8	2.0 ± 0.4	4/4 (0)
	100 μg	5	+2, +4, +6, +8, +10	2.7 ± 0.7	3/3 (0)
	1 mg	3	+4, +6, +8	1.7 ± 1.1	1/3 (67)
	1 mg	5	+2, +4, +6, +8, +10	0.5 ± 0.2**	3/13** (77)
	5 mg	1	+6	2.0 ± 0.6	2/3 (33)
Saline			+4, +6, +8	2.5 ± 0.2	22/22 (0)

Lewis rats were injected with MBP in CFA on day 0 and then at the indicated times fed unconjugated or CTB-conjugated MBP. Results are expressed as in Table 1.

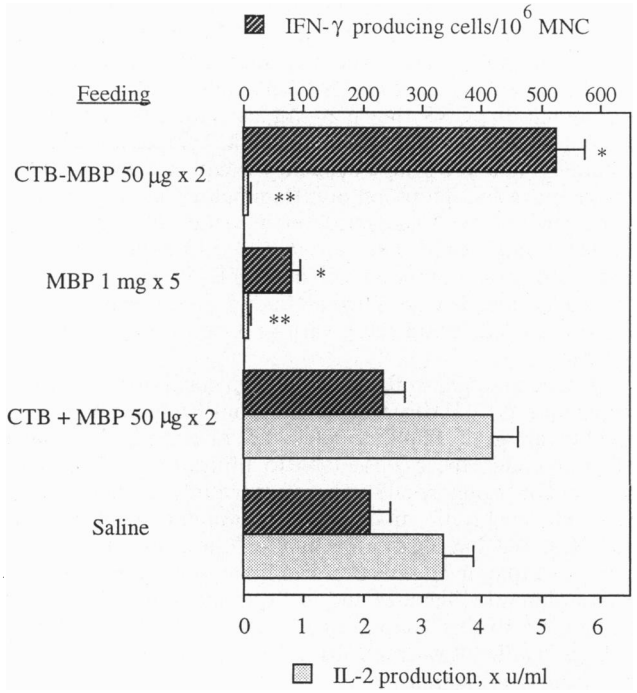

FIG. 1. Reciprocal effects of oral CTB–MBP treatment on IL-2 and IFN-γ production by lymph node cells from Lewis rats. Animals were immunized with MBP in CFA on day 0 and fed with CTB–MBP conjugate, a mixture of unconjugated MBP and free CTB, or five consecutive high doses of unconjugated MBP. On day 15, popliteal lymph node cells were harvested and cell-free IL-2 levels and numbers of IFN-γ-producing cells were determined after 24 hr and 48 hr, respectively. IL-2 levels and mean numbers of IFN-γ-secreting cells were determined on six animals per experimental group and are expressed as the mean ± SD. Significant differences (Wilcoxon rank test) with saline-fed animals are indicated (*, $P < 0.05$; **, $P < 0.01$).

amounts of MBP, although the later animals had a significantly reduced ear skin DTH reactivity to MBP compared with saline-fed control animals.

Rats Protected from EAE by Oral CTB–MBP Treatment Have Fewer Infiltrating Leukocytes in Their Spinal Cords. Histological examination of spinal cord specimens from rats injected with MBP in CFA and subsequently treated with oral CTB–MBP showed a very marked reduction in the magnitude of leukocyte infiltration. Few if any MNC infiltrate could be seen in these animals (Fig. 3). Occasional minor perivascular infiltrates were observed in spinal cord tissue specimens from CTB–MBP fed animals, and in most instances the white matter

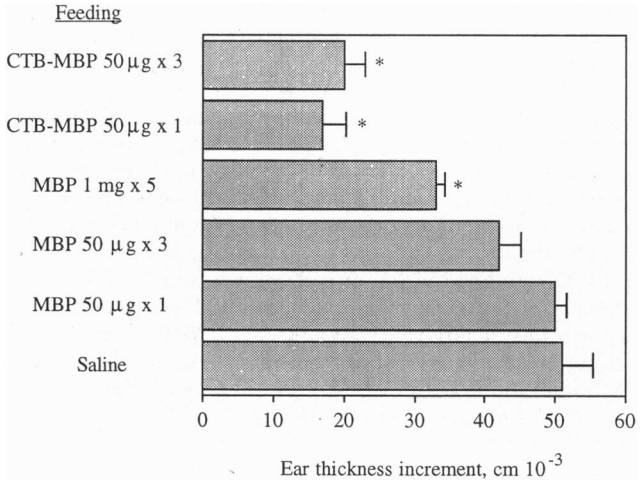

FIG. 2. Low-dose oral CTB–MBP treatment reverses specific systemic DTH reactivity in sensitized Lewis rats. Animals were immunized on day 0 with MBP in CFA and fed with one or three low (50 μg) doses of CTB-coupled MBP or five consecutive high (1 mg) doses of unconjugated MBP. On day 18, ear skin DTH reactivity was determined 24 hr after ear challenge with MBP. Results are expressed as mean ear thickness increment × 10^{-3} cm (±SD), determined on groups of 8–10 animals. Significant differences (Wilcoxon rank test) with saline-fed animals are indicated (*, $P < 0.05$).

was judged free of inflammatory cells. In contrast, numerous perivascular infiltrates and diffuse accumulations of MNCs in the spinal cord white matter were observed in specimens examined from saline-fed animals (Fig. 3). Furthermore, in animals fed repeated large doses of free MBP, spinal cord specimens displayed focal collections of MNCs located around blood vessels and clusters of inflammatory cells in the white matter, although the density of infiltrating cells was relatively lower in animals fed large doses of MBP compared with saline-fed control rats (Fig. 3). Thus, protection from EAE afforded by oral CTB–MBP conjugate is associated with a marked decrease in the level of encephalomyelitis.

DISCUSSION

Previous experiments from this laboratory (12) have shown that oral administration of microgram amounts of particulate or soluble antigens conjugated to the nontoxic mucosa-binding molecule CTB can suppress peripheral DTH reactivity in immunologically naive and systemically sensitized mice. The data presented in this study are consistent with and further extend our previous work (12) with model antigens by showing

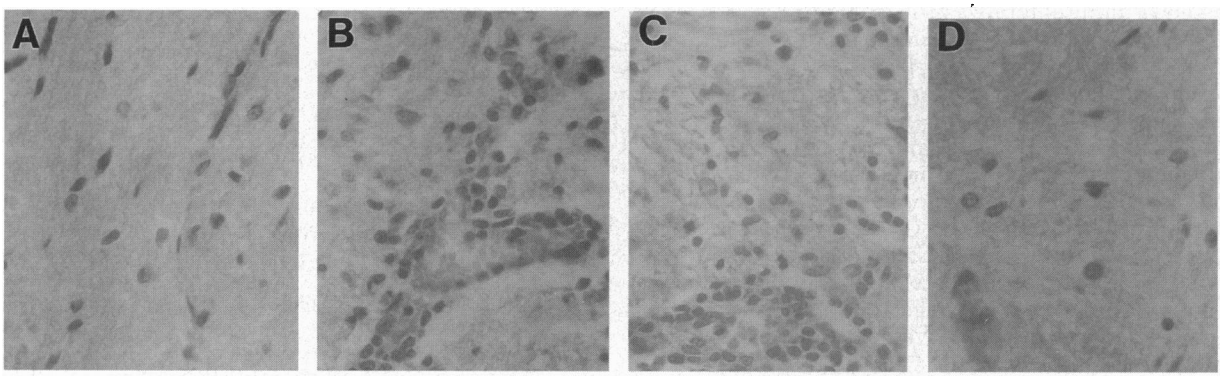

FIG. 3. Oral CTB–MBP treatment suppresses leukocyte infiltration into the CNS. Representative specimens of spinal cord tissue obtained from rats injected with MBP in CFA and subsequently treated with two oral low doses (50 μg) of CTB–MBP conjugate (*A*) or repeated high doses (five 1-mg doses) of unconjugated MBP (*B*). Perivascular cuffs and diffuse accumulations of mononuclear cells are seen in the white matter of animals fed saline (*C*) and in animals treated with high doses (five 1-mg doses) of MBP alone (*B*). Note the absence of such infiltrates in CTB–MBP-treated animals (*A*) and in control (CFA-injected) animals (*D*). (Hematoxylin/eosin staining. ×35.)

that targeting the autoantigen MBP to the gut-associated lymphoid tissue by chemical linking to CTB not only reduces systemic DTH reactivity to MBP but also can prevent as well as reverse the induction of EAE in Lewis rats.

Earlier studies have documented the potential of oral administration of autoantigens to prevent EAE or other experimental autoimmune diseases (3–9). In most instances, the doses of antigens employed have been 100–800 times higher than the doses of MBP now found to be effective when coupled to CTB, and in general the tolerizing effects have been partial (5, 6). Depending on the dose of antigen fed, oral tolerance appears to involve different mechanisms. Thus, oral administration of very large (20 mg) doses of MBP administered repeatedly have been shown to prevent EAE by either direct deletion of autoreactive cells (20) or anergy of MBP-reactive Th1 cells producing IL-2 and IFN-γ (21, 22). On the other hand, Weiner *et al.* (23, 24) have shown that relatively lower yet copious doses (several of milligrams) of MBP administered repeatedly can also protect animals from EAE and have proposed a mechanism involving the recruitment from the gut into the CNS of Th-2-like regulatory cells capable of producing cytokines (transforming growth factor β, IL-4, and/or IL-10) known to antagonize Th-1-driven cell-mediated immune responses (25). Since we did not attempt to measure Th-2 cytokines (e.g., IL-4 or IL-10), the reduced levels of IL-2 and IFN-γ observed in cultures of MBP-stimulated lymph node cells from animals fed large doses of myelin autoantigen could still be compatible with either of the above scenarios.

However, a quite unexpected finding in this study was that, in contrast to proliferative responses and IL-2 production, which were suppressed to a similar extent after repeated feeding with large doses (five 1-mg doses) of free MBP or with even a single dose of 50 μg of CTB-conjugated MBP, IFN-γ production was not suppressed in CTB–MBP-fed animals, at variance with animals fed unconjugated MBP. In fact, there was instead a significant increase in IFN-γ production in cultures of MBP-stimulated lymph node cells from CTB–MBP-fed animals compared with nonfed animals. Since IFN-γ is believed to play an essential role in the recruitment of lymphocytes at sites of inflammation (26), this observation is relatively intriguing. However, it has recently been described (27) that parenterally induced immunological tolerance with model antigens is associated with decreased IL-2 production and elevated IFN-γ (and IL-4) production in draining lymph nodes, a finding similar to the one we observed when feeding CTB–MBP conjugate. Although *in vitro*-induced IFN-γ production may not reflect the situation at sites of potential tissue injury, it is worth recalling (*i*) the protective effects of IFN-γ administration in EAE (28) and the disease-enhancing effects of anti-IFN-γ antibody treatment (29, 30), (*ii*) the known antiproliferative properties of IFN-γ (31, 32), (*iii*) the observation that IFN-γ can promote the release of active transforming growth factor β (33), a cytokine known to protect animals against EAE (34, 35), and (*iv*) recent findings indicating that *in vitro* neutralization of IFN-γ restores and even increases autoantigen-induced proliferation of lymph node cells from animals orally tolerized with CTB–MBP (unpublished data). We have also shown that in humans oral administration of CTB-containing vaccines leads to increased IFN-γ production in the gut (36) and to the generation of circulating T cells capable of producing IFN-γ but not IL-2 when challenged *ex vivo* with fed antigen (37). It thus appears that the cytokine pattern associated with protection against clinical EAE after feeding MBP alone or conjugated to CTB differs. These observations not only suggest that at least partly distinct mechanisms govern the protective effects of these different treatment regimens but also support the notion that IFN-γ acts as an antiinflammatory mediator in EAE.

Another surprising finding was the almost lack of infiltrating leukocytes in the spinal cord of CTB–MBP fed rats. If we assume that mucosally derived regulatory cells with an unusual cytokine repertoire, unlike Th-1 cells and Th-2 cells, do not migrate into the CNS, they may still leave the gut via draining (mesenteric) lymph nodes and enter the circulation and peripheral lymphoid organs where they would interfere with the recruitement and migration of inflammatory leukocytes from the peripheral lymphoid compartment to the CNS. The finding that large numbers of IFN-γ-producing cells appear in lymph nodes from animals treated with oral CTB–MBP is compatible with such a mechanism. Further studies on the expression of adhesion molecules on these various T-cell subsets could be valuable.

We conclude that oral treatment with small doses of MBP-conjugated to CTB can protect rats from EAE and induces a modification in the cytokine repertoire of encephalitogenic T cells and renders these cells unable to infiltrate the CNS. Given these encouraging results, we are currently evaluating this approach in chronic models of autoimmune demyelinating disorders. We have also documented the efficiency of this strategy of tolerance induction in animal models of spontaneous autoimmune diabetes and in experimentally induced autoimmune arthritis (unpublished results). These promising findings in animals warrant further studies on the efficiency of this strategy in humans.

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