

Antigen-driven Bystander Suppression after Oral Administration of Antigens

By Ariel Miller, Ofer Lider, and Howard L. Weiner

From the Center for Neurologic Diseases, Division of Neurology, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Summary

Suppression of experimental autoimmune encephalomyelitis (EAE) in Lewis rats by the oral administration of myelin basic protein (MBP) is mediated by CD8⁺ T cells that can be isolated from the spleens of MBP-fed animals. These cells adoptively transfer protection to naive animals subsequently immunized with MBP and complete Freund's adjuvant (CFA) and suppress *in vitro* MBP proliferative responses. Using a transwell system in which the modulator spleen cells from MBP-fed animals are separated by a semipermeable membrane from responder cells, MBP, or OVA-specific T cell lines, we have found that cell contact is not required for *in vitro* suppression to occur. *In vitro* suppression is dependent, however, upon antigen-specific triggering of modulator T cells. Once antigen-specific triggering occurs, suppression across the transwell is mediated by an antigen-nonspecific soluble factor that equally suppresses an MBP line or an ovalbumin (OVA) line. This phenomenon of antigen-driven bystander suppression was also demonstrated *in vivo*. Specifically, Lewis rats fed OVA which were then immunized with MBP/CFA plus OVA given separately subcutaneously were protected from EAE. Animals fed OVA and then immunized with MBP/CFA without OVA given subcutaneously were not protected. The protective effect of feeding OVA could be adoptively transferred by CD8⁺ T cells from OVA-fed animals into MBP/CFA plus OVA-injected animals. Feeding bovine serum albumin (BSA) or keyhole limpet hemocyanin did not suppress EAE in animals immunized with MBP/CFA plus OVA. EAE was suppressed, however, if BSA was fed and animals then immunized with MBP/CFA plus BSA given subcutaneously. Antigen-driven bystander suppression appears to be an important mechanism by which antigen-driven peripheral tolerance after oral administration of antigen is mediated, and presumably occurs in the microenvironment accounting for the antigen specificity of suppression generated by oral tolerization to antigens.

A variety of mechanisms have been described for the maintenance of self-tolerance in the host. These include clonal deletion (1), clonal anergy (2, 3), and active suppression (4; and reviewed in reference 5). We have been studying antigen-driven active suppression after oral administration of antigens as a tolerance mechanism and as a method to down-regulate autoimmune diseases in experimental animals (6–11). In the experimental autoimmune encephalomyelitis (EAE)¹ model, oral administration of myelin basic protein (MBP) suppresses clinical, histologic, and immune parameters of the disease (6, 12). This effect is mediated by CD8⁺ T cells, which can adoptively transfer protection and suppress *in vitro* proliferative responses (7). Although various mechanisms of active suppression have been demonstrated (5, 13), the precise mechanism(s) by which T cells mediate suppression re-

mains to be defined. The present study uses both an *in vitro* and *in vivo* model system to examine the mechanism of suppression mediated by T cells after oral administration of antigen. Our results demonstrate that *in vitro* such cells are triggered in an antigen-specific fashion but mediate their effect via an antigen-nonspecific suppressor factor. Based on these *in vitro* findings, antigen-driven bystander suppression has also been demonstrated *in vivo* in the EAE model.

Materials and Methods

Animals. Female Lewis rats 6–8 wk of age were obtained from Harlan-Sprague Dawley Inc. (Indianapolis, IN). Animals were housed in Harvard Medical School animal care facilities and maintained on standard laboratory chow and water *ad libitum*. Animals were maintained in accordance with the guidelines of the Committee on Care of Laboratory Animals of the Laboratory Research Council.

Antigens. Guinea pig MBP was purified from brain tissue by a method modified from Deibler et al. (14) and purity checked by gel electrophoresis. OVA and BSA were purchased from Sigma

¹ Abbreviations used in this paper: DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; PPD, purified protein derivative.

Chemical Co. (St. Louis, MO) and KLH from Calbiochem Behring Corp. (La Jolla, CA).

Immunization of Animals. Animals were immunized with 25 μ g of MBP in the footpad, emulsified in an equal volume of CFA containing 4 mg/ml of mycobacterium tuberculosis (Difco Labs, Detroit, MI). For in vivo bystander suppression experiments, 50–300 μ g of the secondary antigens OVA, BSA, or KLH were injected subcutaneously in the same footpad in 100 μ l PBS 8 h after primary immunization with MBP/CFA.

Clinical Evaluation. Animals were evaluated in a blinded fashion every day for evidence of EAE. Clinical severity of EAE was scored as follows: 0, no disease; 1, limp tail; 2, hind limb paralysis; 3, hind limb paraplegia, incontinence; 4, tetraplegia; 5, death. Mean maximal clinical severity was calculated as previously described for each experimental group (7). Statistical analysis was performed using a one-tailed student's *t* test or a χ^2 analysis for comparing incidence between groups.

Induction of Oral Tolerance. Animals were fed 1 mg MBP, OVA, BSA, or KLH dissolved in 1 ml PBS or PBS alone, by gastric intubation using an 18-gauge stainless steel animal feeding needle (Thomas Scientific, Swedesboro, NJ). Animals were fed five times (total dose of 5 mg), at intervals of 2–3 d with the last feeding 2 d before immunization.

Delayed Type Hypersensitivity (DTH) Testing. DTH was tested by injecting 50 μ g of MBP or OVA in PBS, subcutaneously into the ear. MBP was injected in the left ear and OVA in the right ear in the same animal. Thickness, in units of 0.01 inch, was measured in a blinded fashion, before and 48 h after challenge, using micrometer calipers (Mitutoyo, Utsunomia, Japan). Change in ear thickness before and after challenge was recorded for each animal, and results were expressed as the mean for each experimental group \pm SEM; each group consisted of five animals.

Transwell Cultures. A dual chamber transwell culture system (Costar, Cambridge, MA), which is 24.5 mm in diameter and consists of two compartments separated by a semi-permeable polycarbonate membrane, with a pore size of 0.4 μ m, was used. The two chambers are 1 mm apart, allowing cells to be coincubated in close proximity without direct cell-to-cell contact. To measure in vitro suppression of proliferative responses in transwell cultures, 5×10^4 MBP- or OVA-specific line cells, raised and maintained as previously described (15), were cultured with 10^6 irradiated (2,500 rad) thymocytes, in 600 μ l of proliferation media in the lower well. Spleen cells from orally tolerized rats or controls (fed PBS) were added to the upper well (5×10^5 cells in 200 μ l). Spleen cells were removed 7–14 d after the last feeding, and a single cell suspension was prepared by pressing the spleens through a stainless steel mesh. MBP and OVA (50 μ g/ml) were added in a volume of 20 μ l. Because modulator cells are separated from responder cells by a semi-permeable membrane, they do not require irradiation. In some experiments, modulator cells were added in the lower well together with responder cells, and in these instances modulator cells were irradiated (1,250 rad) immediately before being placed in culture. Proliferation media consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2×10^{-5} M 2-ME, 1% sodium pyruvate, 1% penicillin and streptomycin, 1% glutamine, 1% HEPES buffer, 1% nonessential amino acids, and 1% autologous serum. Each transwell was performed in quadruplicate. The transwells were incubated at 37°C in a humidified 6% CO₂ and 94% air atmosphere for 72 h. After 54 h of culture, each lower well was pulsed with 4 μ Ci of [³H]thymidine and at 72 h split and reseeded to three wells in a round-bottomed 96-well plate (Costar) for harvesting onto fiberglass filters and counting using standard liquid scintillation techniques. Percent suppression = $100 \times$

$(1 - \Delta \text{ cpm responders cultured with modulators} / \Delta \text{ cpm of responders})$.

Purification of T Cell Subsets. Depletion of T cell subsets was performed by negative selection using magnetic beads according to the modified method of Cruikshank et al. (16). Spleen cells were incubated with a 1:100 dilution of mouse anti-rat CD8, or CD4, mAbs (clones OX/8 or W3/25 Serotec/Bioproducts, Indianapolis, IN) for 30 min on ice, washed twice, and then added to prewashed magnetic particles, with an average diameter of 450 μ (M-450) with goat anti-mouse IgG covalently attached (Dynal Inc., Fort Lee, NJ). The quantity of magnetic beads used was calculated as being 10 times the estimated target cell population. The cells were incubated with the beads in 0.5 ml of RPMI 1640 supplemented with 10% FCS in a 10 ml round-bottomed test tube (Nunc, Roskilde, Denmark) for 30 min on ice with gentle shaking every 5 min. After incubation, the bead/cell suspension was washed with 5 ml of medium and cell-mAb-bead complexes were separated from unlabeled cells in a strong magnetic field using a magnetic-particle concentrator (Dynal-MPC-1) for 2 min. The supernatant was removed, and the procedure repeated twice to obtain the nonadherent fraction. The T cells in the depleted population were >95% CD4⁺CD8⁻ or CD8⁺CD4⁻ as demonstrated by indirect flow cytometry.

Adoptive Transfer of Disease Suppression. Donor rats were fed either 1 mg MBP, OVA, or KLH, five times at 2-d intervals and killed 7–14 d after the final feeding. Spleen cells were harvested, and incubated in vitro with the homologous antigen (50 μ g/ml) in proliferation medium, for 72 h. Cells were injected intraperitoneally: 10^8 cells for whole spleen populations or $5\text{--}6 \times 10^7$ cells for CD8- or CD4-depleted populations. Recipient animals were irradiated (250 rad) before adoptive transfer, immunized with MBP/CFA 6 h after adoptive transfer, and challenged 8 h later with 50 μ g OVA.

Results

Cognate Recognition Is Not Required for In Vitro Suppression. We have previously shown suppression of an MBP line, when the line was co-incubated with splenic T cells from an animal orally tolerized to MBP (7). To determine whether cell-to-cell contact is required for such in vitro suppression to occur, a transwell system was used. As shown in Table 1, when irradiated splenocytes from MBP-fed animals were incubated together with an MBP line in the lower well, there was suppression of proliferation (line 2), while no suppression was observed with splenocytes from PBS fed animals (line 3). Virtually identical suppression was observed when modulator cells were separated from responder cells by the semipermeable membrane (lines 4 and 5). Thus, suppression appears to be mediated by a soluble factor or factors that diffuse through the transwell membrane.

In Vitro Suppression Is Triggered in an Antigen-specific Fashion but Is Antigen Nonspecific in its Effect. To determine whether the in vitro suppression observed in the transwell system requires identical antigen specificity between modulator and responder cells, an OVA line was placed in the lower well. As shown in Table 2, modulator cells from MBP-fed animals placed in the upper well were able to suppress an OVA line in the lower well, in the presence, but not in the absence, of MBP (lines 2 and 3). MBP added to modulator cells from

Table 1. *Suppression of an MBP T Cell Line by Spleen Cells from MBP-fed Donors in Transwell System*

Upper well	Lower well	Δ cpm	Percent suppression
1. -	MBP line	37,809 \pm 3,326	-
2. -	MBP line + MBP-fed modulators	18,412 \pm 1,867	51
3. -	MBP line + PBS-fed modulators	34,631 \pm 3,994	8
4. MBP-fed modulators	MBP line	15,620 \pm 2,294	59
5. PBS-fed modulators	MBP line	34,043 \pm 3,731	10

5×10^4 MBP line cells + MBP (50 μ g/ml) were placed in the lower well with 10^6 irradiated (2,500 rad) thymocyte as APC. Splenic modulator cells (5×10^5) from MBP- or PBS-fed animals were added to either the upper or lower well. Modulator cells added to the lower well were irradiated (1,250 rad). Background counts of the MBP line without MBP added were between 1,000 and 2,000 cpm.

Table 2. *Suppression of an OVA or MBP T Cell Line by Spleen Cells from MBP- or OVA-fed Donors in Transwell System*

Modulator (upper well)	Responder (lower well)	Δ cpm	Percent suppression
1. -	OVA line + OVA	62,761 \pm 3,881	-
2. MBP-fed	OVA line + OVA	65,868 \pm 3,989	-5
3. MBP-fed + MBP	OVA line + OVA	30,974 \pm 3,450	51
4. PBS-fed + MBP	OVA line + OVA	61,132 \pm 2,967	<1
5. -	MBP line + MBP	71,503 \pm 4,581	-
6. OVA-fed	MBP line + MBP	67,075 \pm 2,904	6
7. OVA-fed + OVA	MBP line + MBP	37,778 \pm 3,780	47
8. PBS-fed + OVA	MBP line + MBP	68,104 \pm 4,832	5

5×10^4 MBP or OVA line cells were placed in the lower well with 10^6 irradiated (2,500 rad) thymocytes as APC. Modulator cells (5×10^5) from MBP-, OVA-, or PBS-fed animals were added to the upper well. Background counts of the MBP and OVA lines without MBP or OVA added were between 1,000 and 2,000 cpm.

animals fed PBS did not suppress the OVA line (line 4). Conversely, suppression of an MBP line was seen with modulator cells from OVA-fed animals in the presence of OVA (line 7). Of note is that soluble antigen added to the transwell in either well diffuses across the membrane and thus is present in both wells.

In Vivo Bystander Suppression. To determine the relevance of the above in vitro bystander suppression to the in vivo situation, a series of experiments were conducted in the EAE model. Rats were fed OVA (1 mg, five times over a 10-d period), then immunized with MBP/CFA in the footpad and given OVA 8 h later in the same footpad. As shown in Fig. 1 A, injecting OVA in the footpad 8 h after immunization with MBP/CFA had no effect on EAE. Mean maximal clinical disease severity was 3.9 ± 0.2 for MBP/CFA immunized and 3.8 ± 0.1 with OVA given subcutaneously. However, if animals were fed OVA before immunization with MBP/CFA after which OVA was given subcutaneously in the footpad, suppression of EAE occurred in an analogous fashion to feeding MBP (Fig. 1 B); disease severity in OVA fed plus OVA given subcutaneously is 0.9 ± 0.2 , in MBP fed is 1.1 ± 0.1 , and in the OVA fed and KLH given subcutaneously control group

is 3.9 ± 0.1 ($p < 0.001$, OVA and MBP fed vs. control). No suppression of EAE was observed in animals fed OVA in whom KLH was given after MBP/CFA immunization. Feeding BSA or KLH had no effect on EAE in animals immunized with MBP/CFA plus OVA subcutaneously (Fig. 1 C), disease severity is 3.7 ± 0.1 and 3.8 ± 0.2 , respectively. These experiments demonstrate an in vivo effect similar to that seen in vitro in the transwell system. Specifically, modulator cells generated by oral tolerization to one antigen can suppress cells of a different antigen specificity when the tolerizing antigen is present.

DTH Responses Associated with In Vivo Bystander Suppression. We have previously found a correlation between DTH responses and the elicitation of suppression of EAE (8). To determine whether a similar correlation existed in the in vivo bystander system and to determine the degree of sensitization that occurs in association with the bystander effect, DTH responses were measured. Suppressed DTH responses to MBP were observed both in animals fed MBP and those fed OVA that were subsequently immunized with the MBP/CFA plus OVA (Fig. 2). Oral administration of other antigens, such as KLH or BSA, had no effect on DTH responses to MBP

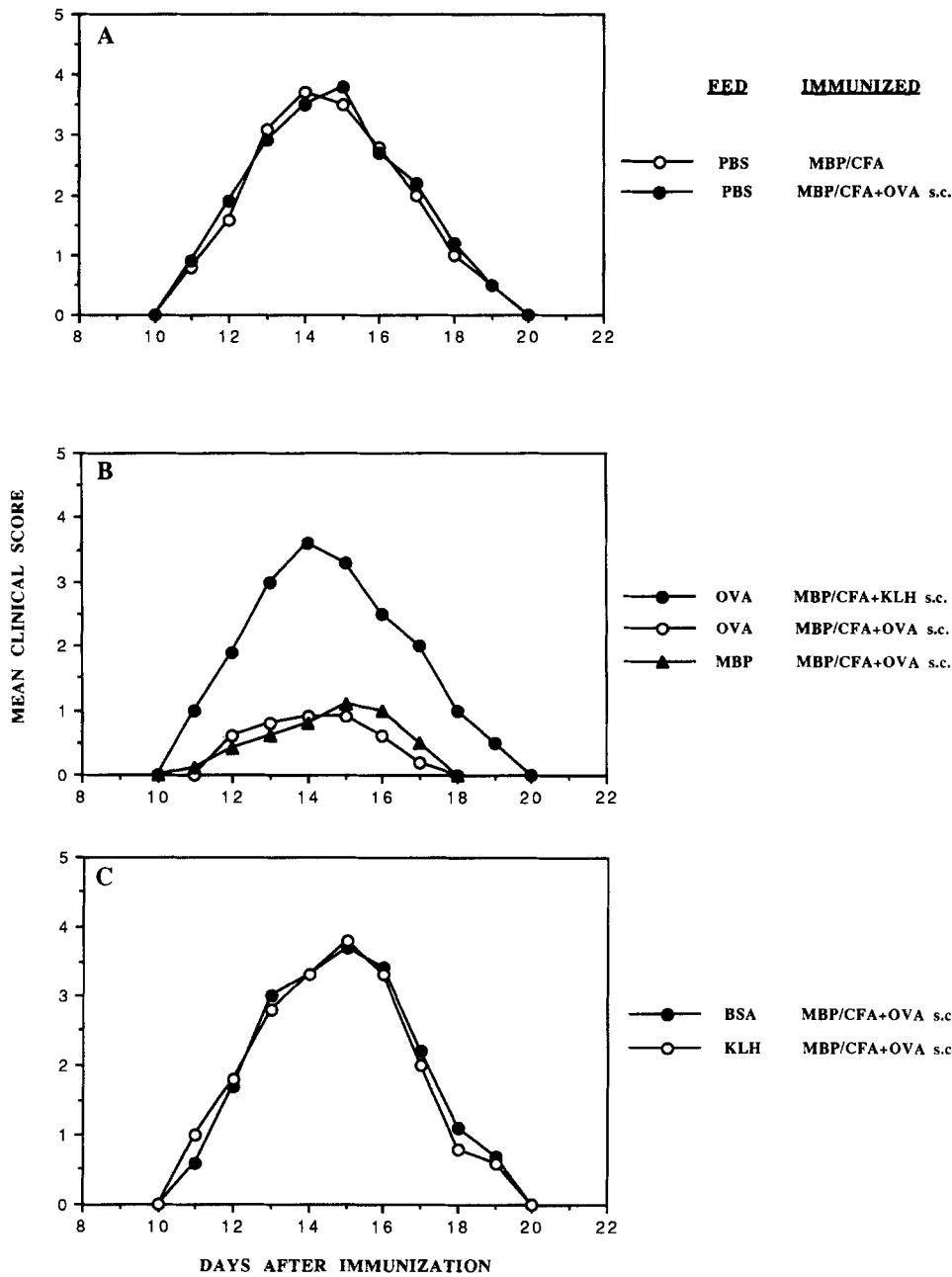


Figure 1. In vivo bystander suppression. Lewis rats were fed 1 mg MBP, OVA, BSA, KLH, or 1 ml PBS five times over a 10-d period then induced for EAE as described in Materials and Methods. 8 h later, the animals were injected subcutaneously with 50 μ g of OVA or KLH, in 100 μ l PBS, in the same footpad in which primary immunization with MBP/CFA occurred. Clinical signs of EAE were monitored as in Materials and Methods. Mean maximal clinical disease severity for control animals fed OVA and given KLH subcutaneously was 3.9 ± 0.1 vs. 0.9 ± 0.2 , and 1.1 ± 0.1 for animals fed OVA or MBP, respectively ($p < 0.001$).

in these animals. Feeding OVA followed by the injection of OVA subcutaneously in association with MBP/CFA did not generate an immune response to OVA as measured by DTH.

In Vivo Bystander Suppression Associated with BSA. To rule out the possibility that something unique to OVA was responsible for the in vivo bystander suppression observed, similar experiments were conducted in which BSA was fed and then given subcutaneously after MBP/CFA immunization. As shown in Fig. 3, oral administration of BSA prior to immunization with MBP/CFA followed by BSA given subcutaneously suppressed EAE in an analogous fashion as that seen with OVA. Of note is that suppression of EAE associated with BSA was observed only when the secondary antigen

was given subcutaneously at a dose of 300 μ g, whereas with OVA, suppression occurred at a dose of 50 μ g.

Adoptive Transfer of Bystander Suppression. We have previously shown that protection after oral tolerization to MBP can be adoptively transferred by CD8⁺ T cells (7). As shown in Fig. 4, spleen cells from MBP- or OVA-fed animals adoptively transferred protection into naive recipients, which were immunized with MBP/CFA and given OVA subcutaneously. Furthermore, adoptively transferred suppression was abrogated by depletion of CD8⁺, but not by depletion of CD4⁺ cells. No protection was observed with the adoptive transfer of spleen cells from KLH-fed animals to animals immunized with MBP/CFA plus OVA.

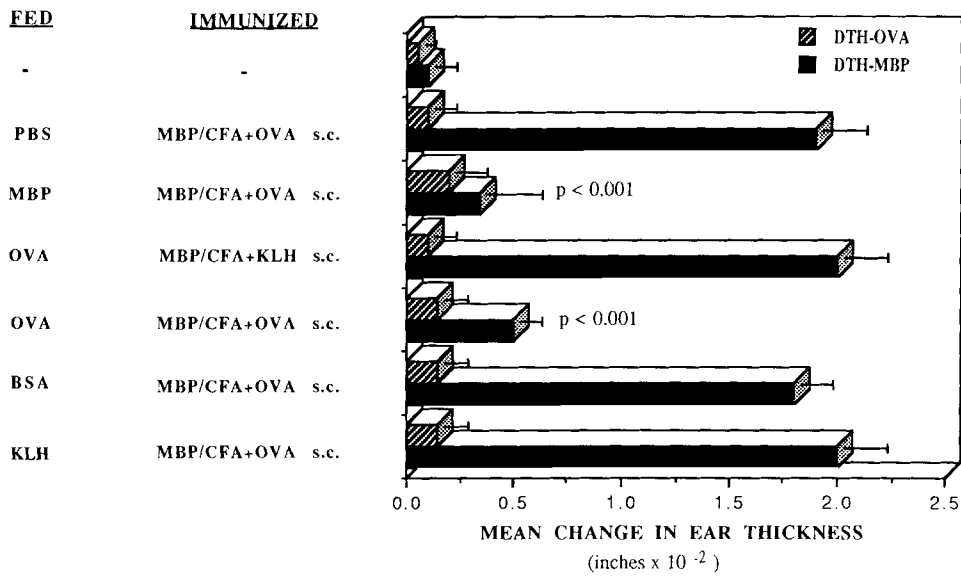


Figure 2. DTH responses associated with in vivo bystander suppression. DTH responses to MBP and OVA were measured 20 d after immunization with MBP/CFA + OVA in association with the experiment illustrated in Fig. 1. Statistical significance was calculated using the student's *t* test. Feeding MBP or OVA vs. feeding PBS, $p < 0.001$.

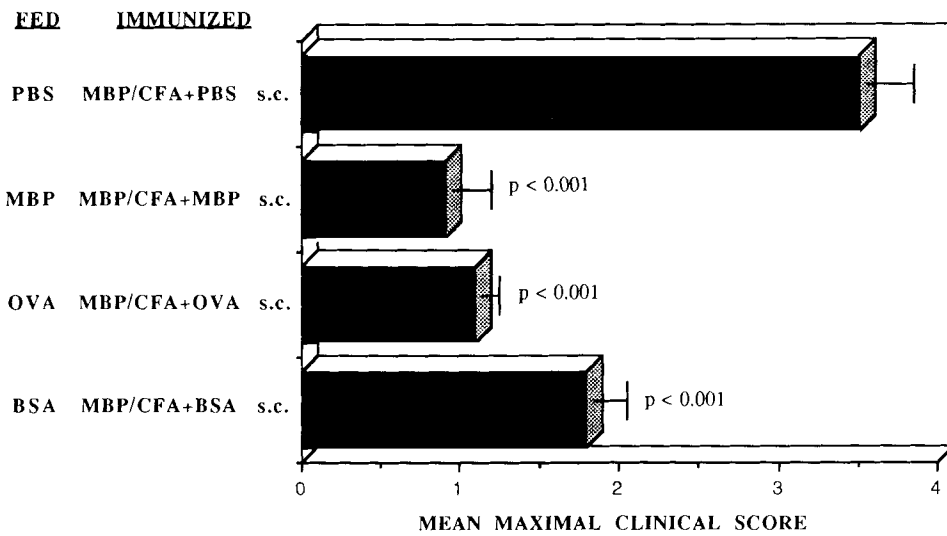


Figure 3. In vivo bystander suppression associated with BSA. BSA, OVA, or MBP were fed 1 mg five times over a 10-d period, then animals immunized with MBP/CFA and 8 h later challenged with 300 μ g of BSA, OVA, or MBP, respectively, at the site of primary immunization.

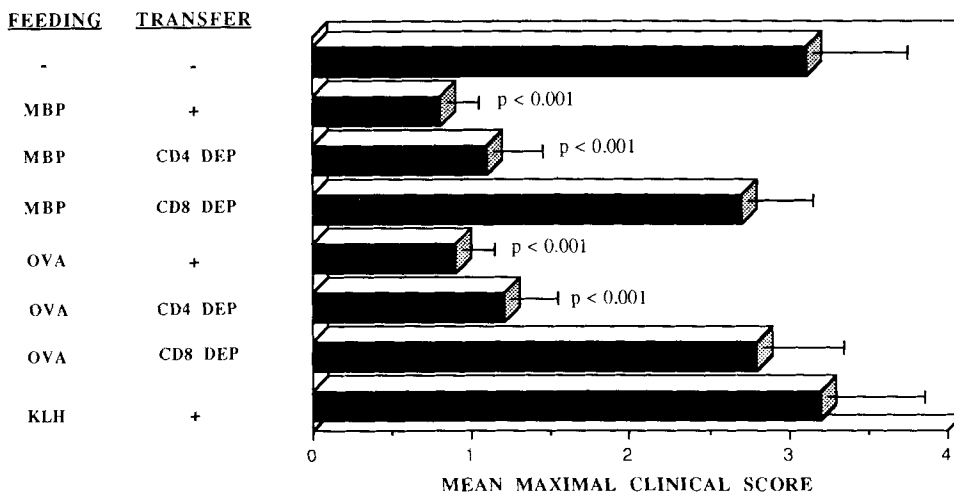


Figure 4. Adoptive transfer of bystander suppression. Donor rats were fed either 1 mg MBP, OVA, or KLH, five times at 2-d intervals, and spleen cells were harvested 7-14 d after the final feeding and incubated in vitro with the homologous antigen (50 μ g/ml) for 72 h before adoptive transfer. Animals were immunized with MBP/CFA 6 h after adoptive transfer, and 8 h later were injected with OVA (50 μ g subcutaneously), as described in Fig. 1. The mean maximal severities of EAE for each experimental group (five animals/group) \pm SEM are presented.

Discussion

In the present report, we have begun to define the mechanism by which cells generated after oral administration of antigen suppress immune responses *in vitro* and induce a form of immunological tolerance via active suppression *in vivo*. T cells generated after induction of oral tolerance are triggered in an antigen-specific fashion and release antigen nonspecific factors that mediate suppression. We have termed this mechanism "antigen-driven bystander suppression". Bystander inflammatory immunologic processes have been described both for EAE and in animal models of arthritis. Bystander central nervous system inflammation occurs in animals sensitized to purified protein derivative (PPD) by immunization with PPD (17) or inoculation with PPD lines when PPD is injected into the brain (18). Similarly, bystander arthritis can be induced by active immunization with various antigens followed by intra-articular challenge with the specific antigen (19–21). Our results are the first demonstration of a bystander immunologic process that suppresses or downregulates an autoimmune inflammatory response rather than enhances it.

Bystander suppression of EAE does not appear to be related to the generation of a competing immune response to OVA, as no DTH responses to OVA develop *in vivo* after either feeding or subcutaneous injection of OVA in PBS. Moreover, adoptive transfer experiments demonstrate bystander suppression involves an active mechanism mediated by antigen-specific CD8⁺ cells. Gautam and Glynn (22) reported that emulsifying OVA with MBP in CFA suppressed EAE by inhibiting activation of MBP-specific T cells, presumably by competition for antigen presentation. We observed no inhibition of EAE when OVA was given subcutaneously in PBS, 8 h after immunization with MBP/CFA. Furthermore, *in vivo* bystander suppression of EAE is not unique to OVA, as we have observed similar suppression with another antigen (BSA) tested in this fashion.

Oral tolerance to autoantigens is both disease and antigen specific. Feeding MBP suppresses EAE but does not affect experimental autoimmune uveitis or adjuvant arthritis (10, 11). Similarly, feeding type II collagen suppresses arthritis models but not EAE (11, 23, 24). Thus, the secretion and action of antigen-nonspecific factors by regulatory cells induced by oral tolerance must occur in the local microenvironment of the lymphoid tissue where the immune response is generated, along migratory pathways of the effector cells and/or at the inflamed site in the target organ where the autoantigen is present. Studies are currently in progress to further elucidate the temporal sequence and location of these interactions between regulatory and effector cells. Of note is that transfer of serum from orally tolerized animals does not affect immune responses or EAE (unpublished data), indicating that the antigen-nonspecific factors released by modulator cells from orally tolerized animals do not appear to be acting systemically.

Several cytokines exert suppressive activity on different aspects of the immune responses such as cell proliferation, differentiation, and the production and release of other cytokines (25). The factor released by T cells that mediates the immunosuppression after oral tolerization, both *in vitro* and *in vivo*, appears to be related to transforming growth factor β (TGF- β), or a member of the TGF- β family. In recent experiments, we have found the abrogation of suppression by culture supernatants of modulator cells and the abrogation of oral tolerance to MBP in the EAE model by anti-TGF- β neutralizing antibodies (26, and Miller, A., A. Roberts, M. Sporn, O. Lider, and H. L. Weiner, manuscript submitted for publication).

The *in vivo* bystander suppression we have described may have implications for the treatment of other autoimmune diseases by oral tolerization. We have previously found that oral administration of collagen type II suppresses adjuvant arthritis (11). Given our present results, it may be that the mechanism for such suppression relates to the generation of collagen-specific suppressor cells that are generated by feeding and that migrate to the joint where they are triggered by collagen to release antigen-nonspecific suppressor cytokines. We have also recently found suppression of diabetes in the NOD mouse by oral administration of insulin, even though there is no clear evidence that autoreactivity to insulin is involved in the pathogenesis of diabetes in the NOD mouse (27). Thus, the treatment of an organ-specific autoimmune disease by oral tolerization may not require knowledge of the inciting autoantigen, only the oral administration of an autoantigen from the target organ.

Although the mechanisms by which active suppression leads to immunologic unresponsiveness are not completely understood, our findings suggest that a common mechanism may exist among different systems. The characteristics of the cells that mediate active suppression induced by oral tolerance described in this report are similar to those described in human lepromatous leprosy (27), in which CD8⁺ suppressor cell clones are triggered in an antigen-specific fashion, but mediate suppression via an as yet unidentified antigen-nonspecific mechanism. Other forms of antigen-driven peripheral tolerance have been described in the EAE model, including autoantigen given intravenously (28) or coupled to splenocytes (28–30), and a form of bystander suppression has also been described using haptens (32). Whether the mechanisms underlying these forms of antigen-driven suppression are similar to the immunological mechanisms of oral tolerance is yet to be defined.

In summary, our results further characterize the mechanism of action of CD8⁺ cells generated after oral tolerization, and may provide a paradigm to understand the mechanism by which active suppression participates in the generation of tolerance to autoantigens.

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Address correspondence to Howard L. Weiner, Center for Neurologic Diseases, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.

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