Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 388–391, January 1996
Immunology

Oral tolerance in myelin basic protein T-cell receptor transgenic mice: Suppression of autoimmune encephalomyelitis and dose-dependent induction of regulatory cells

(peripheral tolerance/transforming growth factor β/interleukin 4/anergy/suppression)

YOUHAI CHEN*, JUN-ICHI INOBE*, VIJAY K. KUCHROO*, JODY L. BARON†, CHARLES A. JANEWAY, JR.†‡, AND HOWARD L. WEINER*§

*Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115; †Section of Immunobiology, Yale University School of Medicine, New Haven, CT 06520; and ‡Howard Hughes Medical Institute, New Haven, CT 06520

Communicated by David W. Talmage, University of Colorado, Denver, CO; October 4, 1995 (received for review May 26, 1995)

ABSTRACT **Orally administered antigens induce a state of immunologic hyporesponsiveness termed oral tolerance. Different mechanisms are involved in mediating oral tolerance depending on the dose fed. Low doses of antigen generate cytokine-secreting regulatory cells, whereas high doses induce anergy or deletion. We used mice transgenic for a T-cell receptor (TCR) derived from an encephalitogenic T-cell clone specific for the acetylated N-terminal peptide of myelin basic protein (MBP) Ac-1-11 plus I-A^u to test whether a regulatory T cell could be generated from the same precursor cell as that of an encephalitogenic Th$_1$ cell and whether the induction was dose dependent. The MBP TCR transgenic mice primarily have T cells of a precursor phenotype that produce interleukin 2 (IL-2) with little interferon γ (IFN-γ), IL-4, or transforming growth factor β (TGF-β). We fed transgenic animals a low-dose (1 mg × 5) or high-dose (25 mg × 1) regimen of mouse MBP and without further immunization spleen cells were tested for cytokine production. Low-dose feeding induced prominent secretion of IL-4, IL-10, and TGF-β, whereas minimal secretion of these cytokines was observed with high-dose feeding. Little or no change was seen in proliferation or IL-2/IFN-γ secretion in fed animals irrespective of the dose. To demonstrate *in vivo* functional activity of the cytokine-secreting cells generated by oral antigen, spleen cells from low-dose-fed animals were adoptively transferred into naive (PLJ × SJL)F$_1$ mice that were then immunized for the development of experimental autoimmune encephalomyelitis (EAE). Marked suppression of EAE was observed when T cells were transferred from MBP-fed transgenic animals but not from animals that were not fed. In contrast to oral tolerization, s.c. immunization of transgenic animals with MBP in complete Freund's adjuvant induced IFN-γ-secreting Th$_1$ cells *in vitro* and experimental encephalomyelitis *in vivo*. Despite the large number of cells reactive to MBP in the transgenic animals, EAE was also suppressed by low-dose feeding of MBP prior to immunization. These results demonstrate that MBP-specific T cells can differentiate *in vivo* into encephalitogenic or regulatory T cells depending upon the context by which they are exposed to antigen.**

Oral administration of antigen is a classic method of inducing antigen-specific peripheral immune tolerance (1, 2). In addition to its physiologic role of preventing systemic immune responses to ingested proteins, oral administration of autoantigens has recently been employed to treat autoimmune diseases in animals and in humans (3). Using conventional animals, we and others have demonstrated that both CD4 and CD8 regulatory cells which act by secreting transforming growth factor β (TGF-β) or Th$_2$ cytokines interleukins 4 and 10 (IL-4 and IL-10) can mediate oral tolerance (4, 5). Such cells are preferentially generated when low doses of antigen are fed (6, 7).

We have successfully cloned CD4$^+$ T cells from the mesenteric lymph nodes of SJL mice that were orally tolerized to low doses of myelin basic protein (MBP) (4). Some of these clones were structurally identical to disease-inducing encephalitogenic CD4$^+$ Th$_1$ clones in T-cell receptor (TCR) usage, major histocompatibility complex (MHC) restriction, and epitope recognition. However, they produced TGF-β with varying amounts of IL-4 and IL-10 and suppressed experimental autoimmune encephalomyelitis (EAE) upon adoptive transfer. These results suggested that T cells capable of regulating immune responses are not a unique subset of T cells but conventional T cells that differentiate along different pathways depending on how they are exposed to antigen. Based on these results we hypothesized that T cells, originating from the same precursor, would differentiate into cells that induced immunity or suppression depending upon the environment in which they were exposed to the antigen. To directly test this hypothesis, we utilized MBP TCR transgenic mice, in which the vast majority (95%) of T cells bear a single TCR which was originally expressed by an encephalitogenic Th$_1$ clone (clone 19) (8, 9). The data presented here show that the T cells expressing the transgenic TCR can be induced by a low dose of oral antigen to produce regulatory cytokines and that these T cells can down-regulate EAE *in vivo*.

MATERIALS AND METHODS

Mice. The (PL/J × SJL)F$_1$ mice were purchased from The Jackson Laboratory. Mice transgenic for a TCR specific for Ac-1-11 peptide of MBP were generated by introducing the rearranged α and β TCR chain genes into the germline of C57BL/6 mice (10). The transgene-positive mice were extensively backcrossed to PL/J mice. Mice were screened for the expression of MBP-specific TCR by fluorescence-activated cell sorting analysis with anti-clonotypic monoclonal antibody (mAb) 19G. All mice were housed in Harvard Medical School Animal Care Facilities.

Induction of Oral Tolerance. Oral tolerance was induced by either multiple low-dose or single high-dose feeding regimens. Briefly, each mouse was fed with either 1 or 25 mg of hen egg lysozyme (HEL) (Sigma) or mouse MBP dissolved in 0.5 ml of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MBP, myelin basic protein; TGF-β, transforming growth factor β; IL, interleukin; IFN, interferon; TCR, T-cell receptor; EAE, experimental autoimmune encephalomyelitis; HEL, hen egg lysozyme; CFA, complete Freund's adjuvant; mAb, monoclonal antibody; MHC, major histocompatibility complex.

§To whom reprint requests should be addressed.

PBS by gastric intubation with an 18-gauge stainless steel feeding needle (Thomas).

Induction and Clinical Evaluation of EAE. Each mouse received a s.c. injection in the flank of 400 μg of mouse MBP in 0.15 ml of PBS emulsified in an equal volume of complete Freund's adjuvant (CFA) containing 4 mg of mycobacterium tuberculosis H37 RA per ml (Difco). For nontransgenic animals, pertussis toxin (200 ng per mouse per injection) (List Biological Laboratories, Campbell, CA) was given i.v. at the time of immunization and 48 hr later. Animals were scored for EAE as follows: 0, no disease; 1, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb plus forelimb paralysis; 5, moribund.

T-Cell Culture. Nylon-wool-enriched splenic T cells, 5×10^4 each, were cultured with 2×10^5 feeder cells (irradiated syngeneic splenocytes) in 0.2 ml of serum-free medium (X-vivo 20, Biowhittaker) containing various concentrations of MBP Ac-1-11 peptide. For cytokine assays, culture supernatants were collected 40 hr [for IL-2, IL-4, IL-10, and interferon γ (IFN-γ)] or 72 hr (for TGF-β) later. For proliferation assay, 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) was added to each culture 72 hr later. Cells were harvested and radioactivity incorporated was counted 16 hr later using a flatbed β counter (Wallac, Gaithersburg, MD).

Antigens, Antibodies, and Recombinant Cytokines. Ac-1-11 peptide of MBP (Ac-ASQKRPSQRHG) was made using a peptide synthesizer and purified through HPLC. Mouse MBP was prepared from the brain tissue by a modified method of Deibler (11); the purity of the MBP preparation was confirmed by gel electrophoresis. Polyclonal chicken anti-TGF-β1 antibody was purchased from R & D Systems; purified bovine TGF-β1 and mouse anti-TGF-β mAb (clone 1D11.16) were provided by Celtrix Pharmaceuticals (Palo Alto, CA). The following reagents were purchased from Pharmingen: purified rat anti-mouse IL-2 (clone JES-1A12), IL-4 (clone BVD4-1D11), IL-10 (clone JES5-2A5), and IFN-γ (clone R4-6A2) mAb; biotinylated rat anti-mouse IL-2 (clone JES6-5H4), IL-4 (clone BVD6-24G2), IL-10 (clone SXC-1), and IFN-γ (clone XMG1.2) mAb; recombinant mouse IL-2, IL-4, IL-10, and IFN-γ.

ELISA for Cytokines. Quantitative ELISAs for IL-2, IL-4, IL-10, and IFN-γ were performed using paired mAbs specific for corresponding cytokines per the manufacturer's recommendations. TGF-β was determined as follows. Ninety-six-well microtiter plates (Dynatech) were coated overnight at 4°C with 100 μl of chicken anti-TGF-β1 polyclonal antibodies in carbonate buffer (pH 8.0) at a concentration of 5 μg/ml. The plates were then washed three times with PBS containing 0.5% Tween 20, blocked with 3% bovine serum albumin in PBS, washed, and incubated with culture supernatants or TGF-β1 standard overnight at 4°C. The plates were washed again and incubated with mouse anti-TGF-β mAb (clone 1D11, 1 μg/ml) for 1 hr followed by peroxidase-labeled goat anti-mouse IgG (Vector Laboratories) for 45 min at room temperature. Color was developed with a one-component tetramethylbenzidine reagent (Kirkegaard & Perry Laboratories). The sensitivity of this ELISA is ≈50 pg/ml.

Statistical Analysis. Disease severity, cytokine concentration, and thymidine incorporation data were all analyzed by Student's *t* test.

RESULTS AND DISCUSSION

Splenic T cells from naive MBP TCR transgenic mice can differentiate into both IL-4- and IFN-γ-producing cells upon repeated stimulation *in vitro*. As shown in Fig. 1, cells taken directly from TCR transgenic animals produced low levels of IFN-γ but little or no IL-4 upon primary stimulation *in vitro* with MBP Ac-1-11 peptide. However, upon repeated stimulation with the antigen *in vitro*, both IL-4 and IFN-γ production was increased. These results demonstrate that T cells in TCR transgenic mice are predominantly of precursor phenotype and have the potential to differentiate along both T_{h1} and T_{h2} pathways *in vitro*.

To determine the effect of orally administered antigen in MBP TCR animals, they were fed both high and low doses of antigen. To determine whether low-dose feeding could generate regulatory cells (T_{h2} or TGF-β-secreting T cells), even when the TCR expressed was from an encephalitogenic T_{h1} clone in the transgenic mice, groups of transgenic animals were fed 1 mg of MBP on alternate days over a 10-day period. As a control HEL was fed. Two days after the last feeding spleen cells were removed and stimulated *in vitro* with MBP Ac-1-11 peptide. As shown in Fig. 2, low-dose oral administration of MBP (1 mg) induced cells producing significant amounts of IL-4, IL-10, and TGF-β. Administration of a larger dose (25 mg) of MBP did not lead to the induction of either T_{h2} or TGF-β-producing cells (Fig. 2). These results are consistent with the observation that low but not high doses of antigen preferentially induce regulatory T cells that secrete TGF-β, IL-4, and IL-10 (6, 7). We have recently shown in a non-disease TCR transgenic model that multiple doses of large amounts of antigen can lead to anergy and/or deletion (12). In either low- or high-dose-fed animals, the percentage of TCRαβ transgenic T cells (stained positive for anti-clonotypic mAb 19G) remains roughly the same as unfed animals—i.e., 93–96% of CD4$^+$ T cells.

We then investigated the effect of orally administered antigen on T-cell proliferation and secretion of T_{h1} cytokines, IFN-γ and IL-2. As shown in Fig. 2, IL-2 secretion was slightly

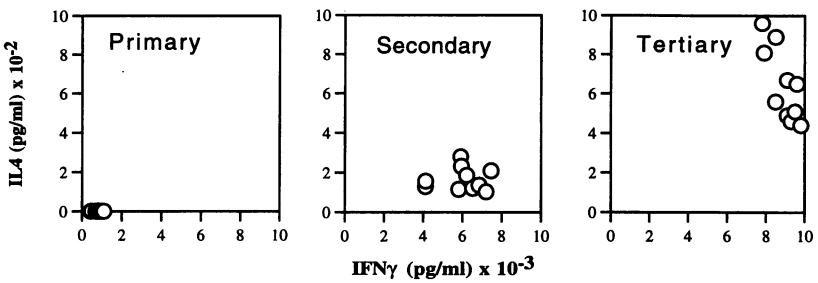

FIG. 1. Splenic T cells from MBP TCR transgenic mice produce both IL-4 and IFN-γ upon repeated stimulation *in vitro*. Spleens were removed from three naive transgenic animals and pooled, and a single cell suspension was prepared. T cells were enriched on nylon wool and five individual cultures containing 1×10^5 splenic T cells and 2×10^6 syngeneic antigen-presenting cells were established in 1 ml of serum-free medium containing 10 μg of MBP Ac-1-11 peptide (primary stimulation). Four days later, cells were expanded in serum-free medium for an additional 3 days, and secondary stimulation was conducted under the same condition as the primary culture. This cycle was repeated another time for tertiary stimulation. Supernatant from each culture was collected 40 hr after the stimulation and cytokine concentration was determined by ELISA. The experiment was then repeated in a second group of three naive animals. The data presented represent the cytokine profile of each of the 10 individual lines generated in these two experiments.

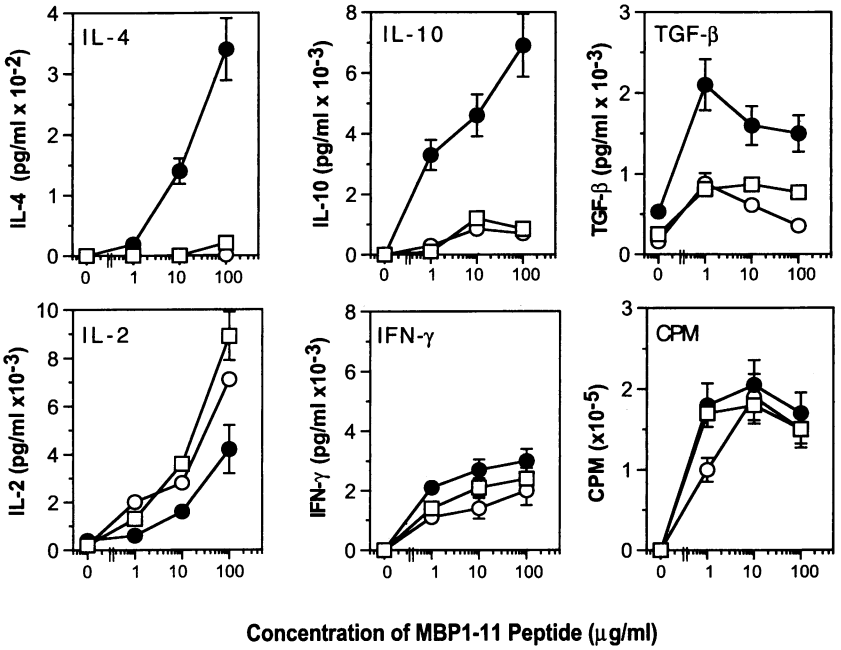

FIG. 2. Oral administration of antigen activates cells producing IL-4, IL-10, and TGF-β. Three groups of MBP TCR transgenic mice, four mice each, were fed with (*i*) 1 mg of HEL (□) or 1 mg of mouse MBP (●) on days 1, 3, 5, 7, and 9 or (*ii*) 25 mg of mouse MBP (○) one time on day 7. On day 11, mice were sacrificed and a single cell suspension of spleen was prepared. The spleens from the four mice in each group were pooled and nylon-wool-enriched splenic T cells, 5×10^4 each, were cultured with 2×10^5 feeder cells in 0.2 ml of serum-free medium containing various concentrations of MBP Ac-1-11 peptide. Culture supernatants were collected at 40 hr for IL-2, IL-4, IL-10, and IFN-γ or at 72 hr for TGF-β. Cytokine concentration was determined by ELISA. Data presented represent mean ± SD of triplicate cultures. For proliferation assay, 1 μCi of [^{3}H]thymidine was added to each culture at 72 hr. Cells were harvested and radioactivity was counted 16 hr later. Data presented represent mean ± SD of triplicate cultures and are representative of three experiments.

reduced in the TCR MBP transgenic animals fed with a low dose of MBP but there was no significant effect on IFN-γ production or proliferation in all three groups. The lack of significant inhibition or anergization of IL-2-secreting cells most probably relates to the fact that the vast majority (95%) of T cells in the transgenic mouse are of a precursor type which secrete IL-2 and proliferate vigorously in response to MBP without prior sensitization. Thus, T-cell anergy is not demonstrable in our transgenic animals fed with either 1 or 25 mg MBP.

These results demonstrate the preferential activation of T_{h2} and TGF-β-secreting cells following orally administered antigen. To establish whether this preferential activation resulted in the generation of cells capable of suppressing EAE, animals were fed and splenocytes were adoptively transferred into nontransgenic (PL/J × SJL)F_1 animals. These animals were immunized with MBP and observed for the development of EAE. As shown in Fig. 3, T cells from animals fed MBP markedly suppressed the development of EAE upon adoptive transfer. T cells from HEL-fed transgenic mice did not suppress EAE. We have also adoptively transferred disease protection from MBP TCR transgenic animals using Peyer's patch cells from MBP-fed animals (J.-I. Inobe and H. L. Weiner, unpublished). Although the cells were transferred from transgenic H-2^u mice and the recipients were (PL/J × SJL/J)F_1, no graft-vs.-host response was evident. *In vitro*, T cells from transgenic animals do not proliferate when cocultured with splenocytes of (PL/J × SJL)F_1 mice in the absence of MBP. This probably reflects the dominance of the transgenic TCR in the donor mice and the lack of reactivity to I-A^s of this receptor (9).

The TCR of transgenic T cells was originally expressed by an encephalitogenic T_{h1} cell clone, 19G (8). While the vast majority of T cells in transgenic animals are of naive phenotype, ≈10% of these mice in our facility develop spontaneous EAE within 12 months of age, suggesting spontaneous generation of T_{h1} cells. Immunization of transgenic animals with MBP in CFA induces EAE in virtually all mice. As a control for oral tolerance, we examined the cytokine phenotype of transgenic T cells after s.c. immunization with MBP/CFA. Virtually no T_{h2} or TGF-β-secreting T cells were detected in

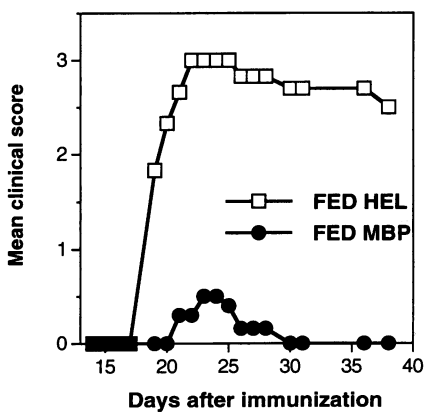

FIG. 3. T cells from MBP-fed mice suppress EAE. Two groups of (PL/J × SJL)F_1 mice, six animals per group, received (*i*) an i.p. injection of 5×10^6 T cells from TCR transgenic mice, (*ii*) a s.c. immunization with 400 μg of mouse MBP in 0.15 ml of PBS emulsified in an equal volume of CFA containing 4 mg of mycobacterium tuberculosis H37 RA per ml, and (*iii*) an i.v. injection of 200 ng of pertussis toxin in 0.2 ml of PBS. Mice received another injection of pertussis toxin (200 ng per mouse) 48 hr later. □, Mice received T cells from transgenic animals fed 1 mg of HEL for five times; ●, mice received T cells from transgenic animals fed 1 mg of MBP for five times. Donor animals were sacrificed 2 days after the last feeding and all T cells were purified from the spleen by positive selection with magnetic beads coupled with anti-mouse CD4 mAb (Advanced Magnetics, Cambridge, MA). The maximal scores of animals receiving cells from MBP-treated transgenic animals are significantly different from those of the control group ($P < 0.001$).

immunized animals (EAE score, 2–4) (data not shown). By contrast, IFN-γ-secreting T_{h1} cells were detected in animals immunized s.c. with MBP and CFA (Fig. 4). These data suggest that, unlike oral feeding, s.c. immunization with CFA activates primarily T_{h1} cells. To test whether oral administration of MBP could ameliorate EAE induced by MBP/CFA immunization in TCR transgenic animals, we fed mice with MBP to induce oral tolerance and challenged with MBP in CFA s.c. As shown in Fig. 5, the clinical score of EAE was significantly reduced by oral administration of MBP as seen between 13 and 29 days after immunization ($P < 0.01$ as compared to HEL-fed mice). The incidence of disease was also reduced from 5/6 in the HEL-fed group to 2/6 in the MBP-fed group.

There has been a great deal of controversy on the structural nature of regulatory T cells that suppress immune responses in an antigen-specific manner (13). A number of previous studies suggested that regulatory T cells were a unique subset of T cells, which recognize a distinct antigenic epitope in the context of a novel MHC molecule (14). However, our studies on cloned $CD4^+$ regulatory T cells from orally tolerized mice demonstrated that they are identical to encephalitogenic T_{h1} cells in TCR usage, MHC restriction, and epitope specificity but differed from the latter in their production of suppressive cytokines such as TGF-β (4). We have taken these studies further by investigating TCR transgenic mice in which the vast majority of CD4 T cells express only one pair of MBP-reactive TCR. Thus, it is clear from the studies reported here that the same TCR-bearing cells, recognizing a particular epitope/MHC combination, can differentiate into regulatory T cells by exposure of antigen via gut. Activation of these T cells by s.c. immunization with CFA leads to the generation of pathogenic autoreactive T_{h1} cells. Oral administration of MBP does not activate cells to become disease inducing even though MBP-reactive cells constitute the major proportion of the peripheral T-cell repertoire in these animals. These data demonstrate that it is not the antigenic epitope, TCR, or MHC that dictates the functional differentiation of a T cell into a regulatory T cell. Our data support the idea that the same T cell could attain the ability to become an encephalitogenic or a regulatory T cell depending upon the context (environment) in which it is exposed to antigen. Furthermore, this differentiation is dose dependent when oral antigen is administered.

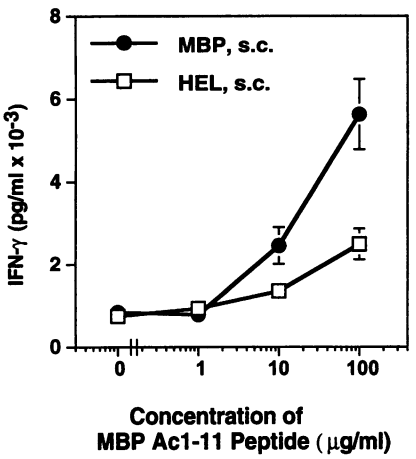

FIG. 4. Immunization with MBP/CFA activates cells producing IFN-γ. Two groups of MBP TCR transgenic mice, three mice per group, were immunized s.c. with 400 μg of HEL (□) or mouse MBP (●) in 0.15 ml of PBS emulsified in an equal volume of CFA containing 4 mg of mycobacterium tuberculosis H37 RA per ml. On day 13, mice were sacrificed and splenic T cells were prepared and cultured as in Fig. 2. The spleens from the three mice in each group were pooled. IFN-γ was determined by ELISA. Data presented represent mean ± SD of triplicate cultures and are representative of two experiments.

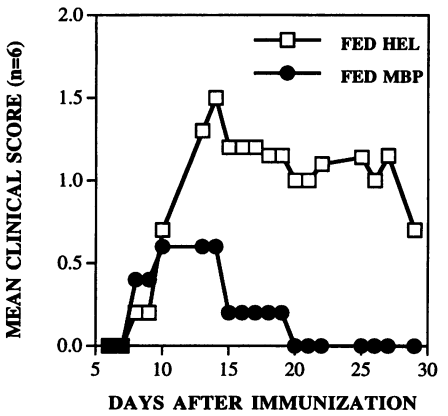

FIG. 5. Oral administration of MBP suppresses actively induced EAE in TCR transgenic mice. Two groups of MBP TCR transgenic mice, six animals per group, were fed with 1 mg of HEL (□) or mouse MBP (●) every other day for a total of five times. Two days after the last feeding mice were immunized for EAE as in Fig. 4. Data presented are representative of two experiments.

This research was supported in part by National Institutes of Health Grants PO1-AI-36529, NS29352, and PO1-AI-35964.

1. Wells, H. G. (1911) *J. Infect. Dis.* **8,** 147–171.
2. Chase, M. (1946) *Proc. Soc. Exp. Biol. Med.* **61,** 257–259.
3. Weiner, H. L., Friedman, A., Miller, A., Khoury, S. J., Al-Sabbagh, A., Santos, L. M. B., Sayegh, M., Nussenblatt, R. B., Trentham, D. E. & Hafler, D. A. (1994) *Annu. Rev. Immunol.* **12,** 809–837.
4. Chen, Y., Kuchroo, V. K., Inobe, J.-I., Hafler, D. A. & Weiner, H. L. (1994) *Science* **265,** 1237–1240.
5. Miller, A., Lider, O., Roberts, A. B., Sporn, M. B. & Weiner, H. L. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 421–425.
6. Friedman, A. & Weiner, H. L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6688–6692.
7. Gregerson, D. S., Obritsch, W. F. & Donoso, L. A. (1993) *J. Immunol.* **151,** 5751–5761.
8. Baron, J. L., Madri, J. A., Ruddle, N. H., Hashim, G. & Janeway, C. A., Jr. (1993) *J. Exp. Med.* **177,** 57–68.
9. Hardardottir, F., Baron, J. L. & Janeway, C. A., Jr. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 354–358.
10. Lafaille, J., Nagashima, K., Katsuki, M. & Tonegawa, S. (1994) *Cell* **78,** 399–408.
11. Higgins, P. & Weiner, H. L. (1988) *J. Immunol.* **140,** 440–445.
12. Chen, Y., Inobe, J.-I., Marks, R., Gonnella, P., Kuchroo, V. K. & Weiner, H. L. (1995) *Nature (London)* **376,** 177–180.
13. Tada, T., Asano, Y. & Sano, K. (1989) *Res. Immunol.* **140,** 291–294.
14. Germain, R. N. & Benacerraf, B. (1981) *Scand. J. Immunol.* **13,** 1–10.