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Direct Measurement of Anergy of Antigen-Specific T Cells Following Oral Tolerance Induction[1]

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T cell tolerance induced by oral administration of Ag may be the result of either deletion or functional inactivation of Ag-specific T cells. OVA p(323–339)-specific TCR transgenic (Tg^+) lymphocytes were transplanted into BALB/c recipients. Chimeric mice were fed OVA and subsequently challenged with the peptide in CFA. Tolerance was then assessed by measurement of lymph node (LN) cell proliferation in response to the peptide, and deletion was assessed by measuring the frequency Tg^+ T cells by flow cytometry. Lymphocytes from chimeric mice fed OVA showed a dose-dependent decline in their proliferative response to the peptide in vitro, compared with immunized control mice that were not fed OVA. Calculation of proliferative potential per Tg^+ cell demonstrates that nonresponsiveness due to feeding Ag results in the induction of anergy in the LN. In addition, analysis of intestinal intraepithelial lymphocytes following feeding of OVA did not show evidence of trafficking of LN T cells to the small intestine intraepithelial nor lamina propria compartments. ***The Journal of Immunology,*** **1996, 157: 1337–1341.**

Animals fed protein Ags may develop Ag-specific nonresponsiveness, yet the mechanisms of this tolerance induction are not known. Oral tolerance has proven effective in preventing autoimmune responses or sensitization to the autoantigen in naive animals. Orally administered Ags are effective in both prevention and suppression of experimental autoimmune encephalitis, collagen arthritis, thyroiditis, and uveitis (1–5). Treatments of several autoimmune diseases in humans are in clinical trials with Ag-specific immune suppression by oral tolerance (6–8).

Oral tolerance has been described primarily as the absence of an Ab or lymphocyte proliferative response to a specific Ag. Assessing whether this lack of response represents deletion or anergy has not been possible, since the Ag-specific T cells could not be phenotypically identified. We used DO11.10 mice that bear transgenic TCR α- and β-chains that when expressed together, recognize OVA peptide p(323–339) presented by MHC I-A^d. This TCR $\alpha\beta$ heterodimer is itself recognized by an anti-clonotypic mAb, KJ126 (9) on $CD4^+$ T cells. Tg^+ cells from DO11.10 mice were transplanted into BALB/c recipients, according to the method of Kearney (10). Chimeric BALB/c mice were examined for the proportion of Tg^+ T cells and for the induction of tolerance following orally administered Ag, and the Tg^+ T cells were tracked by flow cytometry. We observed a rapid and profound induction of anergy following oral OVA, despite incomplete deletion of Ag-specific T cells.

Materials and Methods

Mice

Homozygous DO11.10 breeders were generously provided by Dr. Dennis Loh, and were maintained under specific pathogen-free conditions in the Animal Resource Facility at the University of Texas Medical Branch, Galveston, and were fed an OVA-free diet. Only female DO11.10 mice were used for adoptive transfer studies to avoid minor histocompatibility Ag (H-Y) reactivity in recipient animals. BALB/c female mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN) were used as recipients of Tg^+ lymph node (LN)[3] cells.

Adoptive transfer, feeding, and immunization

Lymphocytes from DO11.10 mice were obtained from axillary, inguinal, popliteal, and mesenteric LN and were dispersed and washed in sterile PBS. An aliquot of cells was analyzed by flow cytometry to measure the percentage of $CD4^+KJ126^+$ (Tg^+) cells. 3×10^6 Tg^+ cells in 100 μl PBS were transplanted into each recipient BALB/c mouse by tail vein injection. Chimeric BALB/c mice containing DO11.10 lymphocytes were fed 0, 25, or 100 mg OVA (Sigma Chemical Co., St. Louis, MO) in a total of 250 μl PBS. OVA p(323–339) was synthesized by the Protein Chemistry Laboratory of the Sealy Center for Molecular Science, Galveston. Indicated mice were immunized s.c. in the back with 125 μg peptide in CFA. Unless otherwise indicated, BALB/c mice received DO11.10 lymphocytes on day 0, fed on day 2, immunized on day 6, and draining LN harvested on day 14. Intestinal intraepithelial lymphocytes (IEL) were prepared by extraction in Ca^{2+}- and Mg^{2+}-free HBSS and Percoll density gradient centrifugation, and lamina propria lymphocytes (LPL) were isolated by further digestion of small intestine fragments with collagenase (Worthington Biochemical Corp., Freehold, NJ) as described (11, 12).

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[3] Abbreviations used in this paper: IEL, intestinal intraepithelial lymphocytes; KJ126, mAb that recognizes the transgenic TCR-clonotype on Tg^+ T cells; LN, lymph node; LPL, lamina propria lymphocytes; Tg^+, $KJ126^+CD4^+$ lymphocytes from DO11.10 mice.

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Monoclonal Abs and FACS analysis

Anti-Tg^+ clonotype Ab (KJ126) was prepared from hybridoma-derived ascites. IgG was purified by protein-G (Pharmacia, Uppsala, Sweden) chromatography and was FITC conjugated. Anti-CD4-Tricolor and control mAbs were purchased from Caltag (South San Francisco, CA). Rat-IgG-FITC was used as a control for KJ126(rat)-FITC, and Rat-IgG-Tricolor as a control for the CD4-Tricolor for lymphocyte staining. Control Abs were run for each tissue type in every experiment. Nonspecific staining in lymphocyte populations in Figure 5 were eliminated by staining with a nonspecific rat IgG-phycoerytherin third Ab. Phycoerytherin-positive cells were electronically gated out. Cell surface expression of the above molecules was detected by staining 10^6 viable cells with the indicated Abs for 20 min at 4°C in PBS/azide, and analyzed on a FACScan (Becton Dickinson). For detection of Tg^+ cells in adoptive transfer recipients, 50,000 lymphocytes were analyzed.

Functional analysis: in vitro proliferation stimulated by p(323–339)

5×10^4 cells were cultured in 200 μl of RPMI with 10% FCS (HyClone, Logan, UT), 50 μM 2-ME, 10 μg/ml folate, and 2 mM glutamine with the indicated concentration of p(323–339). Cells were also cultured on plates with PMA (10 ng/ml) and ionomycin (250 ng/ml), and proliferative responses were measured to assure that cells were viable. Cells were cultured for 3 days, and 1 μCi [^{3}H]-thymidine was added to wells for the last 18 h of culture. Statistical analysis was performed by calculation of standard deviations, and probability values were calculated by t test.

Measure of anergy: calculation of cpm per cell

For each individual mouse, the percentage of Tg^+ cells based on FACS analysis ($KJ126^+CD4^+$) was multiplied by the number of cells per well (5×10^4) to give the number of Tg^+ cells per well. The peptide-specific [^{3}H]-thymidine incorporation (average of triplicate wells) was divided by the number of Tg^+ cells per well to give the cpm per Tg^+ cell for each animal:

$$\frac{\Delta\text{cpm}}{(\%Tg^+/100) \times 5 \times 10^4} = \text{cpm/Tg}^+ \text{ cell}$$

Results

Persistence of antigen-specific T cells following oral administration of Ag

Expansion and deletion of Ag-reactive T cells was measured by tracking Tg^+ T cells in chimeric BALB/c mice transplanted with lymphocytes from DO11.10 mice. DO11.10 mice bear a transgenic TCR that recognizes OVA peptide p(323–339), restricted to I-A^d (9). OVA-specific Tg^+ T cells were detected by reactivity with the mAb, KJ126, which recognizes the clonotypic TCR α- and β-chains. We created chimeric BALB/c mice by transferring 3×10^6 Tg^+ T cells from DO11.10 donors (10). Figure 1 shows two-color flow cytometric analyses of KJ126-FITC and anti-CD4-Tricolor staining of DO11.10, BALB/c, and chimeric mice. The Tg^+ population in the homozygous DO11.10 mice was 50.86% and was undetectable in the BALB/c controls. Seven days after transfer, lymphocytes from the LN of chimeric mice had detectable Tg^+ T cells based on KJ126 and CD4 staining (0.21 ± 0.08). This population was expanded in the LN of chimeras immunized s.c. with p(323–339) 2 days after transfer, and analyzed 5 days after immunization (2.19 ± 0.58). Mice fed 100 mg OVA on day 2 had detectable Tg^+ T cells in their LN at 5 days after feeding (0.42 ± 0.18). LN from mice fed on days 2 and 4 had slightly higher proportions of Tg^+ cells (0.51 ± 0.24%) compared with unfed chimeras or chimeras administered a single feeding.

Feeding OVA induces functional anergy of antigen-specific T cells

BALB/c mice receiving Tg^+ lymphocytes were fed whole OVA. Four days later, fed and control animals were challenged s.c. in the back with OVA peptide p(323–339) in CFA. Draining LN were obtained for measurement of Tg^+ T cell proportion and function.

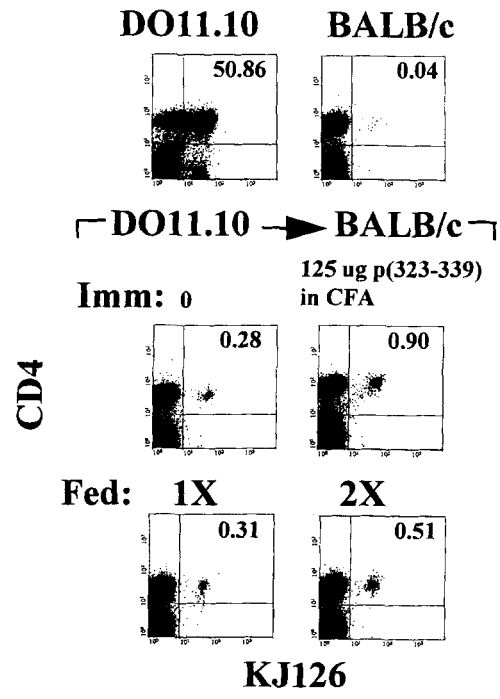

FIGURE 1. Two color flow cytometric analyses of lymphocytes from DO11.10 mice and BALB/c-DO11.10 chimeras following feeding OVA or s.c. immunization. 3×10^6 $KJ126^+CD4^+$ DO11.10 lymphocytes were transferred to 6- to 8-wk-old BALB/c mice on day 0. Groups of 3 chimeric mice were either unmanipulated, immunized with 125 μg p(323–339) in CFA, or fed 100 mg OVA in PBS on day 2. Another group of three animals were fed 100 mg OVA on both days 2 and 4. Draining LN cells (axillary and inguinal) were analyzed on day 7. Fifty thousand lymphocytes were analyzed by flow cytometry for the presence of Tg^+ T cells by staining with KJ126-FITC and anti-CD4-tricolor mAbs.

Heterozygous DO11.10 mice bear 15 to 25% Tg^+ cells in their LN. Figure 2 illustrates a population of Tg^+ cells from immunized chimeric mice. Eight days after immunization, fewer clonotype-bearing T cells were observed in OVA-fed mice immunized with the peptide in CFA, compared with mice that were not fed but immunized. However, more Tg^+ T cells were consistently observed than in mice that were fed but not immunized, as in Figure 1. Tg^+ T cells were not detected in BALB/c mice, whether naive or immunized with the peptide.

The induction of tolerance is defined in this study as a decrease in the ability of immunization to prime in vitro proliferative responses to the Ag. Lymphocytes from LN draining the sites of immunization were analyzed for in vitro proliferative responses to peptide 8 days later. Figure 3 shows that the mice fed OVA before peptide/CFA challenge had greatly diminished responses to the peptide in vitro. Lymphocytes from unchallenged chimeric mice did not respond in vitro to peptide stimulation 14 days after transplantation of Tg^+ T cells. The p(323–339)-specific response was calculated by subtracting the background proliferation from the proliferative response to 10 μM p(323–339) for each animal and averaging the calculated Δcpm's (Fig. 4*B*). For animals fed 25 mg, the proliferative response to peptide was 8% of the immunized but unfed animals, $p < 0.05$. Lymphocytes from animals fed 100 mg OVA did not respond at all above the media control, $p < 0.001$. Thus, we have defined that feeding OVA to animals with OVA-specific T cells induces nonresponsiveness to antigenic challenge.

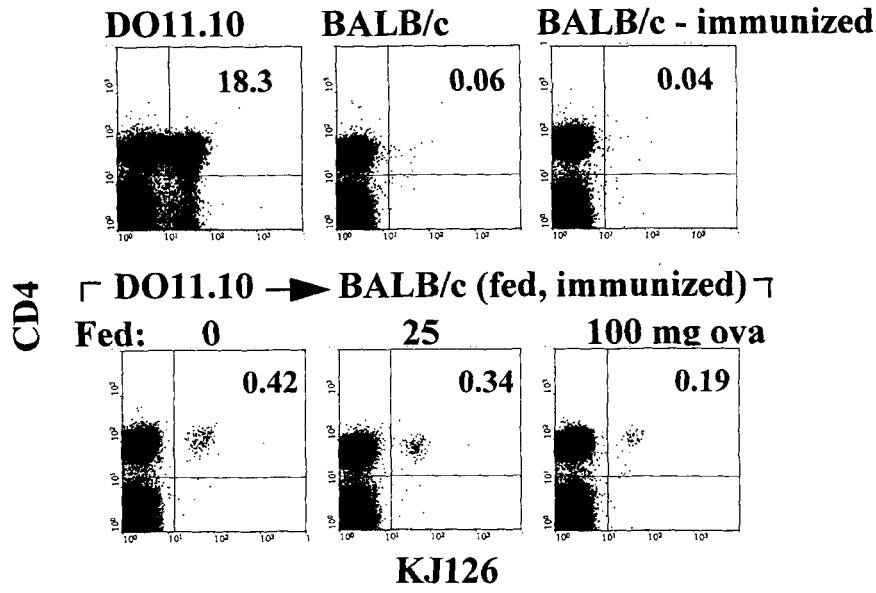

FIGURE 2. Two-color flow cytometric analyses of lymphocytes from DO11.10 mice and BALB/c-DO11.10 chimeras following feeding OVA and s.c. immunization or immunization without prior feeding. 3×10^6 KJ126$^+$CD4$^+$ DO11.10 lymphocytes were transferred to 6- to 8-wk-old BALB/c mice on day 0, as in Figure 1. Groups of three chimeric mice were then fed 0, 25, or 100 mg OVA in PBS on day 2 and immunized with 125 μg p(323–339) in CFA on day 6, where indicated. Lymphocytes from draining LN cells were analyzed by flow cytometry on day 14.

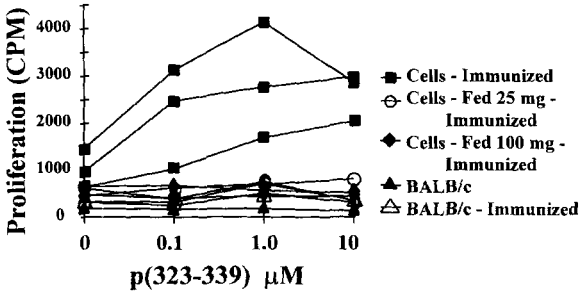

FIGURE 3. Proliferative responses of lymphocytes from chimeric BALB/c mice to p(323–339). Mice were fed 0, 25, or 100 mg OVA in PBS 4 days before s.c. immunization with 125 μg p(323–339) in CFA, and proliferative responses of lymphocytes from draining LN were measured 8 days later, as in Figure 1. Draining LN cells were cultured for 3 days at 5×10^4 cells/well and [^{3}H]-thymidine was added for the last 18 h of culture.

Measurement of anergy

Nonresponsiveness, however, does not distinguish whether the decrease in proliferation is due to deletion or anergy. We can differentiate whether deletion or anergy has occurred by comparing the proliferative response with the number of Tg$^+$ cells retrieved following tolerization (Fig. 4*C*). By multiplying the percentage of clonotype$^+$ cells by the number of cells cultured (5×10^4 cells/well), the total number of Tg$^+$ cells per well was calculated. The proliferative response peptide-specific in cpm was then divided by the number of Tg$^+$ cells in each animal to calculate the cpm per Tg$^+$ cell present in the culture (see *Materials and Methods*). Although a modest decline in the percentage of clonotype bearing cells was detected in lymphocytes from mice fed before immunization (Fig. 4*A*) ($p < 0.1$), a substantial decrease in the proliferative potential was observed (Fig. 3). Calculation of OVA-specific cpm (as in Fig. 4*B*) per input clonotype$^+$ cell illustrates that in mice fed OVA before immunization, the overall proliferative capacity of the Tg$^+$ lymphocytes was reduced (Fig. 4*C*). The presence of detectable Tg$^+$ cells that lack a proliferative response to the peptide demonstrates that these cells must be anergic. These data support the hypothesis that oral tolerance results in the induction of anergy.

Tg$^+$ T cells do not traffic to IEL or LPL following OVA feeding

To determine whether the Tg$^+$ T cells traffic to the small intestine after feeding, we examined the presence of Tg$^+$ cells in the IEL and LPL. Each BALB/c mouse received 6×10^6 Tg$^+$ cells i.v. Two days later, indicated animals were fed 100 mg OVA in PBS, and were analyzed on days 4 or 7. Figure 5 illustrates IEL and LPL stained with KJ126 and anti-CD4 Abs, following OVA feeding. Tg$^+$ cells were detectable in the LN of both control and fed animals on 2 and 5 days following feeding, as well as in unfed animals. Two days after feeding, no Tg$^+$ T cells were detectable in the IEL population. Five days after feeding, negligible numbers of Tg$^+$ cells were detected in the IEL and LPL. Groups of 3 chimeric mice were analyzed per treatment group and time point, with identical results for the IEL and LPL. These data indicate that the Tg$^+$ T cells do not traffic to the intestinal mucosa upon exposure to Ag. Kinetic studies of PBL in chimeric mice demonstrate a typical expansion and contraction of Tg$^+$ lymphocytes in immunized mice over 7 days. Only a gradual rise in Tg$^+$ cells in the PBL were observed following the feeding of OVA (not shown).

Discussion

By tracking Tg$^+$ T cells in chimeric animals, we were able to demonstrate the induction of functional nonresponsiveness to Ag following oral administration of Ag. The expansion of Tg$^+$ T cells observed during the induction of nonresponsiveness indicates the development of a state of anergy in the Ag-reactive T cells. This anergy was not broken by subsequent immunization with peptide in CFA, indicating that further antigenic insult is not sufficient to reactivate proliferative responses. No deletion of Tg$^+$ lymphocytes was observed 5 days after feeding OVA. However, there were fewer remaining cells in animals fed 100 mg OVA on day 2 after transfer and immunized on day 6, and analyzed on day 14. Thus, some deletion may be occurring after challenge. Further kinetic studies, and analysis of cell cycle and DNA degradation, will clarify the relative contribution of deletion to the final levels of Tg$^+$ lymphocytes after feeding OVA.

The specific site of tolerance induction has not been clearly distinguished. Our data indicate that the Ag travels to the peripheral lymphoid organs, as opposed to the T cells traveling to the gut. This is supported by studies of Peng et al. (13) who have measured

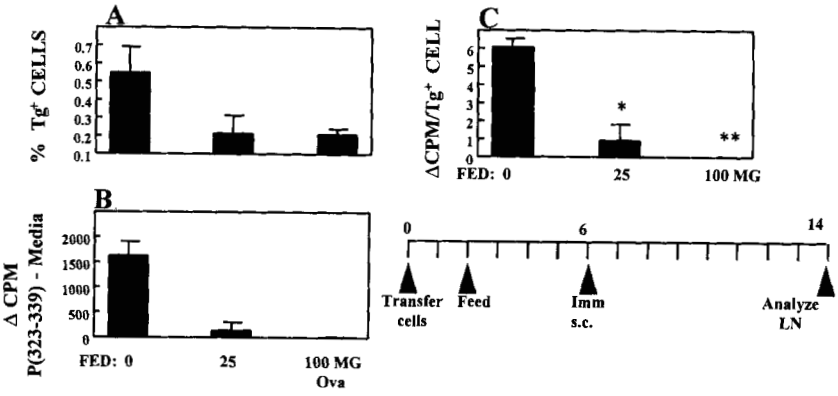

FIGURE 4. Measurement of Ag-specific anergy following oral administration of Ag. Chimeric BALB/c mice were fed 0, 25, or 100 mg OVA in PBS and subsequently challenged with p(323–339) in CFA, as in Figure 2. *A*, Proportion of Tg^+ cells recovered from draining LN. *B*, Proliferative response to 10 μM p(323–339) in vitro. *C*, Proliferative response to 10 μM peptide as a function of the proportion of Tg^+ T cells in culture. See *Materials and Methods* for calculation. $*p < 0.05$, $**p < 0.001$.

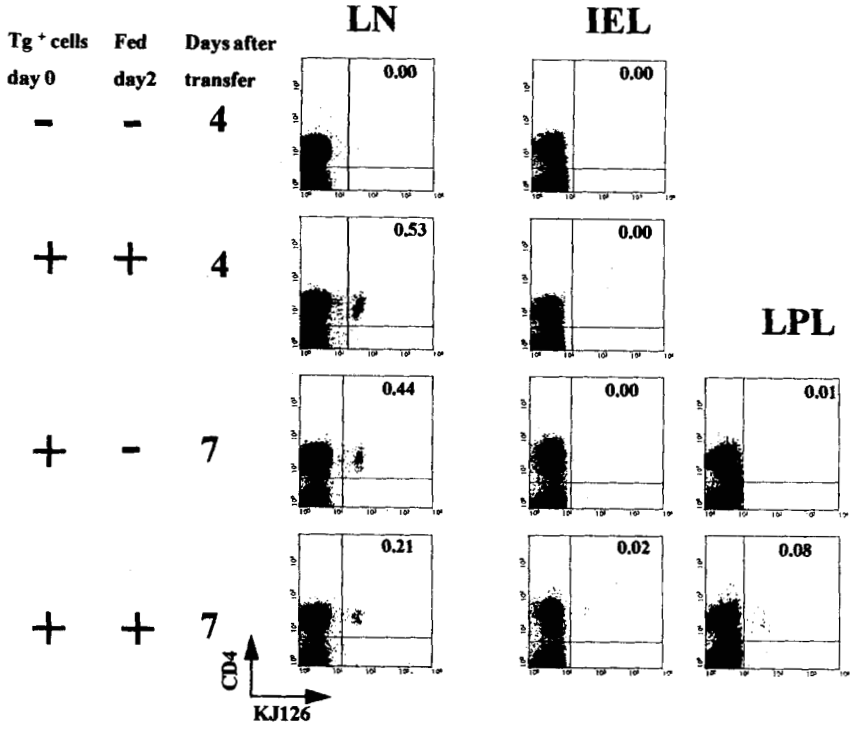

FIGURE 5. Ovalbumin-specific T cells do not traffic to the small intestinal epithelium nor lamina propria following feeding. Groups of three mice were transplanted with 3×10^6 Tg^+ T cells on day 0 and fed 100 mg OVA on day 2. LN and IEL were examined for presence of Tg^+ T cells on day 4 (2 days post-feed), and LN, IEL, and LPL were examined on day 7 (5 days post-feed).

OVA in the circulation as early as 2 min following oral administration to BALB/c mice, peaking at one h. Thus the mechanism of oral tolerance may be similar to that suggested for i.v. tolerance (10) where Ag encountered in the absence of activated Ag presenting cells would favor the induction of anergy rather than activation. It has been suggested that the expression of CD1 Ags in the intestinal epithelium may contribute to the generation of oral tolerance by favoring Th2 responses upon stimulation of $NK1.1^+$ T cells to produce IL-4 (14, 15). The peptide and MHC specificity of the DO11.10 T cells in this system would argue against this route as a means of tolerance induction. Although CD1 is expressed on intestinal epithelial cells, the resident IEL do not express NK1.1 in C57BL/6 mice (J. She, Beth Israel Hospital, personal communication), which are also capable of oral tolerance induction (16, 17). Dendritic cell Ag presentation is also equivalent from the oral mucosa and from sensitizing sites, suggesting also that tolerance induction occurs at the level of the LN and not the epithelium (18).

The correlation of IL-4 production with tolerance induction suggests that tolerance is the result of a preferential Th2 response (19, 20). Whether the Ag-specific T cells themselves produce IL-4 remains to be determined. The lack of in vitro proliferative responses does not exclude differentiation of Ag-specific T cells to become memory Th2 cells. In vitro studies of Th1 and Th2 populations indicates the process of polarization is reversible only for a short period of time, and results from the expansion of a small population of uncommitted precursors (21). The induction of oral tolerance in vivo may parallel this system, as the persistence of Ag at the site of immunization may contribute to the chronic stimulation of the Th2 committed lineage.

We have shown that oral Ag administration can result in functional anergy of Ag-specific T cells. The kinetics of this response may also involve deletion of some of the Ag-specific T cells, as has been shown (22). However, we observed that the population of T cells remaining after resolution of the immune response includes these Ag-specific T cells. These T cells are no longer responsive to Ag in vitro. Whether this nonresponsiveness is solely due to anergy rather than the generation of additional suppressive factors cannot be determined by these studies. Preliminary in vitro mixing experiments did not indicate

significant suppression of sensitized T cells by tolerized T cells. This is in agreement with Whitacre et al. who found that myelin basic protein fed rats were susceptible to induction of experimental autoimmune encephalomyelitis induced by transfer of cells of an encephalogenic T cell clone (1). The tolerant state resulted from inactivation of potentially reactive T cells, rather than the generation of regulatory cells that would be able to suppress additional reactive T cells when introduced. Although we are able to demonstrate the prevention of sensitization through the induction of anergy, it is not yet clear whether oral tolerance induction will serve to reverse existing immune responses, such as is the case in autoimmune disease.

Acknowledgments

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