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J Immunol 1999; 162:5868-5875; ;
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Antigen-Specific T Cell Activation and Proliferation During Oral Tolerance Induction

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One of several routes of achieving immunologic tolerance is through functional inactivation of Ag-specific T cells. Oral administration of Ag can allow survival of the Ag-specific T cells that are functionally anergic. The aim of this study was to investigate whether functional inactivation of Ag-specific T cells is directed through an activation process and to further define the differentiative pathways and functional characteristics of anergic T cells. Mice were transplanted with OVA-specific TCR-transgenic T cells and either fed OVA or immunized s.c. with the OVA peptide 323–339 in CFA. OVA-specific T cells from OVA-fed mice were unresponsive to restimulation in vitro within 48–72 h after treatment. In vivo, however, T cell proliferation was detected by 5,6-carboxy-succinimidyl-fluoresceine-ester intensity changes in OVA-specific T cells. The mesenteric lymph nodes (LNs) from OVA-fed mice more frequently contained OVA-specific dividing cells in vivo than those in the peripheral LNs, and the reciprocal was observed following s.c. immunization of the OVA peptide in CFA. The induction of anergy in OVA-fed mice was accompanied by rapid up-regulation of CD69 and CTLA-4, later down-regulation of CD45RB on OVA-specific T cells, and a marked decrease in T cell secretion of IL-2, IL-10, and IFN-γ after OVA restimulation in vitro. Results from this study indicate that the inductive phase of oral tolerance is preceded by Ag-specific T cell activation in vivo, proliferation in the regional draining LNs, and differentiation into a memory-like state. These results indicate that Ag-directed differentiation occurs as a part of T cell tolerance through anergy. *The Journal of Immunology, 1999, 162: 5868–5875.

The route by which an Ag is administered affects the type and magnitude of the ensuing immune response. Ags injected s.c. with adjuvant generally elicit strong immune responses, while those infused directly into the circulation tend to induce tolerance. Ags administered to the gastrointestinal tract, however, frequently elicit a local Ab response in the intestinal lamina propria, yet produce a state of systemic unresponsiveness. Understanding the immune consequences of administering Ag orally impacts approaches to prevention of food allergy and the development of mucosal vaccines. Ags entering the body by different routes encounter distinct types of APCs and are distributed to different lymphoid tissues. The special characteristics of regional immune systems such as the gut-associated lymphoid tissues (GALT) and other mucosal-associated lymphoid tissues are not yet fully defined. In mice, orally or i.v. administered OVA results in tolerance (anergy) and deletion of T cells, whereas s.c. injection leads to vigorous T cell responses (1, 2). To date, the precise means by which the route of Ag administration controls the nature and the intensity of the response is not known.

In the present study we focused on the induction phase of peripheral tolerance resulting from orally encountered Ag. Due to a low T cell precursor frequency in vivo, T cell proliferation in response to OVA usually cannot be detected in immunized hosts. Therefore, we adopted a system described by Kearney et al., in which limited numbers of OVA-specific cells were transferred into syngeneic recipients (1). We transferred lymph node (LN) cells from OVA TCR-transgenic (Tg) mice into syngeneic BALB/c mice. As a result, 1–2% of the cells in the LNs of recipient mice bear the clonotypic TCR encoded by the TCR-α and -β transgenes that together recognize OVA peptide 323–339, and their presence was easily detected by a clonotypic mAb KJ126 (1, 3). The responses of recipient mice immunized s.c. with OVA peptide in CFA were compared with responses from mice fed with OVA in PBS. At several time points after Ag encounter, lymphocytes from mesenteric and peripheral (axillary and inguinal) LNs were harvested. Changes in the expression of cell surface activation markers, proliferative responses, and lymphokine synthesis of the Tg T cells were measured in response to OVA stimulation. Results from this study demonstrate that during the inductive phase of oral tolerance, anergy is preceded by Ag-specific T cell activation, and that regional draining LNs participate in Ag uptake and tolerance induction.

Materials and Methods

Mice

DO11.10 (OVA-TCR) breeders were obtained from D. Y. Loh (4). They were maintained under specific pathogen-free conditions in the Animal Resource Facility at the University of Texas Medical Branch (Galveston, TX) and were fed an OVA-free diet. BALB/c mice (6–10 wk old from Harlan Sprague-Dawley, Indianapolis, IN) were used as recipients of Tg T cells. The university animal use and care committee approved all procedures.
Adoptive transfer and 5,6-carboxy-succinimidyl-fluorescein-ester (CFSE) labeling of donor cells

Peripheral (axillary, inguinal, and popliteal) and mesenteric LN lymphocytes from DO11.10 (OVA-TCR) Tg mice were isolated and washed in sterile PBS containing 1% normal mouse serum. An aliquot of cells was analyzed by flow cytometry to determine the percentage of CD4+ and Tg+ cells. Tg+ cells (3 × 10^5 in 100 μl of PBS) were transplanted into each recipient BALB/c mouse by tail vein injection. To directly follow the donor cells in recipient mice, CFSE (Molecular Probes, Eugene, OR) labeling was performed as previously described (5, 6). Briefly, lymphocytes from OVA TCR-Tg mice were resuspended in PBS containing 0.1% BSA (Sigma, St. Louis, MO) at 10^7 cells/ml and were incubated in a final concentration of 10 μM CFSE for 10 min at 37°C. Labeled cells were washed twice with PBS containing 0.1% BSA before they were resuspended in PBS for tail vein injection.

Feeding and immunization

Mice transplanted with Tg+ cells were divided into three groups. One day following the transfer, one group of mice was fed 100 mg of OVA (Sigma) in 250 μl of PBS. The second group was immunized s.c. with 125 μg of OVA peptide 323–339 in CFA. The peptide was synthesized by the Protein Chemistry Laboratory of the Sealy Center for Molecular Science (Galveston, TX). The control group received neither OVA preparation. Mice from each group were sacrificed at the indicated time intervals following Ag administration.

Proliferation assay

To quantify the in vitro proliferative response to OVA peptide 323–339, 10^7 lymphocytes from peripheral and mesenteric LNs were cultured in 200 μl of complete medium with 10% FCS (HyClone, Logan, UT), 50 μM 2-ME, 10 mg/ml folate, and 2 mM glutamine with the indicated concentrations of OVA peptide. Cells were cultured for 3 days, and 1 μCi of [3H]thymidine was added to the wells for the last 18 h of culture. Statistical analysis was performed by calculation of SDs, and p values were calculated by Student’s t test.

Determination of anergy was performed as described previously (2).

Since [3H]thymidine uptake per input cell reflects the intensity of proliferation rather than the total number of Tg+ cells, the counts per minute per input Tg+ cell was calculated to assess the level of anergy in T cells. For each individual mouse, the percentage of Tg+ T cells, based on FACS analysis (KJ126 + CD4+ ) was multiplied by the number of cells per well (10^6) to give the total number of Tg+ cells per well. The specific [3H]thymidine incorporation (average of triplicate wells) was divided by the number of Tg+ T cells per well to give the counts per minute per Tg+ cell for each animal: Δcpm/[% Tg+ (100)] = cpm/Tg+ T cell.

mAbs and FACS analysis

Anti-transgene (Tg+) clonotypic mAb (KJ126) (3) was prepared from hybridoma cells. IgG purified by protein G chromatography (Pharmacia, Uppsala, Sweden) was FITC conjugated. Affinity-purified mouse IgG-FITC was used as a control for KJ126-FITC. Anti-CD4-TRICOLOR (clone CT-CD4) and control Rat-IgG2a Tricolor were purchased from Caltag (South San Francisco, CA). Anti-CD69 (clone H1.2F3), CD45RB (clone 16A), and CTLa-4 (clone UC10-4F10-11) were purchased from Pharmingen (San Diego, CA). Hamster IgG-PE (clone A19-3) was used as an isotype control for intracellular staining of CTLA-4.

Most cell surface molecules were detected by staining 10^6 viable cells with the indicated conjugates for 20 min at 4°C in PBS/0.1% azide/1% BSA. Intracellular CTLa-4 (along with an isotype control mAb) was detected following 0.3% saponin permeabilization (7). Data from 50,000 size-gated lymphocytes were acquired on a FACSscan (Becton Dickinson, Mountain View, CA) and analyzed with CellQuest software from Becton Dickinson.

ELISA for cytokines

IL-2 and IL-10 were measured by ELISA using paired mAbs specific for corresponding cytokines according to the manufacturer’s recommendations (PharMingen). Paired mAbs clones specific for IL-2 and IL-10 were JES5-2A5/SXC-1, respectively. IFN-γ was determined as follows: 96-well microtiter plates (Dynatech, Chantilly, VA) were coated overnight at 4°C with 5 μg of purified anti-IFN-γ (HB170) from PharMingen in 100 μl of carbonate buffer, pH 8.0. The plates were washed with PBS containing 0.5% Tween-20, blocked with 5% FBS in PBS, washed, and then incubated with culture supernatants or rIFN-γ standard (PharMingen) overnight at 4°C. The plates were washed again and incubated with rabbit anti-IFN-γ Ab (1/1000) for 1 h followed by peroxidase-conjugated goat-anti-rabbit IgG (1/5000) for 45 min at 37°C. The enzyme activity was determined colorimetrically using O-phenylenediamine dihydrochloride reagent (Sigma) and was read at 492 nm.

Results

Lymphocytes from OVA-fed mice are responsive to restimulation at 18 h after feeding, but are anergic by 48 h

When T cells from OVA TCR-Tg mice were transferred into normal histocompatible BALB/c mice, a small population of transgenic donor cells (CD4+ KJ126+) persisted in the LNs of recipient animals for an extended period (2, 8). We previously observed the induction of anergy following oral OVA feeding of the recipient mice. In the present study, we explored the early differentiative events and the role of regional draining LNs in the development of anergy.

BALB/c mice received 3 × 10^6 Tg+ cells from OVA TCR-Tg mice (DO11.10) and were divided into groups that were OVA/CFA immunized as positive controls, OVA-fed, or untreated. Mice from each group were sacrificed at the indicated time intervals following antigenic exposure, and their responses to antigenic re-stimulation in vitro were determined using proliferation assays. Mice from all three groups responded initially to OVA peptide restimulation 18 h after antigenic exposure (Fig. 1). Lymphocytes from OVA-immunized mice proliferated vigorously in response to antigenic exposure in vitro. By 72 h after antigenic stimulation, lymphocytes from OVA-immunized mice were significantly more responsive than LN cells from animals receiving OVA orally. No significant differences between OVA-fed and control lymphocytes were observed until 96 h, when lymphocytes from the OVA-fed mice were significantly less responsive to restimulation in vitro than lymphocytes from untreated mice (p < 0.05). The lack of response in the OVA-fed group persisted despite increases in the proportion of Tg+ T cells following feeding (Fig. 2A). The percentages of Tg+ cells from pooled peripheral and mesenteric LNs in all three groups were relatively uniform before cellular expansion (18 h). The proportion increased steadily in OVA-immunized mice thereafter. In OVA-fed animals, however, the proportion of Tg+ cells peaked at 72 h and then began to decline (Fig. 2A).
Unresponsiveness following antigenic priming may occur through different mechanisms, including clonal deletion and anergy (Fig. 1). During the early priming phase T cells were not significantly deleted in OVA-fed animals (Fig. 2A). To help elucidate the mechanism(s) involved in the OVA-tolerance model, we devised a method for measuring anergy by normalizing the T cell proliferation for the number of Tg T cells. Error bars represent SEMs. Asterisks denote those groups with a significance of $p < 0.05$ compared with their respective control groups.

When proliferation was expressed as a function of input Tg cells in the in vitro cultures restimulated with 1 $\mu$M peptide as a function of the input of OVA-TCR Tg cells. Error bars represent SEMs. Asterisks denote those groups with a significance of $p < 0.05$ compared with their respective control groups.

Unresponsiveness following antigenic priming may occur through different mechanisms, including clonal deletion and anergy (Fig. 1). During the early priming phase T cells were not significantly deleted in OVA-fed animals (Fig. 2A). To help elucidate the mechanism(s) involved in the OVA-tolerance model, we devised a method for measuring anergy by normalizing the T cell proliferation for the number of Tg cells in each well (total counts per minute/total number of Tg cells) (2). We reasoned that the reduced proliferation per input Tg T cells should indicate a state of anergy.

When proliferation was expressed as a function of input Tg cells in the in vitro cultures restimulated with 1 $\mu$M OVA peptide 323–339, Tg cells from immunized mice responded to a greater extent than those of control and OVA-fed animals throughout the study (Fig. 2B). While the proliferative responses of cells from all three groups of animals gradually declined after the initial antigenic priming, proliferation in OVA-fed mice decreased more quickly than that in the immunized animals. These data suggest that oral priming alone is sufficient to induce anergy as early as 48 h after priming.

OVA-specific T cells undergo Ag-specific activation and proliferation in vivo in response to OVA feeding

Since activated T lymphocyte blasts are larger than resting T lymphocytes, we examined the distribution of forward light scatter (FSC) by T cells from recipient chimeric mice. Donor T cells that were CD4+ Tg+ demonstrated a considerable increase in frequency of large cells in response to OVA (Fig. 3A). Most lymphocytes from the LNs that were derived from the recipient animal (Tg−) did not respond with an increase in size whether they were CD4+ or CD4− (Fig. 3A). Kinetic analysis revealed that the FSC increased in donor T cells (CD4+ Tg+) at 18, 48, and 96 h following antigenic exposure (Fig. 3B). Recipient cells did not exhibit these changes at those time points.

To directly monitor the expansion of transplanted donor cell in the recipient mice, we labeled donor (DO11.10) lymphocytes with a fluorescent chloromethyl derivative, CFSE, and then transferred them into recipient (BALB/c) mice (5, 6). Upon Ag-stimulated proliferation, the two daughter cells contain approximately one-half of the original fluorescence, and their progeny contain one-quarter, and so on. Thus, cells that divide a number of times exhibited proportionally reduced fluorescence intensity, as detected by flow cytometry. In these experiments, only Tg+ cells from either OVA-immunized or OVA-fed animals exhibited evidence of cell division by their reduced CFSE fluorescence intensity (Fig. 4 and Table I). Transgene-negative donor cells in both immunized and fed groups served as internal controls and failed to show cell division. Unprimed control mice did not respond to the Ag with cell division. Thus, using both ex vivo and in vivo approaches in this study, we were able to show that T cell activation and proliferation accompanied functional unresponsiveness and was highly specific to OVA (Figs. 3 and 4 and Table I).

The results from this study further suggest a regional difference in the response of cells in the draining LNs during tolerance induction, depending on the route and form of Ag introduced. In this

![FIGURE 2. Measurement of OVA-specific anergy following OVA stimulation. OVA-TCR-reconstituted BALB/c mice were immunized with 125 $\mu$g of OVA peptide 323–339 in CFA or were fed 100 mg of OVA in PBS as described in Fig. 1. Each data point represents an average of three mice. A. Proportion of OVA-TCR Tg cells detected by flow cytometry in LNs. Error bars represent SDs. B. Proliferative response to 1 $\mu$M peptide as a function of the input of OVA-TCR Tg T cells. Error bars represent SEMs. Asterisks denote those groups with a significance of $p < 0.05$ compared with their respective control groups.](http://www.jimmunol.org/)

![FIGURE 3. Activation of T cell subsets in recipient mice following administration of OVA. A. The top plot illustrates three gates drawn based on their CD4 and Tg TCR intensities. The lower panels are histograms demonstrating the size, based on FSC profiles, of lymphocytes from CD4+ Tg+, CD4+ Tg−, and CD4− Tg− compartments 96 h post-OVA-exposure. CD4+ Tg TCR+ T cells in recipient mice were donor cells, and those from Tg TCR− compartments were mostly of recipient origin. Only CD4+ Tg TCR− cells in OVA-immunized and OVA-fed mice developed to activated T blasts. B. Proportion of activated T cell blasts from donor and recipient origins on sequential days following OVA− exposure. Each data point represents an average of two or three mice.](http://www.jimmunol.org/)
were analyzed by flow cytometry. (CFSE low OVA-TCR high) over total number of animals. Data were pooled from two independent experiments with three animals per group in each experiment. T cells were also determined by flow cytometry (Fig. 5) expression of CD69 and CD45RB. Intracellular CTLA-4 levels in specific T cells by flow cytometric analysis of the cell surface.

Since the state of anergy is induced during the early priming phase, feeding OVA Rapid induction of T cell activation and memory markers after systemic immunization. Pheno-
tic changes in cell activation markers were determined on OVA-specific T cells by flow cytometric analysis of the cell surface expression of CD69 and CD45RB. Intracellular CTLA-4 levels in T cells were also determined by flow cytometry (Fig. 5A).

We found, based on CD69 expression, that activation of the OVA-specific T cells occurred in both peripheral and mesenteric LNs within 3–6 h after oral administration of OVA (Fig. 5B). The expression of this early T cell marker subsided gradually after 18 h in OVA-imunized and OVA-fed mice (Table II). A subsequent decrease in the CD45RB level also suggested a prior activation of T cells in both groups of OVA-primed mice. Changes in CD69 and CD45RB in control animals were not significant. These data suggest that T cells from both OVA-fed and OVA-immunized animals were progressing to a phenotype of memory-like T cells. CTLA-4 is known to be a negative regulator of cell-mediated immune responses (9–13) and is normally expressed on activated T cells. Since a large proportion of CTLA-4 is expressed intracellularly, Ag detection was enhanced when saponin-permeabilized cells were stained with an anti-CTLA-4 mAb as gray overlays. The isotype control mAb was used as a control. CTLA-4 intensities were measured on gated OVA-specific, Tg+ cells (Fig. 5A). Intracellular CTLA-4 levels were near background in all three groups 18 h after antigenic exposure (Table II). While CTLA-4 remained undetectable in control animals, its levels were markedly increased in OVA-immunized and OVA-fed animals at 48 h after priming. Kinetic analysis of CTLA-4 expression indicated that the up-regulation of CTLA-4 was transient and returned to its initial level 96 h following antigenic exposure in all groups. In summary, data from these experiments revealed rapid T cell activation shortly after feeding. Sustained, but regulated, T cell activation led these cells to progress to a memory-like phenotype. No phenotypic differences between the OVA-immunized and OVA-fed mice were detected that would aid in differentiation between the early events in oral tolerance and systemic immunization.

Table I. In vivo proliferation of OVA-TCR Tg cells in recipient BALB/c mice following OVA priming

<table>
<thead>
<tr>
<th>Mice Exhibiting Proliferation of Tg+ Cells</th>
<th>Peripheral LNs</th>
<th>Mesenteric LNs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Immunized</td>
<td>6/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Fed</td>
<td>2/6</td>
<td>5/6</td>
</tr>
</tbody>
</table>

* As in Fig. 4, mice were sacrificed at 3 days postantigenic exposure, and cells were analyzed by flow cytometry.

* Data are expressed as number of animals exhibiting proliferation of Tg+ cells (CFSE low OVA-TCR high) over total number of animals. Data were pooled from two independent experiments with three animals per group in each experiment.
Production of cytokines during anergy induction

Since the proliferation and differentiation of resting T cells are driven by IL-2 and are also influenced by many other cytokines (e.g., IFN-γ), the most important change in anergic T cells is their inability to produce IL-2 when they encounter an Ag. To further examine the early events after antigenic stimulation, we compared the cytokine production by lymphocytes from OVA-immunized and -fed mice. T cells from peripheral and mesenteric LNs (or pooled) were restimulated in vitro with the OVA peptide. The production of IL-2, IFN-γ, and IL-10 was detected by sandwich ELISA and was sensitive down to approximately 32 pg/ml for IL-2, 45 pg/ml for IFN-γ, and 120 pg/ml for IL-10.

T cells from peripheral LNs in OVA-primed mice secreted large amounts of IL-2 18 h following in vitro restimulation (Fig. 6A), whether fed OVA or immunized s.c. with the peptide in CFA. In OVA/CFA-immunized animals, IL-2 reached its peak concentration 72 h after priming and then declined. By comparison, cells from OVA-fed mice produced much less IL-2 at 72 h, resulting in levels no more than those in control animals. While the kinetics of IL-2 secretion in mesenteric LNs were reminiscent of those in peripheral LN responses, mesenteric T cells from OVA-fed animals were more responsive to restimulation at 72 and 96 h following feeding compared with their peripheral counterpart. When T cells were pooled from peripheral and mesenteric LNs, reduced IL-2 production upon restimulation was also observed in OVA-fed mice compared with that in OVA/CFA-immunized mice (data not shown).

At 48 h following immunization, T cells from OVA/CFA-immunized mice were able to secrete IFN-γ after in vitro restimulation, reaching a peak concentration at 72 h, after which IFN-γ production declined (Fig. 7). LN cells from OVA-fed mice produced much less IFN-γ than the immunized mice, although the kinetics of IFN-γ production in the OVA-fed mice was similar to that of their immunized counterparts.

T cells from peripheral LNs in OVA-immunized mice produced significantly increased amounts of IL-10 4 days after immunization (Fig. 8). T cells from control and OVA-fed mice did not secrete or accumulate great amounts of IL-10 in response to restimulation. While mesenteric T cells in OVA-immunized and fed animals appeared to secrete slightly more IL-10, the difference between these groups and the control group was not significant by Student’s t test. In another experiment lymphocytes were pooled from peripheral and mesenteric LNs. In that case, again, only OVA-immunized animals produced increased amounts of IL-10 (data not shown).

These experiments demonstrate that T cells from systemically immunized mice secrete a transient, but substantial, amount of IL-2, IFN-γ, and IL-10 upon in vitro restimulation. While both OVA-immunized and -fed animals underwent T cell activation and proliferation shortly upon priming, LN T cells from OVA-fed mice secreted much less IL-2, IFN-γ, and IL-10 than immunized mice (Figs. 6–8). Normalization for variations in the proportions of TG T cells prior to restimulation did not alter the results (data not shown).

![Figure 6](http://www.jimmunol.org/) IL-2 production in vitro upon restimulation with 1 μM OVA peptide. OVA-TCR-reconstituted BALB/c mice were immunized with 125 μg of OVA peptide 323–339 in CFA or were fed 100 mg of OVA in PBS as described in Fig. 1. A, IL-2 concentration from peripheral LN cells. B, IL-2 concentration from mesenteric LN cells. Each data point represents an average of three mice, and vertical bars denote SEMs. No statistically significant differences were found at any time points by Student’s t test (p > 0.05).

![Figure 7](http://www.jimmunol.org/) Production of IFN-γ in vitro upon restimulation with 1 μM OVA peptide. OVA-TCR-reconstituted BALB/c mice were immunized with 125 μg of OVA peptide 323–339 in CFA or were fed 100 mg of OVA in PBS. Cells were pooled from peripheral and mesenteric LNs in BALB/c recipient mice at several time points following OVA exposure as described in Fig. 1. Data from an independent experiment with comparable results are not shown.

Table II. Kinetic analysis of T cell activation marker on OVA-specific cells

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control CD69</th>
<th>Immunized CD69</th>
<th>Fed CD69</th>
<th>Control CD45RB</th>
<th>Immunized CD45RB</th>
<th>Fed CD45RB</th>
<th>Control CTLA-4 MFI</th>
<th>Immunized CTLA-4 MFI</th>
<th>Fed CTLA-4 MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>152 ± 94</td>
<td>549 ± 285*</td>
<td>417 ± 205*</td>
<td>2887 ± 804</td>
<td>2942 ± 712</td>
<td>3150 ± 813</td>
<td>0.52 ± 0.04</td>
<td>1.38 ± 1.31</td>
<td>1.36 ± 0.01*</td>
</tr>
<tr>
<td>48</td>
<td>171 ± 58</td>
<td>280 ± 112</td>
<td>316 ± 38*</td>
<td>2458 ± 244</td>
<td>1601 ± 827</td>
<td>1826 ± 800</td>
<td>0.95 ± 0.56</td>
<td>6.91 ± 0.84*</td>
<td>4.49 ± 1.57</td>
</tr>
<tr>
<td>72</td>
<td>166 ± 58</td>
<td>163 ± 61</td>
<td>293 ± 355</td>
<td>2582 ± 390</td>
<td>1597 ± 49*</td>
<td>1057 ± 278*</td>
<td>0.39 ± 1.79</td>
<td>1.79 ± 0.01</td>
<td>1.31 ± 1.36</td>
</tr>
<tr>
<td>96</td>
<td>107 ± 23</td>
<td>108 ± 19</td>
<td>122 ± 25</td>
<td>1959 ± 612</td>
<td>1026 ± 337</td>
<td>713 ± 336</td>
<td>1.23 ± 0.78</td>
<td>1.30 ± 0.39</td>
<td>1.79 ± 0.01</td>
</tr>
</tbody>
</table>

* The intensity of cell surface CD69 and CD45RB on OVA-TCR Tg cells was detected by flow cytometry in draining LNs (mean ± SD). Asterisks denote those groups with a significance of p < 0.05. Each data point represents an average of three mice, and vertical bars denote SEMs. No statistically significant differences were found at any time points by Student’s t test. In another experiment lymphocytes were pooled from peripheral and mesenteric LNs. In that case, again, only OVA-immunized animals produced increased amounts of IL-10 (data not shown).
Evidence of activation led to the question of the sustained phenotype of the remaining anergic T cells. CD45RB is a cell surface phosphatase expressed at high levels on naive T cells. Expression is decreased on memory and effector CD4+ T cells compared with that on their naive progenitors (16, 17). In our study OVA-activated T cells progress to a memory phenotype by virtue of the reduced CD45RB expression (17). Costaining of CFSE with CD45RB on Tg+ gated T cells from OVA-fed mice suggested that the loss of CD45RB expression is due to dilution during cell division, as cells with the least CFSE intensity exhibited concomitantly lower CD45RB expression (not shown).

The results from this study also reflect on the site where T cell tolerance takes place after OVA administration to the gastrointestinal tract. While the specific site of tolerance induction has not been clearly defined, the GALT, specifically Peyer’s patches (PP) and lamina propria, has been proposed (18). PP of OVA TCR-Tg mice have increased synthesis of Th2 cytokines IL-4, IL-10, and TGF-β, indicating a predominately Th2-type response rather than unresponsiveness in GALT (19). However, these studies were performed in animals that had a high precursor frequency of OVA-specific T cells and thus differ from the environment in the DO11.10-BALB/c chimeras, which have only about 1% Tg+ T cells in lymphoid organs and even fewer in PP. In a normal precursor environment, tolerance induction may instead occur within local draining (mesenteric) LNs. We previously found no evidence of trafficking of OVA-specific CD4+ T cells to the small intestine intraepithelial or lamina propria compartments (2) or to PP (N. Van Houten unpublished observations). In the present study antigenic priming clearly took place in both mesenteric and peripheral LNs shortly after OVA feeding. Yet, T cell activation and proliferation in these sites were not accompanied by the production of IL-2 and IL-10 upon restimulation. This observation is consistent with the report that orally administered Ag can travel systemically (20) and induce tolerance at sites other than the GALT. Recent work using Cyt c TCR transgenic mice demonstrated that the orally administered Cyt c enters the bloodstream and is rapidly distributed to secondary lymphoid organs (mesenteric LNs and the spleen), where it can trigger T cell responses (15). Peripheral tolerance that is induced by orally administered OVA could also be explained by splint tolerance, where Ags introduced solely to the gastrointestinal tract have distinctive local effects. Oral Ag frequently elicits a local Ab response in the intestinal lamina propria while at the same time producing a state of systemic tolerance manifest as a diminished response to the same Ag if it is administrated in immunogenic form elsewhere in the body. This unique antigenic tolerance may be important in avoiding allergy to Ags in food, while the inhibition of systemic response prevents self destruction of the host tissues processing Ag.

The mechanisms of oral tolerance induction are probably due to the nature of Ag presentation. Although dendritic cells are generally accepted as immunostimulatory APCs in the initiation of immune responses, the role of these cells in T cell sensitization and tolerance remains controversial. OVA-specific T cells can be sensitized by exposure to adoptively transferred OVA-loaded dendritic cells (21, 22). However, in situ expansion of dendritic cells in mice after administration of Flt3 ligand enhances tolerance induction (23). There is accumulating evidence that it is the qualitative nature of the interaction between APC and T cells that determines whether tolerance is induced. The role of B cells in T cell sensitization has also been examined (24). OVA-specific T cells can be sensitized by HEL-specific B cells supplied with Ag containing both T and B cell epitopes (OVA/HEL); however, this T/B interaction does not occur when Ags are administered in separate soluble forms (24). The form, site, and dose of the Ag dictate...
the fine microenvironmental influence of T cell migration and sensitization.

The two-signal theory of T cell stimulation (25) has led to the investigation of CD28 and its counterpart CTLA-4 in the induction of T cell signaling and tolerance (9). Given the immunosuppressive role of CTLA-4 engagement in T cell stimulation (26, 27), we hypothesized that the kinetics or quantity of expression of CTLA-4 would differ between anergic and sensitized T cells. Although blockage of CTLA-4 signaling prevents tolerance induction in i.v. and oral tolerance (28, 29), we found that the expression of CTLA-4 on OVA-specific T cells after in vivo exposure was similar in groups of animals that were fed OVA or sensitized with the Ag in adjuvant. Thus, it appears that the decision between anergy and sensitization in vivo depends on the level of the signal delivered and not on variation in the expression of the molecule receiving the signal.

It has not been established exactly how cytokines participate in the decision of T cells to become anergic or sensitized in vivo. The differentiated anergic or sensitized T cells may express differential cytokine gene products or cytokine receptors once the functional commitment has been made. Although IL-10 has been associated with immunosuppressive activity in some systems (30–33), it can also restore proliferative responses in tolerized T cells (34). IL-10 is synthesized in the spleens of OVA-TCR recipient mice that were fed low, but not high, doses of OVA (35). It can also be expressed in PP in OVA TCR-Tg mice fed OVA (19). Conversely, oral tolerance has been achieved in mice treated with anti-IL-10 (36). Thus, our findings that IL-10 is not induced in peripheral LNs upon restimulation in lymphocytes from orally tolerized mice are consistent with its potential immunostimulatory role in immune responses (37, 38).

The most remarkable difference in lymphocytes between OVA-fed and -immunized animals was the production of IFN-γ after restimulation in vitro. Lymphocytes from OVA-immunized animals produced considerable levels of IFN-γ upon restimulation in vitro, while those from OVA-fed animals produced significantly less. In a study of splenocytes from OVA-TCR recipient mice (35), IFN-γ was detected at 2 days after feeding but was at much lower levels at 10 and 20 days. Their most striking finding was the production of TGF-β in the spleens of animals fed both high and low doses of Ag.

We have found that the mechanism of oral tolerance induction is parallel in many ways to that of i.v. induced tolerance. This finding provides a potential opportunity to draw from the results of other studies of i.v. tolerance in further defining oral tolerance. However, it should be appreciated that oral exposure is routine for dietary Ags and is being explored extensively for protective immunizations and potential tolerance against autoantigens and transplantation Ags (39–42). Thus, further studies of the mechanisms of induction of oral tolerance have extensive practical implications in the development and application of mucosal vaccines and in the treatment of inflammation.

Acknowledgments

We thank Dr. Dennis Loh for the DO.11 mice and KJ126 mAb, Dr. Rolf Konig for sharing the peptide for these studies, Drs. Marc K. Jenkins and Randall M. Goldblum for critical review of this manuscript, and Marjelle Susman for helping to prepare the manuscript.

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