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In Situ Immune Response in Gut-Associated Lymphoid Tissue (GALT) Following Oral Antigen in TCR-Transgenic Mice

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Oral administration of Ag results in systemic hyporesponsiveness termed oral tolerance. The regulatory cells induced by oral Ag are effective in the suppression of Th1-type autoimmune diseases. We examined the cytokine microenvironment in gut-associated lymphoid tissue in response to orally administered OVA in OVA TCR-transgenic mice. Mice were fed a low (0.5 mg) or high (500 mg) dose of OVA one time or five times. Immunohistochemical analysis demonstrated increased IL-4, IL-10, and TGF-β in the gut within 6 h of a low-dose feeding and after five low-dose or high-dose feedings. IFN-γ and IL-2 either decreased or showed no change, with the exception of a small transient increase in IL-2 at 6 h after a low dose. Increases in IL-4 and IL-10 were found in the dome of the Peyer’s patch, and increases in TGF-β were observed in the interfollicular region and the villi. IL-10 was also substantially increased in the villi. IL-4 and IL-10 were produced predominately by CD4 T cells. TGF-β was found predominately in macrophages and CD4+ T cells. Peyer’s patches had a marked up-regulation of TGF-β mRNA as measured by RT-PCR. These results demonstrate the differential activation of cytokine production in discrete regions of gut-associated lymphoid tissue. The induction of cytokines known to inhibit autoimmune disease at the site of Ag absorption indicates an important role for the mucosal immune system in the establishment of oral tolerance.

One of the consequences of oral Ag administration is the induction of oral tolerance. Oral tolerance is evidenced by the suppression of humoral and cell-mediated responses to immunization with Ag following oral administration of the same Ag (5, 6). Depending upon the dose of Ag that was fed, oral tolerance may be mediated by anergy, deletion, or the induction of regulatory T cells (7, 8, 20). Regulatory T cells generated during the course of oral tolerance function by producing suppressive cytokines such as IL-4, IL-10, and TGF-β (9, 10). TGF-β is an important gut-associated cytokine, as it provides help for IgA production, and regulatory T cells induced by oral Ag preferentially secrete TGF-β. In the rat, TGF-β-secreting regulatory cells can be found in Peyer’s patches that are removed 24 to 48 h after one feeding of myelin basic protein (MBP) and can adoptively transfer protection in vivo (11). In murine models, CD4+ regulatory cells from mesenteric lymph nodes (MLNs) which secrete TGF-β, IL-4, and IL-10 have been cloned (9). Oral tolerance has been used to successfully treat cell-mediated autoimmune diseases in animals and is currently being tested in humans (12). In the present study, we used OVA TCR-transgenic mice to examine the cytokine milieu in the Peyer’s patch and lamina propria in response to orally administered OVA. Low-dose and high-dose feeding was compared. The transgenic model enabled us to characterize the T cell response to feeding in vivo; such a response could not be defined in conventional animals without immunization due to the low frequency of Ag-specific T cells. In addition, the transgenic model allowed us to examine immunologic events associated with the generation of immune responses in GALT.

Materials and Methods

Antibodies

Abs were obtained as follows: rat anti-mouse IL-2 (clone S4B6) and rat anti-mouse IL-10 (clone JES5-2A5) were purchased from PharMingen (San Diego, CA); rabbit anti-porcine TGF-β was obtained from R&D Systems (Minneapolis, MN); rat anti-mouse IL-4 (BVD6.24G) was provided by Dr. A. Lichtman (Harvard Medical School, Boston, MA); and hamster anti-mouse IFN-γ was purchased from Genzyme (Cambridge, MA).
specificities of these Abs were established by bioassays and by blocking staining with recombinant cytokines.

Ab concentrations were as follows: IL-10, IL-2 and IFN-γ (5 μg/ml), IL-4 (hybridoma supernatant); TGF-β (2 μg/ml).

The secondary Abs used for immunofluorescence staining of IL-4 and TGF-β were R-phycocerythrin (R-PE)-conjugated goat anti-rat IgG (Caltag, Burlingame, CA) and FITC-conjugated goat anti rabbit IgG (Caltag), respectively. IFN-γ was labeled with FITC-conjugated goat anti-hamster IgG (Caltag). For double staining, FITC- or R-PE-labeled anti-murine CD4 or CD8 were obtained from PharMingen. Macrophages (Mφ) were labeled with rat anti-mouse F4/80 (Caltag) followed by R-PE-labeled anti-rat IgG (Caltag). Abs for immunofluorescence were used at a concentration of 5 μg/ml.

**Mice**

OVA-specific TCR transgenic mice (BALB/c) expressing the Vα1.3/ Vβ8.2 TCR on 97% of peripheral T cells (20) were fed 0.5 mg or 500 mg of OVA either one or five times on alternate days. Tissues were taken from the jejunum of the small bowel. A section of bowel (8–12 cm) that was localized 2 to 3 cm beyond the pyloric sphincter was examined. Peyer’s patches were harvested at 1, 2, 4, 6, 24, and 48 h after a single low-dose feeding and at 48 h after five low-dose feedings. Tissues from animals given a single high-dose feeding or five high-dose feedings were harvested 48 h after the last feeding. Control mice that had been designated unfed were given PBS instead of OVA. Some controls were fed hen egg lysozyme (HEL). In an initial series of experiments, we fed animals PBS or equivalent amounts of HEL or OVA and found no difference between HEL- and PBS-fed animals in terms of immunohistochemistry or the release of cytokines after secondary in vitro stimulation.

**Preparation of cryosections**

Peyer’s patches and villus regions from OVA TCR-transgenic mice were excised, rinsed in PBS, immersed in OCT freezing medium (Miles, Elkhart, IL), and snap frozen in liquid nitrogen-cooled isopentane. Tissues were sectioned at −20°C, fixed for 5 to 10 s in chilled acetone, and stored at −70°C.

**Immunohistochemistry**

Frozen sections were thawed and fixed in acetone for 2 min. Endogenous peroxidase activity was quenched by incubation in periodic acid (0.003 M in water, pH 2.45) for 10 min followed by washing in PBS and immersion in 0.003 M sodium borohydride for 30 min (13). After washing, tissues were incubated for 20 min in diluted normal blocking serum that was prepared from the species from which the secondary Ab is made. Endogenous biotin was blocked by 1-h incubations in avidin D followed by biotin (vector blocking kit, Vector Laboratories, Burlingame, CA). Sections were rinsed in PBS and immersed in primary antisera that had been diluted in blocking serum for incubation overnight. Next, sections were washed for 10 min in PBS and incubated in biotinylated, secondary Ab for 1 h. After washing, sections were incubated in Vectastain Elite avidin-biotin complex reagent, washed again, and incubated in peroxidase substrate solution containing 3,3′-diaminobenzidine (DAB Substrate Kit, Vector Laboratories) for 5 to 7 min. Sections were washed for 5 min in tap water, counterstained in hematoxylin, cleared, and mounted.

Frozen sections were prepared for immunofluorescence microscopy by fixation in acetone for 2 min followed by incubation for 20 min in diluted normal blocking serum as described above. To block nonspecific staining by fluorochrome-conjugated Abs that were mediated by FcRs, sections were incubated with 2.4G2 Ab (PharMingen, 10 μg/ml) that was directed against FcγRII/IIIIs. For cytokine labeling, sections were incubated overnight in primary antisera. After washing for 10 min in PBS, sections were incubated in R-PE- or FITC-labeled secondary Ab for 1 to 2 h. For double staining, sections were incubated in FITC- or R-PE-labeled anti-C4D (PharMingen) or CD8 (PharMingen) for 1 h. Mφ were incubated with rat anti-mouse F4/80 for 1 to 2 h followed by R-PE-labeled anti-rat IgG (Caltag) for 1 h. Sections were washed in PBS and mounted in Vectashield mounting medium (Vector Laboratories). The specificity of fluorescence staining was demonstrated by preincubation of the Ab with its target Ag or by incubation of tissue with unlabeled anti-cytokine Ab before staining with the fluorochrome-conjugated form of the same anti-cytokine Ab. Nine sections from three animals (three per animal) were examined both for animals fed 0.5 mg once (or 500 mg) or five times. No staining was observed following preincubation of the Ab with its target Ag (100-fold excess) or following incubation of tissue with unlabeled anti-cytokine Ab (100-fold excess) before incubation with the fluorochrome-conjugated form.

**RT-PCR for detection of cytokine mRNAs**

Total RNA (10 μg) was quantitated by spectrophotometer and reverse transcribed using oligo(d1)18. (Life Technologies) and RNaseH-reverse transcriptase (Superscript II, Life Technologies) at 42°C for 2 h. The reverse transcriptase was inactivated at 95°C. To ensure that each sample had the same amount of cDNA, the hypoxanthine phosphoribosyltransferase (HPRT) cDNA concentration of each sample was first determined using HPRT-specific primers. Then cDNA samples were amplified with IFN-γ- and TGF-β1-specific primers, and products were separated on agarose gels and stained with ethidium bromide. PCR reactions were incubated for 36 cycles (denaturation for 30 s at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C). The sequences of primers for the cytokine genes are as follows: IFN-γ forward, 5′CACACTGACATCTTGGGCTT-3′;

![FIGURE 1. IL-10 and IL-4 quantitation in the dome of the Peyer’s patch of OVA TCR-transgenic mice that were fed a low dose of OVA. Tissues were harvested at 1, 2, 4, and 6 h as well as at 24 and 48 h after a low dose.](http://www.jimmunol.org/)

![FIGURE 2. Immunohistochemical localization of IL-4 in the Peyer’s patch of HEL-fed (a) and OVA-fed (b) OVA-transgenic mice at 6 h after a low-dose feeding. HEL-fed mice are virtually devoid of IL-4–positive cells in the dome, while OVA-fed mice show the presence of many positive cells. Both HEL- and OVA-fed animals show the presence of IL-4 in the corona. D, Dome; C, Corona. Stained cells are indicated by an arrow.](http://www.jimmunol.org/)
Quantification

Tissues from two experiments were examined. Two animals were fed per dose in one experiment, and one animal was fed per dose in a second experiment. Nine sections (three per animal) were randomly selected and quantitated for each feeding regimen. The number of stained cells in one centimeter field per section was counted under ×400 magnification. Data are expressed as the average number of cells per nine high-power fields ± SD and were obtained by the Student t test.

Results

Effect of oral Ag on the cytokine microenvironment in the GALT of OVA TCR-transgenic mice.

Dome. The dome of the Peyer’s patch is the primary site of Ag entry after being taken up by M cells. Within 6 h, OVA TCR-transgenic mice that were given a single low-dose feeding of 0.5 mg of OVA had a large increase in both IL-10- and IL-4-stained cells in the dome of the Peyer’s patch (Fig. 1). IL-10 was significantly increased by 1 h (from 1.7 ± 4.0 to 16.3 ± 10.1, p < 0.001) and increased further by 6 h (61.8 ± 5.7, p < 0.001). By 24 h, the number of IL-10-secreting cells had returned to control levels. IL-4 also increased in the dome after a single low-dose feeding, although not as rapidly as IL-10. Significant increases were observed at 6 h (from 9.7 ± 3.1 to 26.1 ± 1.1, p < 0.002). Unlike IL-10, which returned to control levels by 24 h, the number

FIGURE 3. Cytokine profiles in the dome of the Peyer’s patch of OVA-transgenic mice. Mice were fed a low dose, and tissues were harvested at 6 h after feeding. The number of cells that positively stained for IL-4, IL-10, TGF-β, IFN-γ, and IL-2 were quantitated. Both unfed and fed mice were compared. Increases were found for IL-4 (p = 0.003) and IL-10 (p ≤ 0.001), while IFN-γ decreased (p = 0.001).

IFN-γ reverse, 5′-ACTCCITTTCGCTTT-3′; TGF-β1 forward, 5′-CTTTAGGAAGGACCTGGGTT-3′; TGF-β1 reverse, 5′-CAGGAGCGCA CAATCATGTT-3′; HPRT forward, 5′-CTCGAAGTGTTGGATACAGG-3′; HPRT reverse, 5′-TGGCCTATAGGCTCAT AGTG-3′.

FIGURE 4. IFN-γ and IL-2 staining in the Peyer’s patch of unfed and low-dose-fed (five times) OVA TCR-transgenic mice. a, Hamster IgG control. D, Dome; C, Corona. b, IFN-γ-stained cells are scattered in the dome and corona of an unfed animal. A stained cell is indicated by an arrow. c, A similar staining pattern is seen in the dome and corona after low-dose feeding. No increase in IFN-γ staining was detected. d, Rat IgG control. e, IL-2 staining in the dome and corona of an unfed animal. IL-2-stained cells are localized beneath the epithelium in the dome of the Peyer’s patch (large arrow). A few IL-2-stained cells are found in the corona. A subepithelial localization of IL-2-positive cells (small arrow) is also found in the villi (V). f, IL-2 staining after low-dose feeding is similar to unfed animals. No increase in IL-2 was detected.
of IL-4-positive cells increased at 24 h (28.9 ± 10.1, p = 0.014) and returned to control levels by 48 h. Figure 2 shows the immunohistochemical localization of IL-4 in the Peyer’s patch dome of animals that were fed OVA vs those that were fed HEL. No increase in IL-4 or IL-10 was observed in animals that were fed single or multiple high doses (500 mg) of OVA or a control protein (HEL). There was no expression of TGF-β at 6 h in the dome of the Peyer’s patch in animals that were fed a single dose of OVA (Fig. 3). In fact, there was little expression of TGF-β at any of the time points examined (1–48 h), regardless of whether animals had five feedings of 0.5 mg of OVA or high-dose (500 mg) feedings of OVA one or five times.

IFN-γ and IL-2 were expressed in the dome of control animals; IFN-γ decreased at 6 h after a single low-dose feeding, whereas there was no change in IL-2 (Fig. 3). The expression of IFN-γ or IL-2 in the dome did not significantly change at other time points or as a result of animals receiving multiple low- or high-dose feedings of OVA (Fig. 4). Thus, the primary change in the dome area involved the marked increase in expression of IL-10 and IL-4 after low-dose feeding.

**Corona**

The expression of cytokine in the corona, the area in the Peyer’s patch just underneath the dome, was similar to that seen in the dome itself, with the exception that a large increase in IL-4 was observed after multiple low-dose feedings. As in the dome, there was a rapid increase in the expression of IL-10 after a single low-dose feeding (within 1 h) that decreased by 24 h. There was virtually no expression of TGF-β at any time point or with any feeding regimen. IFN-γ decreased and IL-2 increased slightly after a single low-dose feeding, and both were similar to controls after other feeding regimens. The levels of IL-10 in unfed mice (0.1 ± 0.3) increased in the corona by 1 h (10.8 ± 9.3) after a low-dose feeding, and increases were also evident at 4 (14.4 ± 8.3) and 6 (24.4 ± 18.5) h. By 24 h after feeding, IL-10 had returned to the levels found in unfed animals. IL-10 levels remained similar to the levels seen in unfed animals at 48 h after other feeding protocols.

In the corona, levels of IL-4 showed a tendency to increase, with a large SD by 6 h (30.4 ± 6.7), but returned to unfed levels (19.4 ± 6.8) at 24 and 48 h after a single low-dose feeding. However, a large increase (2.5-fold) in IL-4 was found following a five low-dose feedings (45 ± 3.3). Amounts similar to those seen in unfed mice were found with other feeding regimens. No significant changes for TGF-β were detected in the corona (data not shown).

For IFN-γ, a decrease from unfed levels (16.6 ± 8.1) was seen at 48 h after a single low-dose feeding (5.1 ± 3.9). The results from all other feeding regimens were similar to those found in unfed mice. IL-2 showed a small increase from unfed mice (5.2 ± 2.3 to 12.9 ± 2.9) at 6 h after a low-dose feeding and then returned to unfed levels at 24 and 48 h. All other feeding regimens had levels that were similar to unfed levels.

**Germinal centers**

Germinal centers are B cell-rich areas in the Peyer’s patch. The primary change observed in germinal centers was an increase in the expression of IL-4 (Fig. 5). This increase was seen at 24 h after a single-dose feeding but subsequently returned to control levels. No changes in IL-4 were seen at high doses. There was no increase in TGF-β or IL-10 staining.

For Th1-type cytokines, low-dose feeding resulted in a decrease in IL-2 staining from control levels of 17 ± 7.5 to 7.0 ± 2.5 after five low-dose feedings. In contrast, a single high-dose feeding resulted in an increase in IL-2 (33.5 ± 6.6), and five feedings resulted in an increase with a large SD (26.6 ± 17.3). For all feeding regimens, IFN-γ staining showed no change compared with control levels, with the exception an increase with a large SD (from...
48.4 ± 12.9 to 70.7 ± 12.4) that was seen at 48 h after a low-dose feeding.

Interfollicular region

The interfollicular area was notable for a marked expression of TGF-β. This peaked at 6 h and was seen at 24 and 48 h as well (Figs. 6A and 7). An increase in IL-2 was also observed at 6 h after a single low-dose feeding (Fig. 6B). No changes were observed in the expression of IL-4, IL-10, or IFN-γ in the interfollicular region.

Lamina propria

A marked increase in TGF-β was observed in the lamina propria of fed mice. This was observed at 6 and 48 h after a single low-dose feeding and was even more pronounced after five low-dose feedings (Figs. 8A and 9). A single high dose did not induce TGF-β expression, whereas five high-dose feedings resulted in increased TGF-β expression.

IL-10 increased at 6 h after a single low-dose feeding and at 48 h after a single low-dose feeding or multiple low-dose feedings (Fig. 8B). In contrast, no significant change was detected after a single high-dose feeding, while increased IL-10 was detected after five feedings. Of particular note, constitutive staining of the epithelium for IL-10 was routinely observed (Fig. 10).

IL-4 increased at 2 and 4 h after a single low-dose feeding (Fig. 7). IL-2-stained cells were localized predominately underlying the epithelium and increased transiently at 6 h after a single low-dose feeding (Fig. 11). No change was seen following a single high-dose feeding. IFN-γ-stained cells were scattered throughout the lamina propria, and no change in IFN-γ was found after either low- or high-dose feedings.
Cytokine profiles from the lamina propria of mice at 6 h after a single low-dose feeding showed a large increase in IL-10 and TGF-β compared with control mice (Fig. 12A), while other cytokines were similar to controls. Similarly, IL-10 and TGF-β increased at 48 h after five low-dose feedings, with TGF-β showing a greater increase than that seen at 6 h after a single feeding (Fig. 12).

**FIGURE 9.** Immunohistochemical localization of TGF-β in villi. *a.* Unfed mice show the virtual absence of TGF-β-positive cells. A single positive cell is indicated by an arrow. *b.* Increase in TGF-β-stained cells after a five low-dose feedings. *c.* No staining is found in the rabbit IgG control.

**FIGURE 10.** Immunohistochemical localization of IL-10 and IL-4 in villi. *a.* Controls incubated in rat IgG showed the absence of staining. *b.* IL-10-stained cells are located under the epithelium and scattered in the lamina propria. A stained cell is indicated by an arrow. The epithelium (E) also stains for IL-10. *c.* IL-4-stained cells are localized in the lamina propria. No staining of the epithelium was observed.
Thus, increased expression of IL-10 and TGF-β are dominant responses to oral Ag in the lamina propria of the villi.

Identification of cytokine-producing cells

At 6 h after a low dose of OVA for IL-4 and IL-10 and after five feedings for TGF-β, CD4⁺ and CD8⁺ T cells and Mφ were examined for the production of cytokines by double-label immunofluorescence microscopy. A 6-h period was chosen for IL-4 and IL-10 production because it is a significant period for cytokine expression in the lamina propria.

**FIGURE 11.** Kinetic analysis of IL-2 and IFN-γ in the lamina propria of transgenic mice that were fed a low dose of OVA. IL-2 increased by 6 h but returned to naive levels by 48 h. IFN-γ levels were not significantly different from those of naive mice.

**FIGURE 12.** Cytokine profile from the lamina propria of OVA-TCR transgenic mice. **A,** Unfed and fed mice at 6 h after feeding. **B,** 48 h after five low-dose feedings.

**FIGURE 13.** Localization of IL-4 and CD4⁺ T cells in the Peyer’s patch at 6 h after the oral administration of 0.5 mg of OVA by immunofluorescence microscopy. **A,** IL-4 in the dome of the Peyer’s patch was labeled with primary rat anti-murine IL-4 IgG and visualized with FITC-conjugated (green) goat anti-rat IgG (arrow). **B,** CD4⁺ T cells were labeled with R-PE conjugated (red) goat anti-murine CD4 (arrow). **C,** Colocalization (yellow) of IL-4 and CD4⁺ T cells (arrow).
IL-10, since up-regulation of these cytokines in the dome of the Peyer’s patch (Fig. 1) occurs at 6 h after feeding. As shown in Figure 13, IL-4-producing cells in the dome of the Peyer’s patch and in the lamina propria of the villi were predominately CD4$^+$ T cells at 6 h. IL-10 staining in the dome, corona, and villi was also found predominately in CD4$^+$ T cells (data not shown). Only occasional staining of CD8$^+$ T cells for IL-4 and IL-10 was found in the Peyer’s patch and villi. As shown in Figure 14, TGF-$\beta$ up-

**FIGURE 14.** TGF-$\beta$ staining in the Peyer’s patch after five feedings of 0.5 mg of OVA. 

- **a.** TGF-$\beta$-stained cells (seen in the interfollicular region underlying the villi) were labeled with primary rabbit anti-human TGF-$\beta$ IgG and visualized with FITC-conjugated (green) goat anti-rabbit IgG (arrow). 
- **b.** Mφ were labeled with rat anti-mouse F4-80 followed by R-PE-labeled (red) goat anti-rat IgG (arrow). 
- **c.** TGF-$\beta$-producing Mφ (yellow) are indicated by an arrow. 
- **d.** TGF-$\beta$-producing Mφ (yellow) in the lamina propria (arrow). 
- **e.** TGF-$\beta$-producing CD4$^+$ T cells (yellow) at the periphery of the Peyer’s patch (arrow). 
- **f.** Occasional TGF-$\beta$ CD8$^+$ T cells (yellow) are seen within the Peyer’s patch (arrow).
regulation in the interfollicular region and the lamina propria after five low-dose feedings was predominately due to Mφ and CD4+ T cells. An occasional staining of CD8+ T cells was also observed (Fig. 14). At 6 h after one feeding of 0.5 mg, it was found that staining of TGF-β occurred predominately in Mφ and CD4+ T cells. Some IFN-γ and IL-2 staining of CD4+ T cells was detected in the Peyer’s patch and in the lamina propria (data not shown).

**RT-PCR for TGF-β and IFN-γ**

Isolated Peyer’s patch cells were examined for response to oral Ag by RT-PCR. Levels of TGF-β and IFN-γ mRNA were examined in Peyer’s patch cells from OVA- and PBS-fed OVA TCR-transgenic mice at 6 h after a single feeding of 0.5 mg. As shown in Figure 15, a marked increase in TGF-β mRNA was found in OVA-fed animals when compared with PBS-fed animals. A slight increase in IFN-γ expression was also observed in OVA-fed animals.

**Discussion**

Our results demonstrate that within 6 h of administering a single low dose (0.5 mg) of OVA, an increase in IL-4 and IL-10 expression occurs in the dome, and IL-10 increases in the corona of the Peyer’s patch. The dome is the region just below M cells that functions to facilitate Ag absorption from the gut lumen, and the corona is just below the dome region. By 48 h, the level of IL-4 and IL-10 in the dome of the Peyer’s patch returned to control levels. With multiple low-dose feedings there was an enhanced expression of IL-4 that occurred specifically in the corona. The only change we observed in the germinal centers of the Peyer’s patch was an increased expression of IL-4, which was observed at 24 h after a single feeding of 0.5 mg.

TGF-β is an important gut-associated cytokine, as it serves as a switch factor for IgA production and may also be involved in the homing mechanism of cells to high endothelial venules. Little expression of TGF-β was observed in the dome, corona, or germinal center of the Peyer’s patch, but there was a marked increase seen in the interfollicular region at 6 h after a single low-dose feeding. The interfollicular region is a T cell migration area. TGF-β-secreting cells can be found in the Peyer’s patch at 24 to 48 h after feeding of MBP (11). In addition, there was a marked increase in the expression of TGF-β in the lamina propria at 6 h after a single feeding of 0.5 mg.

Although it is well established that absorption of Ag, especially particulate Ag, occurs via the M cells overlying the Peyer’s patch, soluble Ag may also be absorbed into the lamina propria via the villous epithelium. In addition to an increase of TGF-β in the lamina propria, we also observed increased expression of IL-4 and IL-10 in the lamina propria after a single feeding of 0.5 mg of OVA. IL-4 increased at 2 and 4 h after feeding but returned to control levels by 48 h, whereas IL-10 increased after 1 h and remained elevated at 48 h. The area of the Peyer’s patch that most resembles the cytokine increases found in the lamina propria is the interfollicular region, a T cell migration region.

T cells in lymphoid organs drained by mucosal sites secrete IL-4 as a primary T cell growth factor, whereas those drained by non-mucosal sites secrete IL-2 (14). IL-4 is an important growth factor for the generation of Th2-type and TGF-β-secreting CD4+ T cells (15, 16, 34, 35). As described above, we found marked increases of IL-4 in several anatomic regions of GALT. There were minimal changes in terms of IL-2 expression, with a slight increase in the expression of IL-2 in the corona, interfollicular region, and lamina propria at 6 h after a single low dose. Notably, we did not observe an increased expression of IFN-γ by immunohistochemistry in any of the regions examined, apart from a slight increase in the germinal center. In fact, there was a decrease in IFN-γ expression in the dome of the Peyer’s patch after a single feeding, which is consistent with the increased expression of IL-4 and IL-10 that occurs in this region. These observations are consistent with studies of Peyer’s patches from wild-type mice. Activated T cells from the Peyer’s patches produced IL-4 as the major T cell growth factor (14). Peyer’s patch CD8+ T cells purified by flow cytometry have been shown to contain transcripts for IL-4, IL-5, and IL-10 (17). Also, oral immunization with sheep RBCs has been shown to result in predominately Th2-type cells in the Peyer’s patch (18). Nonetheless, other investigators have observed increased IFN-γ secretion in Peyer’s patch cells after secondary stimulation in vitro (19). In these studies, patches were taken from the entire small bowel of OVA TCR-transgenic animals after three feedings of 250 mg each. A similar phenomenon has been observed after multiple low-dose feedings when patches taken from the entire small bowel are used (J. Inobe and H. L. Weiner, unpublished observations). However, when we examined patches from the proximal bowel of OVA TCR-transgenic mice given three 250-mg feedings, we did not observe the up-regulation of IFN-γ by immunohistochemical analysis. These results suggest that the observed in vitro IFN-γ secretion occurs only upon secondary stimulation or when distal patches are included.

**RT-PCR analysis of Peyer’s patch cells taken 6 h after feeding with 0.5 mg of OVA was undertaken to determine the response to feeding at the level of mRNA production. TGF-β mRNA showed a marked increase at 6 h that was also evident at 6 h and after five low-dose feedings according to immunohistochemistry. There was not a marked change in IFN-γ mRNA, although there was a suggestion that a slight increase occurred. This finding is consistent with the increase in IFN-γ seen by immunohistochemistry in the germinal center at 48 h.**

The mechanism of immune hyporesponsiveness following the oral administration of Ag is dose dependent, with higher doses inducing anergy and deletion. We previously reported that five high-dose (500 mg) feedings in OVA TCR-transgenic animals led to the deletion of cells in the dome region of the Peyer’s patch and to a decrease in IFN-γ and IL-4/IL-10 production in the spleen following in vitro stimulation with Ag. It should be noted that there was no decrease in TGF-β secretion (20). Our immunohistochemical studies of GALT are consistent in that there was increased TGF-β expression in both the villi and the Peyer’s patch interfollicular region even with multiple high-dose feedings. IL-4 and IL-2 expression decreased. Significantly, we observed increased IL-10 secretion in the villi with high-dose feeding.

We found constitutive expression of IL-4 and IL-10 in the lamina propria before the administration of Ag. There was little expression of TGF-β in any of the areas of the GALT before feeding.
suggesting the importance of Ag in inducing TGF-β. Animals were not fasted before the oral administration of OVA. IL-10 staining of the epithelium as well as in underlying lymphoid tissue before feeding was a prominent finding. Whether IL-10 is produced by intestinal absorptive cells or absorbed from underlying lymphoid tissue is not known. The results of Pania et al. (21) suggest that the intestinal epithelium may produce IL-10. They used density gradient centrifugation and RT-PCR to show that IL-10 production segregated with intestinal epithelial cells and not with contaminating T cells. The importance of IL-10 for the intestine is indicated by studies indicating that IL-10-deficient mice develop chronic enterocolitis (22), and that tolerance to resident intestinal flora is abrogated in experimental colitis and restored by treatment with IL-10 (23). In other studies, we have found that IL-10 can prime naive dendritic cells to induce IL-4 secretion, and that IL-10 down-regulates IL-12 production (36). Thus, IL-10 may be an important cytokine in GALT that serves to prime for Th2 responses, inhibiting IL-12 and, consequently, Th1 responses.

The predominance of cytokine changes in the gut that involve the increased expression of IL-4, IL-10, and TGF-β is consistent with the host preventing potentially harmful Th1-type responses. Indeed, oral tolerance may broadly be defined as the inhibition of Th1 responses in the periphery. Factors that affect Th1-type responses would be expected to affect oral tolerance, and the administration of IFN-γ abrogated oral tolerance (24), whereas anti-IL-12 enhanced it (19). Consistent with this observation, we observed a decrease in IFN-γ and an increase in IL-4 and IL-10 in the dome of the Peyer’s patch at 6 h after feeding.

It has been shown that orally administered LPS enhances protection by orally administered MBP; this protection is not seen when LPS is given systemically (25). The LPS synergistic effect was found to be associated with the expression of IL-4 in the brain of orally tolerized animals (26). It is possible that LPS in the gut enhances the Th2 response to orally administered OVA. The effect of LPS may be mediated by the polysaccharide o-side chain, which has been shown to be transported across the intestinal epithelium (27). In support of this hypothesis, it has been shown that oligosaccharide from schistosoma-infected mice is a potent inducer of IL-10 production by B220 cells (28). Studies of IL-4-deficient mice concluded that IL-4 and probably Th2 cells are required for the induction of gut mucosal Ab responses, since the administration of Ag to IL-4-targeted mice failed to stimulate Ag-specific T cell responses even though switch differentiation from IgM to IgA was not impaired (29).

LPS has also been found to mediate immunization via the gut as well as oral tolerance. The expression of dietary protein in Escherichia coli renders it strongly antigenic to GALT as measured by biliary IgA Abs (30–32). Whether LPS is involved in tolorization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of TGF-β following antigen-specific triggering. Proc. Natl. Acad. Sci. USA 89:421.


Whether cells secreting TGF-β and Th2 cytokines are initially generated in the Peyer’s patch, lamina propria, or MLNs is not definitively known. Previous studies of the MLNs of orally tolerized (SJL×PLJ)F1 mice found that both CD8+ T cells which secrete TGF-β and CD4+ cells which secrete IL-4 and IL-10 in addition to TGF-β are induced by oral tolerization (9). MBP-specific CD4+ T cell clones generated from the MLNs of MBP-fed SJL mice secrete TGF-β, IL-4, and IL-10 but not IFN-γ. Other investigators have shown by immunohistochemical studies of murine Peyer’s patches that both CD4+ and CD8+ cells are found in the interfollicular region of the patch (33). Whether TGF-β-secreting cells of the Peyer’s patch also secrete Th2 cytokines was not examined. Other cell types may also contribute to the cytokine milieu of GALT.

In summary, we have found that oral Ag induces Th2-type and TGF-β responses in the Peyer’s patches and villi of OVA TCR-transgenic mice. The mechanisms by which a predominately Th2 environment is induced in the subepithelial dome and TGF-β is induced preferentially in the interfollicular region and in the villi are not well understood. However, our finding of differential cytokine induction in specific anatomic locations in GALT provides a basis for understanding the mechanisms of mucosal immune responses.

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References


