# Peyer's patches are required for oral tolerance to proteins

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**To clarify the role of Peyer's patches in oral tolerance induction, BALB/c mice were treated *in utero* with lymphotoxin β-receptor Ig fusion protein to generate mice lacking Peyer's patches. When these Peyer's patch-null mice were fed 25 mg of ovalbumin (OVA) before systemic immunization, OVA-specific IgG Ab responses in serum and spleen were seen, in marked contrast to low responses in OVA-fed normal mice. Further, high T-cell-proliferative- and delayed-type hypersensitivity responses were seen in Peyer's patch-null mice given oral OVA before systemic challenge. Higher levels of $CD4^+$ T-cell-derived IFN-γ, IL-4, IL-5, and IL-10 syntheses were noted in Peyer's patch-null mice fed OVA, whereas OVA-fed normal mice had suppressed cytokine levels. In contrast, oral administration of trinitrobenzene sulfonic acid (TNBS) to Peyer's patch-null mice resulted in reduced TNBS-specific serum Abs and splenic B cell antitrinitrophenyl Ab-forming cell responses after skin painting with picryl chloride. Further, when delayed-type hypersensitivity and splenic T cell proliferative responses were examined, Peyer's patch-null mice fed TNBS were unresponsive to hapten. Peyer's patch-null mice fed trinitrophenyl-OVA failed to induce systemic unresponsiveness to hapten or protein. These findings show that organized Peyer's patches are required for oral tolerance to proteins, whereas haptens elicit systemic unresponsiveness via the intestinal epithelial cell barrier.**

Oral administration of proteins or haptens may induce two distinct types of immune responses. Oral immunization with protein Ag and an appropriate mucosal adjuvant such as cholera toxin or with a recombinant (r) *Salmonella* delivery system induces Ag-specific secretory IgA (S-IgA) and serum IgG Ab responses (1, 2). Studies of induction of Ag-specific S-IgA Ab responses at mucosal surfaces have suggested a common mucosal immune system that interconnects distinct mucosal inductive and effector tissues (1, 2). These studies have shown that IgA inductive tissues include the gut-associated lymphoreticular tissues (GALT) or Peyer's patches in the gastrointestinal tract for mucosal immune responses.

In contrast, oral immunization with large doses or prolonged oral exposure to lower doses of protein or hapten have been shown to induce systemic unresponsiveness. In earlier studies, this type of immunological response was dubbed "oral tolerance" and the concept was used to refer specifically to a failure to induce systemic immune responses after oral delivery of protein (3). It is now generally agreed that oral tolerance is established and maintained at the level of T cells (4–11). Two distinct forms of T-cell unresponsiveness have been proposed as major mechanisms for the induction of oral tolerance. Repeated oral administration of low doses of Ag-induced regulatory T cells to produce suppressive cytokines such as transforming growth factor (TGF)-β, IL-4, and IL-10 (11). In contrast, a high dose of Ag elicited clonal deletion and/or anergy that was characterized by an absence of T-cell proliferation and decreased IL-2 and -2R expression (12–14). Studies in ovalbumin (OVA)-transgenic mice showed significant increases in transgenic T cells in Peyer's patches of mice that had been tolerized by low or high OVA doses, suggesting the importance of this mucosal inductive tissue in the development of oral tolerance (15, 16). Further support for a requirement of GALT in oral tolerance induction was provided by *in vivo* treatment with *flt3* ligand. This treatment resulted in an expansion of dendritic cells in Peyer's patches with enhanced oral tolerance induction (17).

The importance of lymphotoxin (LT) signaling pathways in lymphoid tissue development has been studied by using LT-α and -β gene-disrupted mice. LT-α knockout mice do not possess Peyer's patches or associated lymph nodes (18, 19). Mice without LT-β gene expression lack Peyer's patches and peripheral lymph nodes but possess mesenteric, sacral, and cervical lymph nodes (20–22). Recent studies that include those which (*i*) use LT-β-receptor (LTβR) gene knockout mice to block the LTβR signaling pathway (23), (*ii*) use soluble LTβR-Ig (fusion protein of LT-β receptor and Ig) as a treatment (24), and (*iii*) use an agonist Ab to the LTβR (25), all result in aberrant development of peripheral lymphoid organs. Administration of LTβR-Ig during gestation also disrupted the development of peripheral lymph nodes and Peyer's patches; however, mesenteric, sacral, and cervical lymph nodes were intact (24, 26). Thus, the LTβR-Ig fusion protein system provides a unique and useful model to elucidate the role of Peyer's patches in the induction of oral tolerance.

To address precisely the role of Peyer's patches in the induction of oral tolerance, we have generated mice that lack Peyer's patches but that possess brachial, cervical, mesenteric, and sacral lymph nodes, by administration of LTβR-Ig to pregnant mice *in utero*. We have compared the immune responses in offspring of LTβR-Ig-treated and control mice given high oral doses of either OVA or trinitrobenzene sulfonic acid (TNBS) before systemic challenge. The differences in responses to these two forms of Ag are discussed from the standpoint that GALT are major sites for macromolecular protein uptake in maintenance of immunologic homeostasis between the mucosal and systemic compartments of the host.

## Materials and Methods

**Mice.** Timed, pregnant BALB/c mice were purchased from The Jackson Laboratory and were maintained and bred in the

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Abbreviations: OVA, ovalbumin; AFC, Ab-forming cell; DTH, delayed-type hypersensitivity; LT, lymphotoxin; GALT, gut-associated lymphoreticular tissues; LFA-3-Ig, fusion protein of lymphocyte function-associated Ag-3 and Ig; LTβR-Ig, fusion protein of lymphotoxin-β receptor and Ig; TNBS, trinitrobenzene sulfonic acid; TNCB, trinitrochlorobenzene; TNP, trinitrophenyl; Th, T helper; TGF, transforming growth factor.

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experimental facility under pathogen-free conditions in the University of Alabama at Birmingham Immunobiology Vaccine Center. Mice were injected intravenously on gestational days 14 and 17 with 200 $\mu$g of LT$\beta$R-Ig or of lymphocyte function-associated Ag-3 and Ig (LFA-3-Ig), as described (24, 26). The absence of Peyer's patches in mice from LT$\beta$R-Ig but not in LFA-3-Ig-treated dams was confirmed by histopathologic analysis, as described (27).

**Fusion Proteins.** Fusion proteins comprised of the extracellular domain of either murine LT$\beta$R or LFA-3 (which does not bind murine CD2) were fused to the hinge, $C_H2$, and $C_H3$ domains of human IgG1 (LT$\beta$R-Ig and LFA-3-Ig, respectively) and used as experimental and control fusion proteins, respectively (24, 28, 29).

**Immunization.** To establish systemic unresponsiveness to a high dose of oral protein, a standard protocol was used as described (30, 31). Mice were intubated gastrically with 25 mg of hen egg white OVA (Sigma) dissolved in 0.25 ml of PBS. Control mice received PBS only. Seven days later, mice were immunized via the s.c. route with 100 $\mu$g of OVA in 100 $\mu$l of complete Freund's adjuvant (CFA) (OVA/CFA; Difco). OVA-specific T and B cell responses were determined 14 days after s.c. immunization (30, 31). In other experiments, mice were fed 0.25 ml of PBS or PBS containing 10 mg of the hapten TNBS (Research Organics) on days 0 and 7 to induce hapten-specific systemic unresponsiveness (32). In additional experiments, mice were fed 0.5 ml of trinitrophenyl (TNP)-OVA (containing 7 mg of TNP) on days 0, 3, and 7. Seven days later, 0.1 ml of a 7% (vol/vol) picryl chloride (trinitrochlorobenzene, TNCB) solution was painted on the clipped abdomen of orally immunized mice. The delayed-type hypersensitivity (DTH) and T-cell-proliferative responses were examined 7 and 14 days after the abdominal TNCB challenge, respectively.

**DTH Responses.** Ag-specific DTH responses were measured 7 days after systemic challenge with OVA or TNBS as described above. Briefly, PBS (20 $\mu$l) containing 10 $\mu$g of OVA was injected into the left ear pinna of the mice, whereas the right ear pinna received PBS as control (30, 31). In studies of TNP-specific responses, 0.8% of TNCB solution (20 $\mu$l) was painted on the left ear pinna. Ear swelling was measured 24 h later with a dial-thickness gauge (Ozaki Manufacturing, Tokyo, Japan). The DTH response was expressed as the increase of ear swelling after OVA injection or TNCB painting minus the swelling in the control site.

**Ag-Specific T-Cell Responses.** The spleen was removed aseptically and single-cell suspensions were prepared in RPMI 1640 medium (Cellgro Mediatech, Washington, DC) containing Hepes buffer, nonessential amino acids, sodium pyruvate, L-glutamine, penicillin, streptomycin, and gentamycin (incomplete medium; refs. 27, 30, 31, 33). $CD4^+$ T cells were purified by the magnetic activated cell sorter system (Miltenyi Biotec, Auburn, CA) as described (27, 30–32). Cells were passed through the magnetized column after incubation with biotin-labeled anti-CD4 mAb (GK 1.5) and streptavidin-conjugated microbeads. The isolated $CD4^+$ T cells were >97% pure and >99% viable. Purified $CD4^+$ T cells were cultured with 1 mg/ml of OVA in the presence of irradiated (3,000 rads) splenic feeder cells for 5 days. To evaluate TNBS-specific T cell responses, $CD4^+$ T cells were incubated with irradiated TNBS-coupled syngeneic spleen cells for 5 days at 37°C in a moist atmosphere of 5% $CO_2$ in air (32, 34). During the last 18 h of incubation, 0.5 $\mu$Ci of tritiated thymidine (dThd) was added and then the cells were harvested, and the amount of dThd incorporated into the DNA was determined by scintillation counting. T cells cultured without Ag served as controls.

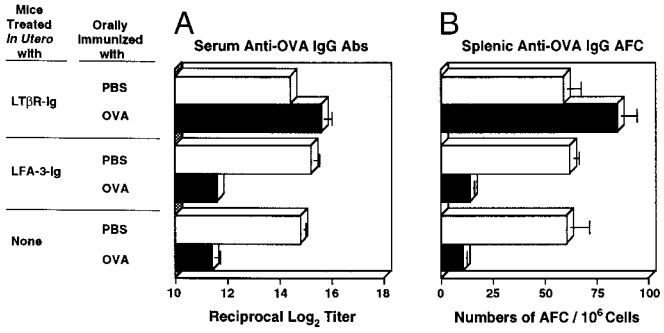


**Fig. 1.** OVA-specific IgG Ab responses (*A*) in serum and IgG AFC (*B*) in spleen of mice from dams treated with LT$\beta$R-Ig, LFA-3-Ig, or untreated BALB/c mice orally immunized with 25 mg of OVA (□) or PBS (■). Both groups of mice were challenged by the s.c. route with 100 $\mu$g of OVA in 100 $\mu$l of complete Freund's adjuvant 7 days after feeding. Fourteen days after s.c. OVA/CFA challenge, serum was assessed for OVA-specific IgG Ab responses. Numbers of splenic OVA-specific IgG AFCs were determined by enzyme-linked immunospot assay. The results represent the mean values ± SEM for 12 mice in each experimental group and from three separate experiments.

**Cytokine-Specific ELISA.** Levels of cytokines in culture supernatants were measured by ELISA. The details of the ELISA for IFN-$\gamma$, IL-2, -4, -5, -6, and -10 have been described (27, 30, 31). The levels of Ag-specific cytokine production were calculated by subtracting the results of control cultures (e.g., without Ag stimulation) from those of Ag-stimulated cultures. This ELISA was capable of detecting 0.8 ng/ml of IFN-$\gamma$, 8 pg/ml of IL-2, 25 pg/ml of IL-4, 0.8 units/ml of IL-5, 200 pg/ml of IL-6, and 4 pg/ml of IL-10.

**Ab Assays.** Ab titers in serum were determined by an ELISA (27, 31, 33). Falcon Microtest assay plates (Becton Dickinson) were coated with an optimal concentration of OVA (100 $\mu$l of 1 mg of OVA/ml) or TNP-BSA (BSA; 100 $\mu$l of 0.1 mg of BSA/ml) in PBS. Endpoint Ab titers were expressed as the last dilution yielding an optical density at 414 nm of >0.1 units above negative control values after a 15-min incubation.

**Enumeration of Ab-Forming Cells (AFCs).** Spleens were removed aseptically and single-cell suspensions prepared in RPMI 1640 medium containing 10% (vol/vol) FCS. An enzyme-linked immunospot assay was used to detect cells producing IgM, IgG, and IgA Abs (27, 31, 33). Ninety-six-well nitrocellulose plates (Millipore) were coated with 1 mg/ml of OVA (100 $\mu$l/well) or with 0.1 mg/ml of TNP-BSA to detect OVA- or TNP-specific AFCs, respectively (27, 30–32).

**Detection of Serum OVA.** To assess OVA levels in the peripheral blood after oral administration, serum samples were collected at 30, 60, and 120 min after OVA feeding and subjected to ELISA. For coating, anti-OVA mAb (OVA-14, Sigma) was used and polyclonal rabbit anti-OVA Abs (Sigma) were used for detection. By using this combination of Abs, the ELISA level of detection was 4 ng of OVA/ml of serum.

**Statistics.** The significance of the differences (e.g., *P* values) between groups was evaluated by the Mann–Whitney *U* test by using a STATVIEW II program designed for Macintosh computers.

## Results

**Oral OVA Fails to Induce Systemic Unresponsiveness in LT$\beta$R-Ig-Treated Mice.** To investigate the potential role of Peyer's patches in oral tolerance, we initially examined the effects of disruption of Peyer's patches but not mucosa-associated lymph nodes on the

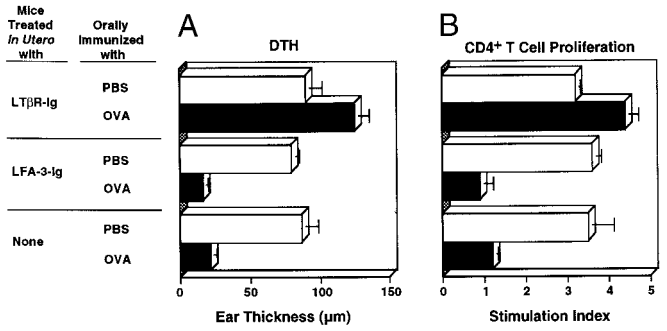


**Fig. 2.** Effects of oral administration of OVA on DTH (*A*) and T-cell-proliferative responses (*B*) in mice from dams treated with LTβR-Ig or LFA-3-Ig, or from untreated BALB/c mice. (*A*) Thirteen days after i.p. immunization, PBS (20 μl) containing 10 μg of OVA was injected into the left ear pinna, and the control right ear pinna was injected with PBS. The DTH response is expressed as the increase in ear swelling after OVA injection over the level of swelling in the control ear pinna receiving PBS. (*B*) Fourteen days after s.c. immunization, splenic $CD4^+$ T cells were cultured with or without OVA for 5 days. The stimulation index was determined as cpm of wells with Ag/cpm of wells without Ag (controls). The levels of thymidine incorporated in each control well were between 500 and 1,000 cpm. The results represent the mean values ± SEM from three separate experiments (triplicate wells/experiment).

efficiency with which oral administration of OVA induces systemic unresponsiveness. When OVA-specific Ab levels in serum of mice from mothers treated *in utero* with LTβR-Ig fusion protein, LFA-3-Ig fusion protein, or untreated BALB/c mice were examined, control groups of mice (LFA-3-Ig-treated or untreated BALB/c) showed lower serum anti-OVA IgG Ab responses than did control mice given oral PBS (Fig. 1*A*, $P < 0.001$). Splenic IgG AFCs were also reduced in both control groups (Fig. 1*B*, $P < 0.001$). In contrast, no reductions in serum IgG anti-OVA Abs or in splenic IgG AFC responses were observed in either PBS- or OVA-fed groups of mice from LTβR-Ig-treated dams (Peyer's patch-null mice). Thus, high levels of OVA-specific Ab responses were seen in Peyer's patch-null OVA-fed mice (Fig. 1 *A* and *B*, $P < 0.01$), suggesting these mice failed to be tolerized orally.

**OVA-Specific T-Cell Responses in Peyer's Patch-Null Mice Fed OVA.** We next determined whether oral administration of a protein Ag would affect subsequent T cell proliferative or DTH responses in Peyer's patch-null mice. Mice from LTβR-Ig-treated mothers and both control groups given PBS orally and then immunized parenterally showed significant DTH responses (Fig. 2*A*). On the other hand, DTH responses were reduced in both control groups given 25 mg of oral OVA before systemic immunization ($P < 0.001$); however, Peyer's patch-null OVA-fed mice showed enhanced DTH responses that were comparable to Peyer's patch-null mice given PBS only (Fig. 2*A*, $P < 0.05$). We also examined splenic OVA-specific T-cell-proliferative responses. Relatively low Ag-specific T-cell responses were seen when splenic $CD4^+$ T cells were taken from either offspring of LFA-3-Ig-treated dams or untreated OVA-fed mice, whereas vigorous proliferative responses were observed with splenic $CD4^+$ T cells isolated from Peyer's patch-null mice given oral OVA (Fig. 2*B*, $P < 0.005$). The T-cell-proliferative responses seen in OVA-fed mice from LTβR-Ig-treated dams were higher than those seen in all groups fed PBS only (Fig. 2*B*, $P < 0.05$). These results show that systemic T-cell responses actually are enhanced in Peyer's patch-null mice, perhaps as a result of a lack of an oral tolerance-inducing pathway in GALT.

**OVA-Specific $CD4^+$ T Helper (Th)1 and Th2 Cytokine Responses.** Because oral administration of OVA to Peyer's patch-null mice induced T-cell responses, it was important to examine the Th1 and Th2 cytokine profiles of these OVA-specific T cells. Both control groups of mice fed OVA showed reduced Ag-specific Th1 (IFN-γ and IL-2)- and Th2 (IL-4, -5, -6, and -10)-type responses, whereas high levels of Th2-type cytokines, and especially IL-4, were seen in mice from LFA-3-Ig-treated dams (data not shown) and normal mice given oral PBS (Table 1). A virtually identical profile of up-regulation of Th2-type cytokine synthesis was seen in the Peyer's patch-null mice after oral administration of PBS. Interestingly, a hyperresponsive Th1- and Th2-type cytokine profile was noted in Peyer's patch-null OVA-fed mice and subsequently challenged with OVA plus complete Freund's adjuvant (Table 1). Similarly, both Th1- and Th2-type cytokine profiles were also up-regulated in brachial lymph nodes of Peyer's patch-null mice fed OVA (data not shown). Taken together, these results show that Peyer's patch-null mice fail to become orally tolerant to OVA.

**Induction of TNP-Specific B-Cell Unresponsiveness in Peyer's Patch-Null Mice.** To assess the role of Peyer's patches in other forms of oral tolerance, we next tested whether oral administration of the hapten TNBS before systemic sensitization would either prevent or reduce contact hypersensitivity to picryl chloride (TNCB). When mice from LTβR-Ig-treated dams and both groups of controls were sensitized with picryl chloride, all groups of mice previously fed PBS showed high-serum anti-TNP IgG Ab responses (Fig. 3*A*). On the other hand, mice fed TNBS contained reduced levels of serum anti-TNP IgG Abs than did mice fed PBS (Fig. 3*A*, $P < 0.001$). In addition, fewer anti-TNP IgG AFC were noted in both control groups of mice given oral TNBS before skin painting of TNCB than in mice fed PBS (Fig. 3*B*, $P < 0.003$). Similarly, reductions in anti-TNP IgG AFC were seen in Peyer's patch-null mice given oral TNBS before systemic challenge (Fig. 3*B*, $P < 0.003$). Interestingly, Peyer's patch-null mice that were not fed TNBS gave significant anti-TNP IgG AFC responses in spleen when sensitized via the skin. These results indicate that

**Table 1. Splenic $CD4^+$ Th1 and Th2 cytokine synthesis in Peyer's patch-null mice orally tolerized with OVA***

| Mouse group treated with | Orally immunized with | Th1 type† | | Th2 type† | | | |
|---|---|---|---|---|---|---|---|
| | | IFN-γ, pg/ml | IL-2, pg/ml | IL-4, pg/ml | IL-5, units/ml | IL-6, ng/ml | IL-10, pg/ml |
| LTβR-Ig | PBS | 21.0 ± 1.3‡ | 320 ± 11 | 1,590.2 ± 186.2 | 57.3 ± 8.7 | 25.3 ± 0.1 | 9.4 ± 1.3 |
| | OVA | 279.2 ± 21.8 | 280 ± 10 | 3,976.5 ± 879.7 | 158.1 ± 33.1 | 22.3 ± 4.2 | 37.9 ± 7.9 |
| None | PBS | 8.2 ± 1.3 | 350 ± 9 | 1,426.5 ± 140.1 | 48.0 ± 3.3 | 16.8 ± 1.1 | ND |
| | OVA | 3.6 ± 0.8 | ND | 27.7 ± 8.9 | 24.3 ± 3.5 | 2.1 ± 0.5 | ND |

ND, not detected.

*Splenic $CD4^+$ T cells ($2 \times 10^6$/ml) from each mouse group were cultured with 1 mg/ml of OVA in the presence of T-cell-depleted and -irradiated splenic feeder cells ($4 \times 10^6$ cells/well).

†Culture supernatants were harvested after 5 days (2 days for IL-2) of incubation and analyzed by the respective cytokine-specific ELISA.

‡The results represent the mean ± SEM of one of three separate experiments.

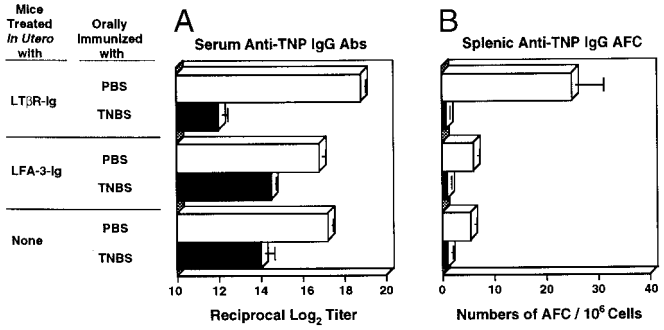


**Fig. 3.** TNP-specific IgG Ab responses in serum (*A*) and IgG AFC in spleen (*B*) of mice from dams treated with LTβR-Ig, LFA-3-Ig, or untreated BALB/c mice. Mice were fed 0.25 ml of PBS or PBS containing 10 mg of TNBS on days 0 and 7. On day 14, mice were sensitized with picryl chloride (TNCB) and 14 days later, TNP-specific serum IgG Ab responses were determined. The numbers of splenic IgG AFCs were determined by enzyme-linked immunospot. The results represent the mean values ± SEM for 12 mice in each experimental group and were representative of three separate experiments.

oral tolerance to hapten can be induced in the absence of organized Peyer's patches in the small intestine.

**Oral Hapten Induces Systemic T-Cell Unresponsiveness in the Absence of Peyer's Patches.** Both experimental and control groups of mice responded to picryl chloride with significant ear swelling (Fig. 4*A*). Contact hypersensitivity responses were reduced significantly in all groups, including Peyer's patch-null mice, after two weekly oral doses of TNBS (10 mg) before systemic sensitization (Fig. 4*A*, $P < 0.008$). To test further for the oral induction of systemic unresponsiveness to TNCB in mice from LTβR-Ig-treated dams, splenic T-cell-proliferative responses were examined. Significantly lower T cell responses were seen in Peyer's patch-null mice fed TNBS than in the groups of control mice fed PBS only (Fig. 4*B*, $P < 0.01$). Collectively, these findings indicate that systemic unresponsiveness to orally administered hapten normally can be established in the absence of GALT.

**Lack of TNP-Specific Oral Tolerance in Peyer's Patch-Null Mice Fed TNP-OVA.** To this point, our results indicate that organized Peyer's patches are not necessary for the induction of oral tolerance to hapten but are required for protein Ags such as OVA. To prove this, we coupled the hapten to OVA and assessed TNP-specific DTH responses in mice from LTβR-Ig-treated dams fed the TNP-OVA conjugate. Mice were fed TNP-OVA that contained

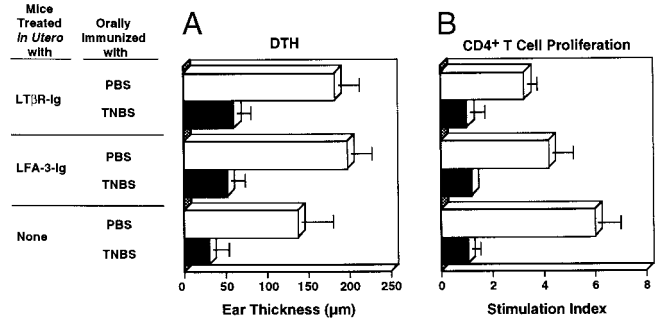


**Fig. 4.** The effects of oral administration of TNBS on DTH (*A*) and T-cell-proliferative (*B*) responses 7 and 14 days after TNCB sensitization. The details of the assays are described in *Materials and Methods*. The stimulation index was determined as described in the legend of Fig. 2. The level of thymidine incorporation for each control well was between 500 and 1,000 cpm. The results represent the mean values ± SEM from three separate experiments (triplicate wells/experiment).

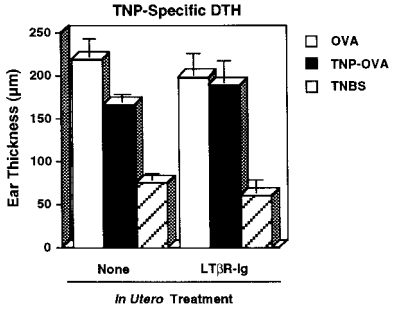


**Fig. 5.** The effects of oral administration of TNP-OVA on TNP-specific DTH responses. Mice from dams treated with LTβR-Ig and untreated BALB/c mice were fed OVA (45 mg), TNP-OVA (containing 7 mg of TNP and 45 mg of OVA), or TNBS (7 mg) on days 0, 3, and 7. The results represent the mean values ± SEM from two separate experiments.

an approximately equal molar ratio of TNBS, e.g., 20 mg of TNBS and 45 mg of OVA. Untreated BALB/c mice fed PBS or OVA (45 mg) showed significantly high TNP-specific DTH responses; however, these responses were reduced when mice were given TNP-OVA orally (Fig. 5, $P < 0.03$). Further, significant reductions in TNP-specific DTH responses were seen also in mice fed TNBS (Fig. 5, $P < 0.02$). In contrast, instead of reduced TNP-specific DTH responses, Peyer's patch-null mice fed TNP-OVA exhibited DTH responses that were essentially equal to those seen in Peyer's patch-null mice fed OVA or PBS only (Fig. 5). These results clearly show that hapten-specific oral tolerance cannot be induced in the absence of Peyer's patches when hapten is coupled to a protein Ag, and further confirm our conclusion that protein Ags, unlike hapten, require organized Peyer's patches for the induction of oral tolerance.

**Levels of Serum OVA in Peyer's Patch-Null Mice.** To determine whether Peyer's patches function mainly as a portal for Ag uptake, serum OVA levels were compared between Peyer's patch-null and control mice. The serum samples were collected from mice given 25 mg of OVA orally after 30, 60, and 120 min, and subjected to sensitive ELISA. After 30 min, serum OVA levels in Peyer's patch-null mice were slightly lower than that of control groups (Fig. 6); however, the concentration of serum OVA reached similar levels after 60 min of feeding OVA regardless of the presence of Peyer's patches (Fig. 6). These results indicate that the uptake of OVA into the systemic circulation occurs somewhat more quickly in the presence of Peyer's patches; however, similar levels are attained within 1 h. Thus, our findings demonstrate that protein Ags can be transported through the intestinal epithelial barrier in the absence of Peyer's patches.

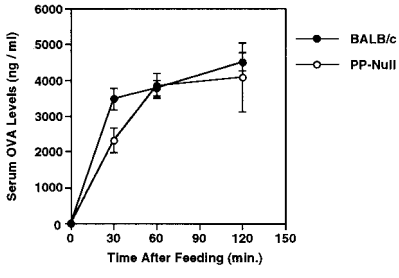


**Fig. 6.** Serum OVA levels after oral administration of OVA. Mice were fed 25 mg of OVA and serum samples were collected at 30, 60, and 120 min after feeding. Serum OVA levels were determined by ELISA as described in *Materials and Methods*. The results represent the mean values ± SEM for 10 mice in each experimental group and were representative of two separate experiments.

## Discussion

The results of the present study provide direct evidence for a distinct role for Peyer's patches in the induction of oral tolerance to protein Ag or hapten. We show that Peyer's patches are essential for the induction of systemic hyporesponsiveness to orally administered protein. Oral administration of OVA before systemic immunization to mice from LT$\beta$R-Ig-treated dams, which lack organized Peyer's patches but which retain other mucosa-associated lymph nodes, resulted in the enhancement of splenic T- and B-cell responses. On the other hand, Peyer's patches were not required for hapten-specific oral tolerance induction. Both Peyer's patch-null and control mice were tolerized identically when these mice were fed TNBS.

Peyer's patches have been studied in detail as a major inductive site for mucosal IgA responses and less intensely in oral tolerance. $CD4^+$ Th cells, including Th1 and Th2 cells, as well as surface IgA-positive ($sIgA^+$) B cells, have been analyzed for their role in the induction of mucosal S-IgA Ab responses (35, 36). In this regard, it is well established that Ag-presenting cells, including dendritic cells, macrophages, and MHC class $II^+$ B cells, are of central importance for mucosal immune responses (35, 36). It has been shown that M cells in the follicle-associated epithelium of Peyer's patches play an essential role in Ag uptake from intestinal lumen and transportation of Ag to lymphocytes folded in pockets in their basolateral surface (35). Oral immunization has been shown to stimulate immunocompetent cells in Peyer's patches that leads to the homing of Ag-specific regulatory Th cells and $sIgA^+$ B cells into IgA effector tissues such as the upper respiratory (nasopharyngeal and tracheal) and gastrointestinal tracts, and exocrine glands (e.g., the salivary and mammary glands) for the actual generation of Ag-specific IgA Abs (1). Our previous studies have shown that oral immunization with tetanus toxoid plus cholera toxin as a mucosal adjuvant elicited distinct Ag-specific Th2-type cytokine responses in Peyer's patches with subsequent induction of both systemic and mucosal IgA Abs (37, 38).

We now postulate that similar regulatory processes in Peyer's patches are required for the initiation of oral tolerance to protein Ags. Thus, uptake into T- and B-cell zones may be required, and Ag-presenting cell presentation to T cells would result in the induction of T- and B-cell unresponsiveness. Ultimately, migration of unresponsive T and B cells would mediate the manifestation of oral tolerance as poor B-cell responses, reduced T-cell proliferation, and lower DTH reactivity. Protein uptake into Peyer's patches in the absence of mucosal adjuvant most likely fails to trigger second signals sufficiently to induce immune responses, whereas protein plus mucosal adjuvant may provide both signals for mucosal and systemic immunity. Although the precise molecular and cellular mechanisms that regulate either oral tolerance or immunity still remain to be elucidated, it should be emphasized that Peyer's patch-derived dendritic cells have been shown to play a central role in the induction of IgA responses and tolerance regulated by Th2- or Th3-type cells (9, 15, 16). In addition, Ag handling by M cells and mucosal epithelial cells may also play an important role in the induction and regulation of these distinct immune responses in Peyer's patches.

Another important finding of the present study was the augmented splenic B- and T-cell responses in Peyer's patch-null mice fed OVA before systemic challenge. Thus, higher levels of serum anti-OVA IgG Abs were associated with increased numbers of OVA-specific IgG AFC in spleen of mice from LT$\beta$R-Ig-treated dams fed OVA than with mice fed PBS. Further, both DTH and $CD4^+$ T cell proliferative responses also were enhanced when Peyer's patch-null mice were given oral OVA before systemic immunization. More strikingly, Th1 (IFN-$\gamma$)- and Th2 (IL-4, IL-5, and IL-10)-type cytokine synthesis by $CD4^+$ T cells from spleen and brachial lymph nodes were up-regulated significantly after *in vitro* stimulation with OVA. On the other hand, both control groups fed OVA showed reduced B and T cell responses. These results suggest that Peyer's patches maintain homeostasis by providing effective down-regulatory responses. They also suggest that protein uptake in the absence of Peyer's patches actually primes for systemic T and B cell responses.

Although the Peyer's patches have been viewed mainly as major mucosal inductive sites for the generation of Ag-specific IgA responses, these tissues may be an important site for dispatching immune-suppressive signals for continuously ingested Ags. Tolerance represents the most common and important response of the host to environmental Ags, including food and commensal bacterial components, for the maintenance of appropriate immunological homeostasis. The continuous ingestion of several thousand different food proteins is but one important example, whereas tolerance to our indigenous microflora that colonize the large intestine represents another major example. It is also important to consider that induction of mucosal and systemic immunity by oral immunization with protein Ag alone is rather difficult and requires the use of potent mucosal adjuvants, vectors, or other special delivery systems (1). Thus, it is logical to consider that Peyer's patches would play a more important role for the maintenance of tolerance in normal situations, instead of induction of Ag-specific IgA responses. To support this view, it has been shown that germ-free mice possessed a profound hypotropy of Peyer's patches (39), and these mice did not elicit systemic unresponsiveness when fed sheep red blood cells for prolonged periods (40). Furthermore, it was shown that the absence of IgA Abs in mother's milk accelerated the development of Peyer's patches in the neonate (41), perhaps because of the induction of tolerance to environmental Ags, which can easily invade the intestinal epithelium in addition to a lack of passive immunity for protection. Nevertheless, it seems that Peyer's patches possess important dual functions for the induction of IgA responses and tolerance. Indeed, TGF-$\beta$ in Peyer's patches has been shown to play a central role in the isotype switching of surface IgM to IgA B cells (42, 43), whereas this cytokine has been detected in Peyer's patches as a key factor in the induction of oral tolerance (15, 16).

Although we found that Peyer's patch-deficient mice lacked oral tolerance to proteins, mice of all groups, regardless of treatment, became orally tolerant to TNBS. The most obvious difference between OVA and TNBS is the molecular size of the oral toleragen. Thus, ingested protein Ags such as OVA require uptake by M cells in Peyer's patches for induction of oral tolerance, despite the fact that some digested protein Ags pass directly through the epithelium and into the lamina propria. This uptake via epithelial cells raised the possibility that lack of oral tolerance in Peyer's patch-null mice may be explained simply by an absence of M cells that prevented Ag uptake into the blood circulation. To directly address this issue, we have compared serum OVA levels between normal and Peyer's patch-null mice. Our results showed no significant differences in serum OVA levels between normal and Peyer's patch-null mice, although slightly reduced levels of OVA were seen in serum of Peyer's patch-null mice at the early time point (30 min after feeding). Taken together with our previous study, which showed that induction of Ag-specific IgA responses occur in Peyer's patch-null mice (27), our present findings strongly suggest that protein Ags can be transported through gastrointestinal-tract epithelial surfaces in the absence of M cells. These findings indicate that not only M cells but also an organized Peyer's patch is essential for the induction of oral tolerance to protein Ags.

The size of Ag alone may not be the only factor involved in oral tolerance, because haptens alone are nonimmunogenic. Even if TNBS were taken up by Peyer's patches, the hapten may fail to stimulate lymphoid cells involved in the induction of oral toler-

ance through active suppression, anergy, or clonal deletion. To support this notion, oral immunization with an immunogenic 10-mer peptide of heat-labile enterotoxin B subunit was found to induce systemic unresponsiveness associated with IFN-$\gamma$, IL-2, IL-4, and TGF-$\beta$ production by $CD4^+$ Peyer's patch T cells (44). To clarify this issue, mice from *in utero* LT$\beta$R-Ig-treated dams currently are being immunized with different lengths of OVA peptide that possess Th-cell epitopes. On the basis of these ongoing experiments, we predict that the initial contact of the intact protein with Peyer's patches may be found to be an important event in the induction of oral tolerance. To support this prediction, several studies using OVA transgenic mice have been conducted already and have suggested important roles for Peyer's patches in the induction of systemic unresponsiveness to ingested OVA (15, 16). Enhanced systemic hyporesponsiveness elicited by high oral doses of OVA with concomitant anti-IL-12 mAb treatment was associated with increased TGF-$\beta$ production by Peyer's patch T cells. Further, orally tolerized transgenic mice showed higher frequencies of apoptotic T cells in both Peyer's patches and spleen (15). These results suggest that Peyer's patches contain TGF-$\beta$-producing T cells, which play a central role in the induction of apoptosis and subsequent oral tolerance induction. *In situ* analysis of Peyer's patches of OVA transgenic mice fed low doses of OVA showed increased numbers of IL-4- and IL-10-producing cells, as well as enhanced mRNA expression for TGF-$\beta$, which are key cytokines for induction of active suppression (11, 16).

The present study directly shows a function for Peyer's patches in the induction of oral tolerance. Although the doses, size, and antigenicity of toleragen may be of some importance in the induction of systemic unresponsiveness, our finding that Peyer's patch-null mice lack OVA-specific oral tolerance clearly shows that GALT provides necessary down-regulatory responses to ingested protein Ags. Because our *in utero* treatment affected only Peyer's patches but not other mucosa-associated lymph nodes, our findings suggest the central importance of Peyer's patches in oral tolerance. The normal oral tolerance to hapten observed in Peyer's patch-null mice again points to significant differences between induction of unresponsiveness to a protein and to a hapten. Thus, use of this LT$\beta$R-Ig-treated mouse model provides essential information for understanding the cellular and molecular mechanisms of Peyer's patches in the induction of oral tolerance.

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