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Oral Tolerance Revisited: Prior Oral Tolerization Abrogates Cholera Toxin-Induced Mucosal IgA Responses

Hirotomo Kato,* Kohtaro Fujihashi,* Rie Kato,* Yoshikazu Yuki,† and Jerry R. McGhee²*

Oral delivery of a large dose or prolonged feeding of protein Ags induce systemic unresponsiveness most often characterized as reduced IgG and IgE Ab- and Ag-specific CD4⁺ T cell responses. It remains controversial whether oral tolerance extends to diminished mucosal IgA responses in the gastrointestinal tract. To address this issue, mice were given a high oral dose of OVA or PBS and then orally immunized with OVA and cholera toxin as mucosal adjuvant, and both systemic and mucosal immune responses were assessed. OVA-specific serum IgG and IgA and mucosal IgA Ab levels were markedly reduced in mice given OVA orally compared with mice fed PBS. Furthermore, when OVA-specific Ab-forming cells (AFCs) in both systemic and mucosal-associated tissues were examined, IgG AFCs in the spleen and IgA AFCs in the gastrointestinal tract lamina propria of mice given OVA orally were dramatically decreased. Furthermore, marked reductions in OVA-specific CD4⁺ T cell proliferative and cytokine responses in spleen and Peyer’s patches were seen in mice given oral OVA but were unaffected in PBS-fed mice. We conclude that high oral doses of protein induce both mucosal and systemic unresponsiveness and that use of mucosal adjuvants that induce both parenteral and mucosal immunity may be a better way to assess oral tolerance. The Journal of Immunology, 2001, 166: 3114–3121.

*Departments of Microbiology and Oral Biology, Immunobiology Vaccine Center, University of Alabama, Birmingham, AL 35294; and †JCR Biopharmaceuticals, San Diego, CA 92121

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²Address correspondence and reprint requests to Dr. Jerry R. McGhee, Department of Microbiology, Immunobiology Vaccine Center, University of Alabama, 761 Bevill Biomedical Research Building, 845 19th Street South, Birmingham, AL 35294-2170. E-mail address: mcghee@uab.edu

Abbreviations used in this paper: S-IgA, secretory IgA; GI, gastrointestinal; DTH, delayed-type hypersensitivity; CT, cholera toxin; LT, labile toxin; CT-B, B subunit of CT; AFC, Ab-forming cell; ELISPOT, enzyme-linked immunospot; KLH, keyhole limpet hemocyanin; TNBS, trinitrobenzene sulfonic acid; CMIS, common mucosal immune system.

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regulation of mucosal immune responses represented by S-IgA Ab production.

Oral tolerance has continued to be defined as systemic unresponsiveness with the maintenance of mucosal Ab responses, and this goes back to studies performed over 20 years ago (2). This finding preceded our knowledge that mucosal adjuvants such as CT reverse oral tolerance and induce potent mucosal and systemic immune responses. Therefore, it was important to revisit this notion and to determine whether oral tolerance also influences mucosal immune responses, because oral tolerance has been mainly assessed by parental boosting with Ag in CFA. Furthermore, studies should address whether potent mucosal adjuvants such as CT can block existing oral tolerance and induce both systemic and mucosal Ab responses. In this study, we addressed these two important issues by gastric administration of OVA followed by an oral immunization protocol with OVA and CT as mucosal adjuvant.

Materials and Methods

Mice

C57BL/6 mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Upon receipt, mice were transferred to microisolators and maintained in horizontal laminar flow cabinets at the University of Alabama at Birmingham Immunobiology Vaccine Center. All mice were free of pathogenic bacteria and viruses as determined by Ab screening and routine histologic analysis of organs and tissues. All experiments were performed using mice between 8 and 14 wk of age.

Induction of oral tolerance

To induce Ag-specific immune unresponsiveness, mice were gastrically intubated with 50 mg of OVA (Fraction V; Sigma, St. Louis, MO) dissolved in 0.25 ml of PBS. On days 7, 14, and 21 after intubation, mice were orally immunized with 1 mg of OVA and 10 µg of CT (List Biological Laboratories, Campbell, CA) as a mucosal adjuvant (21, 22). Both OVA- and B subunit of CT (CT-B)-specific B and T cell responses were determined 7 days after the final oral immunization (day 28).

Sample collection

Blood, fecal extracts, and saliva were collected on day 28. Blood was obtained by use of heparinized Natelson pipettes (Fisher Scientific, Pittsburgh, PA) placed into the suprapubic vein. The blood was centrifuged for 5 min at 5000 rpm, and the serum was collected and stored frozen at −20°C until assayed. Fecal pellets were weighed, added to microcentrifuge tubes, and PBS containing 0.01% sodium azide was used to adjust the volume (100 µg/ml). The pellets were homogenized by continuous shaking for 5 min at 5000 rpm, and the serum was collected and stored frozen at −20°C.

Saliva was obtained from mice following i.p. injection of 100 µl of 1 mg/ml pilocarpine (Sigma).

Lymphoid cell isolation

The spleen was removed aseptically and single-cell suspensions prepared by passage through sterile wire mesh screens as described previously (23, 24). Peyer’s patches were carefully excised from the intestinal wall and were dissociated using the neutral protease enzyme, collagenase type V (Sigma) in RPMI 1640 medium (Cellgro Mediatech, Washington, D.C.) to obtain single-cell preparations (23). Mononuclear cells in the lamina propia were isolated after removal of Peyer’s patches from the small intestine by passage through sterile wire mesh screens as described previously (23, 24). Culture supernatants were collected on day 2 for IL-2 and CT-B was purified from Bacillus brevis strains containing the plasmids for rCT-B (25). Briefly, Falcon Microtest III assay plates (Becton Dickinson, Oxford, CA) were coated with a solution of OVA (1 mg/ml) or with CT-B (5 µg/ml) in PBS and incubated overnight at 4°C. After blocking with 1% BSA in PBS for 2 h at 25°C, serial dilutions of serum or fecal extracts were added to each well. After incubation for 2 h at 25°C, HRP-labeled goat anti-mouse µ, γ, or α heavy chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added to wells and incubated for 2 h at 25°C. For analysis of IgG1 and IgG2a Ab subclasses, biotin-conjugated anti-mouse specific for γ1 (G1-6-5) and γ2a (R19-15) (PharMingen, San Diego, CA) were used. For detection, an HRP-labeled goat anti-biotin Ab (Vector Laboratories, Burlingame, CA) was used. The color reaction was developed with 1.1 mM 2,2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma) in 0.1 M citrate-phosphate buffer (pH 4.2) containing 0.01% H2O2. Endpoint titers were expressed as the last dilution yielding an OD at 415 nm (OD415) of >0.1 U above negative control values after a 15-min incubation.

Enumeration of OVA-specific Ab-forming cells (AFCs)

Mononuclear cells from spleen and GI tract lamina propria were subjected to OVA-specific enzyme-linked immunosupost (ELISPOT) assay for detection of IgM, IgG, and IgA AFCs (23, 24). Briefly, 96-well nitrocellulose plates (Millipitier HA; Millipore, Bedford, MA) were coated with 1 mg/ml of OVA in PBS overnight at 4°C. After blocking with 1% BSA in PBS for 2 h at 37°C, cells were suspended in RPMI 1640 medium containing 10% heat-inactivated FCS, HEPES buffer (15 mM), t-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and gentamicin (80 µg/ml) (complete medium). Aliquots were then added to each well at appropriate dilutions and incubated for 4 h at 37°C in an atmosphere of 5% CO2 in air. The AFCs were detected with HRP-labeled goat anti-mouse µ, γ, or α heavy chain-specific Abs (Southern Biotechnology Associates) and visualized by adding the chromogenic substrate 3-amin-9-ethyl-carbazole (Moss, Pasadena, CA). Spots representing individual AFCs were counted with the aid of a dissecting microscope (SZH Zoom Stereo Microscope System; Olympus, Lake Success, NY).

Measurement of DTH responses

We used a well-characterized technique to assess DTH responses in vivo (23, 24). Briefly, 10 µg of OVA in 20 µl of PBS was injected into the left ear pinna and PBS alone (20 µl) was administered to the right ear pinna as a control. Ear swelling was measured 24 h later with an upright dial thickness gauge (Peacock; Ōzaki, Tokyo, Japan). The DTH response was expressed as the increase in ear swelling after OVA injection following subtraction of swelling in the control site injected with PBS.

CD4+ T cell proliferation assay

CD4+ T cells were purified by the magnetic activated cell sorter system (Miltenyi Biotec, Auburn, CA) as previously described (23, 24). Briefly, splenic or Peyer’s patch cells were incubated with biotin-conjugated anti-mouse CD4 mAb (GK 1.5) and subsequently with streptavidin-conjugated microbeads. The CD4+ T cell population was enriched after passage through a magnetized column. 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 T cell-depleted irradiated (3000 rad) splenic feeder cells from naive syngeneic mice in 96-well tissue culture plates (Corning Glass Works, Coming, NY) for 5 days. An aliquot of 0.5 µCi of [3H]thymidine incorporated into dividing cells was measured by scintillation counting.

Cytokine analysis

Cytokine levels in spleen or Peyer’s patch CT-B+ T cell culture supernatants were determined by a cytokine-specific ELISA as described previously (23, 24). Culture supernatants were collected on day 2 for IL-2 and on day 5 for IFN-γ, IL-4, IL-5, IL-6, and IL-10 for analysis of the secreted cytokine, respectively. The immunoplates (Nunc, Naperville, IL) were coated with monoclonal anti-IL-2 (JES6-1A12), anti-IL-4 (BVD4-1D11), anti-IL-5 (TRFK-5), anti-IL-6 (MP5-20F3), or anti-IL-10 (JES5-2A5) (PharMingen). After blocking with 3% BSA in PBS, serial twofold diluted samples and standards were added to wells and incubated overnight at 4°C. The wells were washed and then incubated with biotinylated monoclonal anti-IL-2 (JES6-3H4), anti-IFN-γ (XMG1.2), anti-IL-4 (BVD6-24G2), anti-IL-5 (TRFK-5), anti-IL-6 (MP5-32C1), or anti-IL-10 (JES5-16E5) mAbs for detection, respectively. After incubation overnight at 4°C, HRP-labeled goat anti-biotin Ab (Vector Laboratories) was added and incubated for 1 h at 25°C. The color reaction was developed...
with 1.1 mM 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma) in 0.1 M citrate-phosphate buffer (pH 4.2) containing 0.01% H2O2. A mouse IL-10 immunoassay kit, Quantikine M (R&D Systems, Minneapolis, MN) was also used to detect IL-10 in the culture supernatants. The minimal detectable level for each cytokine was 1.95 pg/ml for IL-2, 156.25 pg/ml for IFN-γ, 4.69 pg/ml for IL-4, 1.95 pg/ml for IL-5, 39.06 pg/ml for IL-6, and 7.81 pg/ml for IL-10.

**Statistics**

The data are expressed as the mean ± SD and mouse groups were compared with control mice using Student’s t test with StatView software (Abacus Concepts, Berkeley, CA) designed for Macintosh computers. For statistical analysis of cytokine levels below the detection limit, one-half of the minimal detectable levels (e.g., IFN-γ = 78.13 pg/ml) were recorded and analyzed. A p value of <0.05 was considered significant.

**Results**

**Oral OVA plus CT fail to abrogate B cell unresponsiveness**

It is now well established that oral immunization with protein Ags such as OVA with CT as mucosal adjuvant induces immune responses in both the systemic and mucosal compartments (23). However, parenteral immunization with OVA and the adjuvant CFA induces systemic but not mucosal immune responses. We have used a well-established oral immunization protocol of OVA plus CT to assess whether prior OVA feeding affected both systemic and mucosal B cell responses. Groups of C57BL/6 mice were given oral PBS or a high dose of OVA and subsequently orally immunized with OVA and CT on three occasions at weekly intervals. We first determined whether the high oral dose of OVA affected serum OVA-specific Ab responses after oral immunization. OVA-fed mice showed significantly reduced serum IgG Ab responses compared with those of PBS-fed mice (Fig. 1A; p < 0.05). In addition, serum anti-OVA IgA Ab responses were dramatically reduced in OVA-fed mice (Fig. 1A; p < 0.05). In contrast, IgM Ab levels were comparable between the two groups (Fig. 1A). These results clearly show that both serum IgG and IgA Ab responses are down-regulated by feeding of a high dose of OVA. Furthermore, the data suggest that use of CT as oral adjuvant cannot reverse this state of oral tolerance in the systemic immune compartment.

To ensure that reduced IgG and IgA anti-OVA Ab titers were the result of OVA feeding, we also assessed serum Ab responses to CT-B, the immunogenic portion of the CT adjuvant used. Our data showed that significant levels of CT-B-specific serum IgG and IgA Ab responses were induced at comparable levels in both groups of mice regardless of oral OVA, indicating that CT-B-specific Ab responses were unaffected by prior OVA feeding (Fig. 1B). This result shows that reduced serum Ab responses in mice fed OVA were OVA specific.

It has been shown that CT induces Th2-type responses to co-administered Ags given orally and the Th2 cells provide help for B cells producing IgG1, IgA, and IgE Abs (21–23). We assessed serum IgG1 and IgG2a anti-OVA Abs to determine the potential influence of oral immunization with OVA and CT on induction of particular serum IgG Ab subclasses. Consistent with previous reports, oral challenge with OVA and CT induced brisk IgG1 and low IgG2a Ab responses in control, PBS-fed mice. In OVA-fed mice, OVA-specific IgG1 but not IgG2a Ab responses were significantly reduced, indirectly suggesting that OVA-specific Th2-type responses induced by CT as adjuvant were effectively suppressed by prior OVA feeding (Fig. 2A; p < 0.05). The reduction of IgG1 Ab responses observed in OVA-fed mice compared with those in PBS-fed mice were OVA-specific, because IgG1 anti-CT-B Ab responses were elevated and essentially identical in the two mouse groups (Fig. 2B).

**Oral tolerance affects mucosal Ab responses**

Because it has been shown that oral immunization with OVA plus CT as mucosal adjuvant elicited significant Ag-specific mucosal IgA Ab responses, we further determined whether oral tolerance affects mucosal Ab responses in saliva and the GI tract. Interestingly, fecal IgA Ab responses specific for OVA were dramatically reduced in OVA-fed mice compared with PBS-fed mice (Fig. 3A; p < 0.05). In contrast, significant levels of CT-B-specific IgA Ab responses were induced in comparable amounts in fecal extracts of mice regardless of OVA feeding (Fig. 3B). The levels of salivary OVA-specific IgA Abs were undetectable in both groups of mice.

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** OVA-specific (A) or CT-B-specific (B) Ab responses in sera of mice fed OVA (●) or PBS (□) before oral challenge. Mice were fed 0.25 ml PBS or 50 mg of OVA on day 0. On days 7, 14, and 21 after oral feeding, these mice were orally immunized with 1 mg of OVA and 10 μg of CT as a mucosal adjuvant. Ag-specific Ab responses were determined 7 days after the final challenge. The results are expressed as the mean titer ± SD for 20 mice in each experimental group. *, p < 0.05 compared with control mice.
although CT-B-specific IgA Ab responses were comparably induced at low levels in both mouse groups (data not shown). These results clearly indicated that mucosal IgA Ab responses were also susceptible to oral tolerance induction. Furthermore, the data suggest that use of CT as oral adjuvant cannot reverse this state of oral tolerance in mucosal compartments.

The reductions in serum and mucosal Ab responses in OVA-fed mice were also confirmed at the single-cell level by using an Ag-specific ELISPOT assay. When numbers of OVA-specific AFCs in spleen were examined, the frequencies of IgG and IgA but not IgM AFCs were significantly reduced in OVA-fed mice compared with PBS-fed mice (Fig. 4A; p < 0.05). In particular, IgG AFCs in spleen from OVA-fed mice were dramatically decreased (~5/10⁶ cells) compared with those from PBS-fed mice (~70/10⁶ cells) (Fig. 4A; p < 0.05). We also assessed the frequency of OVA-specific AFCs in the lamina propria of mice given either oral PBS or OVA because this is the major mucosal effector site of the GI tract. Of importance, OVA-specific IgA AFCs were markedly diminished in OVA-fed mice compared with control mice (~400/10⁶ cells and ~3500/10⁶ cells, respectively). Furthermore, significant reductions in OVA-specific IgG AFCs (Fig. 4B; p < 0.05) were also seen. This provides direct evidence that mucosal IgA Ab responses are susceptible to oral tolerance. These findings clearly indicate that OVA-specific B cell unresponsiveness is induced for both IgG and IgA Abs in systemic tissues and for IgA Ab responses in mucosal effector sites after a single high oral dose of Ag.

High oral doses of OVA induce T cell tolerance in both systemic and mucosal compartments

We next determined whether a single high oral dose of OVA affected both systemic and mucosal T cell responses induced by subsequent oral challenge with OVA plus CT as adjuvant. We first examined OVA-specific DTH responses in mice given either oral PBS or OVA. OVA-specific DTH responses were induced in mice fed PBS before oral immunization with OVA and CT as adjuvant (Fig. 5). In contrast, significantly lower OVA-specific DTH responses were observed in OVA-fed mice (Fig. 5; p < 0.05). To further characterize T cell responses in these mice, we assessed OVA-specific T cell proliferative responses in vitro. Splenic CD4⁺ T cells from mice fed PBS before oral immunization with OVA plus CT showed marked OVA-specific proliferative responses, although the responses were variable among individual mice (stimulation indices ranging from 3.5 to 9.6) (Fig. 6A). In contrast, splenic CD4⁺ T cells from mice fed OVA before oral immunization were unresponsive to OVA (stimulation indices ranging from 0.9 to 2.1) (Fig. 6A). We also examined CD4⁺ T cell responses in Peyer’s patches of these mice because this tissue is the major mucosal inductive site for immune responses in the GI tract. Of interest, CD4⁺ T cells from Peyer’s patches of mice given oral OVA were essentially unresponsive to the fed Ag (stimulation indices ranging from 1.1 to 1.9) (Fig. 6B). In contrast, Peyer’s patch CD4⁺ T cells from PBS-fed mice exhibited significant OVA-specific proliferative responses (stimulation indices ranging from 1.7 to 4.9) (Fig. 6B). These results indicate that a high oral dose of OVA induces OVA-specific T cell unresponsiveness in mucosal inducive sites in addition to the systemic compartments after subsequent oral challenge with OVA and CT. Furthermore, the data suggest that use of CT as oral adjuvant cannot reverse this state of T cell unresponsiveness.

Diminished OVA-specific cytokine responses by splenic and Peyer’s patch CD4⁺ T cells from OVA-fed mice

It is well established that a single high oral dose of protein induces Ag-specific clonal anergy and/or deletion to subsequent systemic challenge with the same Ag. This was characterized by reductions in T and B cell responses and cytokine production by CD4⁺ T cells (6, 8). It was important to determine whether a single high dose of oral OVA would influence cytokine responses induced by oral immunization. Splenic CD4⁺ T cells from mice fed PBS before oral challenge with OVA and CT produced high levels of the Th2-type cytokines IL-4, IL-5, IL-6, and IL-10, but essentially no IL-2 or IFN-γ (Th1-type) in response to OVA (Fig. 7, A and B). In contrast, these dominant Th2-type cytokine responses by splenic CD4⁺ T cells were markedly decreased when mice were fed a high dose of OVA (Fig. 7B; p < 0.05). In addition, up-regulation of Th1-type cytokine responses were not observed in OVA-fed mice and these cytokine levels remained below detection in these mice. To assess the effects of tolerance on the mucosal T cell cytokine responses, we obtained Peyer’s patch CD4⁺ T cells from mice fed PBS or OVA. Of interest, both Th1- and Th2-type cytokine responses were below detectable levels in Peyer’s patch CD4⁺ T cells from mice given oral OVA (Fig. 8, A and B). In contrast, Peyer’s patch CD4⁺ T cells from PBS-fed mice exhibited significant OVA-specific Th2-type but not Th1-type cytokine responses (Fig. 8, A and B).

Because it was suggested that TGF-β₁ plays an important role in the induction and regulation of oral tolerance, we also measured TGF-β₁ levels in culture supernatants of splenic and Peyer’s patch CD4⁺ T cells from mice fed PBS or OVA. Interestingly, TGF-β₁ in the culture supernatants of OVA-stimulated splenic and Peyer’s patch CD4⁺ T cells in vitro was below detectable levels, although we performed TGF-β₁-specific ELISA using a highly sensitive luminometric assay (data not shown). This suggests that T cell
production of TGF-β was not a major factor in mucosal and systemic unresponsiveness after oral delivery of a large dose of OVA.

Discussion
In this study, we have shown that a single high oral dose of OVA down-regulates both systemic and mucosal immune responses as assessed by a novel oral immunization strategy using OVA and CT as mucosal adjuvant. OVA-specific serum IgG, especially IgG1 subclass, and IgA Ab responses were diminished in OVA-fed mice compared with PBS-fed mice. Furthermore, the numbers of OVA-specific IgG and IgA AFCs in spleen were also dramatically reduced by OVA feeding before oral immunization. Of equal importance, mucosal IgA anti-OVA Abs were also reduced in OVA-fed mice subsequently challenged with OVA plus CT as mucosal adjuvant. The most direct test of the hypothesis that oral OVA would diminish mucosal immunity in the GI tract came from studies that assessed IgA anti-OVA AFCs in lamina propria. Dramatically reduced IgA anti-OVA but normal anti-CT-B AFCs were seen in OVA-fed compared with PBS-fed mice. When we assessed splenic CD4+ T cell responses, significant reductions in proliferative responses and Th2-type cytokine production were observed in mice fed OVA before oral immunization with OVA and CT as mucosal adjuvant. Furthermore, and perhaps more importantly, CD4+ T cell from Peyer’s patches of OVA-fed mice subsequently orally immunized with OVA plus CT exhibited unresponsiveness to OVA, whereas Peyer’s patch CD4+ T cell from PBS-fed mice showed significant OVA-specific proliferative and Th2-type cytokine responses.

Oral tolerance has been characterized as the state of Ag-specific systemic unresponsiveness with the maintenance of mucosal SIgA Ab responses. This concept came from the initial finding that oral administration of a streptococcal Ag or OVA to mice resulted in suppression of Ag-specific systemic immune responses in the presence of reduced but significant salivary SIgA Ab responses (2). Notably, the levels of salivary S-IgA Abs in Ag-fed mice were low, and mucosal immune responses were not induced in systemically challenged control mice (2). Thus, we must reconsider the concept of oral tolerance and address whether mucosal immune responses are susceptible to oral tolerance. To date, nearly all studies of oral tolerance have been assessed with a systemic immunization protocol after oral administration of Ag; however, by using

FIGURE 4. OVA-specific AFCs in spleen (A) and lamina propria (B) of mice fed OVA (■) or PBS (□) before oral challenge. Mononuclear cells isolated from spleen and lamina propria of the GI tract were assessed by OVA-specific ELISPOT assay to determine the numbers of IgM, IgG, and IgA AFCs. The results are expressed as the mean ± SD for 15 mice in each experimental group. *, p < 0.05 compared with control mice.

FIGURE 5. The effects of previous feeding of OVA on DTH responses in mice orally challenged with OVA and CT. An aliquot of 20 μl of PBS or PBS containing 10 μg of OVA was injected into the right or left ear pinna, respectively, of mice fed either OVA (■) or PBS (□). Ear swelling was measured 24 h later and DTH response was expressed as the increase of ear swelling after OVA injection following subtraction of swelling in the control site injected with PBS. The results are expressed as the mean ± SD for 20 mice in each experimental group. *, p < 0.05 compared with control mice.

FIGURE 6. Splenic (A) and Peyer’s patch (B) CD4+ T cell proliferative responses of mice fed OVA (■) or PBS (□) before oral immunization with OVA and CT. Purified CD4+ T cells were cultured with 1 mg/ml of OVA in the presence of T cell-depleted irradiated (3000 rad) splenic feeder cells from naive syngeneic mice in 96-well tissue culture plates for 5 days. An aliquot of 0.5 μCi of [3H]thymidine was added during the last 18 h and thymidine incorporation by cells was measured by scintillation counting. The results represent the mean ± SD. The results represent the individual values from five separate experiments. *, p < 0.05 compared with control mice.
this method, the susceptibility of mucosal immune responses to oral tolerance remains undefined. In this study, we directly addressed this issue by use of a well-established oral immunization protocol with OVA plus CT as mucosal adjuvant. The main advantage to mucosal immunization is that this mode can elicit marked Ag-specific mucosal S-IgA Abs as well as serum Ab responses and one can evaluate the influence of oral Ag on both mucosal and systemic immunity concurrently. To this end, our results provide the first direct evidence that oral tolerance downregulates mucosal as well as systemic immunity.

In this study, we were unable to detect OVA-specific salivary IgA Abs even in PBS-fed mice after oral immunization, although CT-B-specific IgA Ab responses were comparably induced in both PBS- and OVA-fed mouse groups. This is most likely due to the weak immunogenicity of OVA because oral immunization with tetanus toxoid and CT induced tetanus toxoid-specific IgA Ab responses in saliva (26). In addition, oral immunization is suboptimal for induction of salivary IgA Ab responses and one can evaluate the influence of oral Ag on both mucosal and systemic immunity concurrently. To this end, our results provide the first direct evidence that oral tolerance downregulates mucosal as well as systemic immunity.

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TNBS-induced colitis. Furthermore, striking decreases in the numbers of trinitrophenyl-specific IgM, IgG, and IgA AFCs occurred in caudal lymph node, and IgM and IgG but not IgA AFCs decreases were seen in the colonic lamina propria. These results suggested that although oral administration with TNBS inhibited hapten-specific IgG responses in mice with colitis, Ag-specific IgA Ab responses were less affected. It should be noted that the TNBS hapten model is useful for studies of colonic inflammation; however, it is not ideal to determine whether oral tolerance blocks induction of mucosal IgA Ab responses to protein Ags because the hallmark of this response is colonic inflammation. More recently, it was reported that OVA-specific Fcα IgA Ab responses induced by intraperitoneal challenge with OVA in CFA was marginally suppressed by prior OVA feeding in normal BALB/c mice (32). As is well documented, mucosal tissues form an integrated and connected network at the tissue, cell, and molecular levels and mucosal immune responses are regulated by unique pathways termed the common mucosal immune system (CMIS) in mucosal inductive tissues (e.g., Peyer’s patches) and effector sites (e.g., lamina propria) (33). However, the induction of IgA Ab responses in these studies were considered to be a CMIS-independent event and were not initiated in the major mucosal inductive tissues, the Peyer’s patches. In addition, the peritoneal cavity is a major source of B-1 cells, which are distinguishable from conventional B cells (B-2 cells) by cell surface markers; of note, B-1 cells express Mac-1 and not Mac1 (25). In addition, B-1 cells, which are derived from the peritoneal cavity, tend to migrate into lamina propria effector sites of the GI tract and differentiate into IgA-producing plasma cells through a T cell-independent mechanism (36, 37). In contrast, Ag-specific IgA Ab production induced by CT in mucosal effector tissues originates from Th cell-dependent B-2 cells (38, 39). In this study, we showed that prior OVA feeding clearly suppressed both systemic and mucosal immune responses elicited by oral challenge with OVA and CT, indicating that oral administration of a soluble protein Ag blocks the induction of CMIS-dependent mucosal immune responses, including IgA Ab production in the GI tract lamina propria.

As is well documented, CT is an excellent mucosal adjuvant for induction of both systemic and mucosal immune responses, including S-IgA Ab responses in mucosal compartments, and nontoxic derivatives are being evaluated for clinical use (6, 20, 21). Because CT exhibits severe toxicity for humans in its native form, mutants of CT that lack ADP-ribosyltransferase activity and diarrheagenicity but retain adjuvanticity have been developed by using molecular biological techniques to resolve this problem (23). Another possible serious side effect of CT is that mucosal administration of CT may abrogate already established tolerance and lead to mucosal hypersensitivity to food Ags and commensal bacteria. In this study, we showed that a single high-dose feeding of protein Ag induces a state of Ag-specific immune unresponsiveness in mucosal compartments, and CT does not break already established tolerance in either mucosal or systemic compartments. In this regard, our data suggest that CT cannot reverse tolerance to Th2-mediated hypersensitivity responses. Assessment as to whether CT abrogates the maintenance of oral tolerance established by repeated low-dose feeding of Ag is under investigation. In addition, further analyses of oral tolerance in mucosal immune compartments followed by Th1-type immune responses seen when LT is used as mucosal adjuvant or when attenuated Salmonella expressing OVA would provide interesting information on mucosal immune responses in the induction and regulation of oral tolerance.

The mechanisms of oral tolerance induction have been assessed in several models; however, most of these studies only determined whether systemic unresponsiveness was induced. In this study, we have now investigated whether both systemic and mucosal immune responses induced by oral immunization are influenced by prior Ag feeding and we have clearly shown that both systemic and mucosal B and T cell responses were dramatically reduced by a single high oral dose of OVA before oral immunization with OVA and CT. Our results should now change the established notion of oral tolerance that has been defined as suppression of Ag-specific systemic immune responses in the presence of mucosal S-IgA Ab responses. Our results provide a new concept for oral tolerance that oral delivery of protein Ag results in a state of both systemic and mucosal immune unresponsiveness to a subsequent encounter with the same Ag. Our studies also indicate that CT, a potent mucosal adjuvant, does not abrogate already established oral tolerance. Finally, this study has established a new model system to assess orally induced unresponsiveness in both the systemic and mucosal immune compartments.

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