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Effect of Multiple Antigenic Exposures in the Gut on Oral Tolerance and Induction of Antibacterial Systemic Immunity

SANJAY GARG, VINEETA BAL, SATYAJIT RATH, AND ANNA GEORGE*

National Institute of Immunology, New Delhi 110067, India

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We have analyzed oral tolerance of microbial antigens in an experimental model in which mice are treated orally with a single small dose of soluble antigen and challenged systemically with the antigen in complete Freund's adjuvant. We found that, while oral administration of sonicated extracts of either *Leishmania major*, *Leishmania donovani*, or *Staphylococcus aureus* was tolerogenic, as was administration of the nominal antigen ovalbumin or conalbumin, oral administration of *Escherichia coli* or *Salmonella typhimurium* sonicated extract was not. Since *E. coli* is an enteric commensal that colonizes the intestine soon after birth, these data suggested that lack of demonstrable oral tolerance may be related to the frequency of oral exposure to an antigen. In support of this, we found that multiple oral doses of ovalbumin or *S. aureus* or *L. donovani* antigens did not increase systemic hyporesponsiveness beyond that achieved with a single oral dose. We have also tested the ability of mice fed with sonicates of the tolerogenic *S. aureus* or the nontolerogenic *S. typhimurium* to clear a subsequent systemic infection with the homologous bacteria and found that, while clearance of *S. aureus* was unaffected by prior feeding, clearance of *S. typhimurium* was actually enhanced. The data suggest that frequent oral antigenic exposure may eventually lead to induction of systemic immunity in tolerant mice.

The gastrointestinal tract, lined by a layer of simple epithelium, is prey to constant assault from ingested parasites. Protective immune responses are initiated predominantly in Peyer's patches, the organized lymphoid tissue present at discrete intervals along the length of the small and large intestine, and for gastrointestinal immunity to be effective, immune cells generated here have to seed, via circulation, the entire epithelial layer and the lamina propria of the gut. A further layer of complexity is added to gastrointestinal immunity by the fact that absorption of nutrients also takes place at this site, so that a balance has to be struck between the generation of protective antimicrobial immune responses and the nongeneration of harmful immune responses against food antigens. It is known that oral administration of soluble antigens or inert particulate antigens usually leads to antigen-specific systemic hyporesponsiveness. The phenomenon, called oral tolerance, was described several years ago in models of anaphylaxis and experimental drug allergy (4, 46) and, in more recent years, as systemic hyporesponsiveness to a variety of antigens, often following antigen-specific T-cell activation (13) and in the face of a good mucosal immunoglobulin A (IgA) response. The readouts used have been as varied as delayed-type hypersensitivity, passive cutaneous anaphylaxis, serum IgG and IgE levels, enumeration of plaque-forming cells, cytotoxic allograft reactions, T-cell stimulation, induction of autoimmune disease, and measurement of systemic antigen-specific cells (1–3, 7, 9, 16–19, 22, 29, 34, 35, 42, 44).

If oral tolerance is a generalized phenomenon and applicable to all antigens, it raises the possibility that oral exposure to microbial antigens may immunocompromise the host by dampening the generation of subsequent antimicrobial systemic immune responses against the parasite. We have explored this possibility by looking at the ability of a single oral application of microbial sonicates to induce systemic hyporesponsiveness in fed mice, by using in vitro T-cell stimulation assays and in vivo bacterial clearance assays as readouts. We report here our findings on oral tolerance induced by a single, low-dose, oral application of sonicates obtained from enteric and nonenteric microorganisms. We also report the effects of administering multiple oral doses of antigens on oral tolerance.

* Corresponding author. Mailing address: National Institute of Immunology, Aruna Asaf Ali Rd., New Delhi 110067, India. Phone: 91 11 618 3004. Fax: 91 11 616 2125. E-mail: anna@nii.res.in.

MATERIALS AND METHODS

Bacteria. *Escherichia coli* HB101 (American Type Culture Collection) and Stm 754, a clinical isolate of *Salmonella typhimurium* (39), are routinely maintained in the laboratory. *Staphylococcus aureus* was a gift of A. Kapil, All India Institute of Medical Sciences, New Delhi, India. *Leishmania donovani* was a gift of K. P. Chang, Chicago Medical School, and *Leishmania major* was a gift of D. Sarkar, Indian Institute of Chemical Biology, Calcutta, India. Bacterial stocks were stored in glycerol broth at −70°C, and a fresh aliquot was plated out either on salmonella-shigella agar (SS agar; Hi Media, Bombay, India) in the case of *S. typhimurium* or on Luria-Bertani (LB) agar (Hi Media) in the case of *S. aureus* and *E. coli*, for each immunization. *Leishmania* promastigotes were propagated in tissue culture flasks (Falcon; Becton Dickinson Labware, Franklin Lakes, N.J.) at 30°C in M199 medium supplemented with 10% fetal calf serum (Biological Industries, Kibbutz Bet Haemek, Israel), 100 μg of penicillin per ml, and 100 U of streptomycin (Hi Media) per ml. For preparation of bacterial sonicates, overnight cultures of bacteria in LB broth cultures were spun down, washed in phosphate-buffered saline, and killed by treating the cells in a boiling water bath for 45 min. The suspension was sonicated for 15 min in phosphate-buffered saline containing 10 mM phenylmethylsulfonyl fluoride (Sigma Chemical Company, St. Louis, Mo.) as a protease inhibitor. The sonicates were spun at 100,000 × *g* for 60 min to remove insoluble debris and to decrease lipopolysaccharide levels, and the supernatants were filtered and used as soluble antigen for in vitro assays. For preparation of leishmanial antigens, promastigote cultures were spun down, washed, killed in a boiling water bath, and sonicated as described above.

Mice and immunization. Six- to ten-week-old BALB/c mice (The Jackson Laboratory, Bar Harbor, Maine), bred in the Small Animal Facility of the National Institute of Immunology, were used for all experiments. For oral tolerance experiments, mice were fed orally with various doses of antigen in 3.5% sodium bicarbonate (Sigma) and challenged in the footpad with homologous antigen emulsified in complete Freund's adjuvant (CFA). All oral doses were administered with a 21-gauge gavage needle attached to a 1-ml tuberculin syringe. Ovalbumin (OA) and conalbumin (CA) were purchased from Sigma. For bacterial challenge experiments, sonicate-fed and control, unfed, mice were challenged intraperitoneally (i.p.) with 10^5 *S. aureus* or 10^6 *S. typhimurium* organisms, and 5 to 7 days later, their spleens were harvested and appropriate

dilutions were plated out on LB or SS agar. The bacteria were enumerated as CFU per spleen. The limit of detection was 50 CFU/spleen.

T-cell proliferation. Single-cell suspensions from pooled populations of inguinal and popliteal lymph nodes were cultured in triplicate at 3×10^5 cells/well with graded doses of antigen. All assays were done in 200 μl of Click's medium (Irvine Scientific, Irvine, Calif.) containing 10% fetal calf serum (HyClone, Logan, Utah), 100 μg of penicillin per ml, 100 U of streptomycin per ml, and 0.05 mM 2-mercaptoethanol (Gibco BRL, Grand Island, N.Y.) in 96-well flat-bottom plates (Falcon). Proliferation was measured by pulsing the wells with 0.5 μCi of [^{3}H]thymidine (NEN, Boston, Mass.) 72 h after initiation of culture and harvesting the samples 12 to 16 h later onto glass fiber filters for scintillation spectroscopy (Betaplate; Wallac, Turku, Finland). Replicate-well samples were harvested for cytokine estimation by enzyme-linked immunoassay (EIA).

Adoptive transfer. T and B cells were purified from spleens of mice 1 week after feeding by magnetic separation on Midi-MACS columns (Miltenyi Biotec, Hamburg, Germany), with Thy1 and B220 beads, respectively, according to the manufacturer's recommendations. Fractionated cells were analyzed by flow cytometry and were used for adoptive transfer experiments only if enrichment was >95%. Unfractionated cells and purified subsets were transferred into naive recipients intravenously (3×10^7 cells per mouse), and the recipients were challenged with live bacteria i.p. 12 h later.

Cytokine assays. EIAs were performed on culture supernatants with appropriate purified and biotinylated antibody pairs for gamma interferon (IFN-γ) (Genzyme, Boston, Mass.) and interleukin-10 (IL-10) (PharMingen, San Diego, Calif.) according to the manufacturer's protocols. Purified monoclonal anti-mouse IFN-γ or IL-10 was captured onto polystyrene microtiter plates (Nunc, Roskilde, Denmark). Culture supernatants were then added, followed by either biotinylated goat anti-mouse IFN-γ or biotinylated monoclonal rat anti-mouse IL-10. Streptavidin peroxidase, followed by hydrogen peroxide and tetramethylbenzidine (Sigma), was used for detection. Titration curves of recombinant IFN-γ (Genzyme) and IL-10 (PharMingen) were used as standards for calculating cytokine concentrations in the culture supernatants. The limit of detection for both cytokines was 15 to 30 pg/ml.

Fragment cultures. Peyer's patch fragment cultures were set up as described earlier (21). Briefly, Peyer's patches from mice rendered tolerant and control mice not rendered tolerant were recovered by dissection from the small intestines and halved, and groups of four halves were cultured in 2 ml of Dulbecco minimal essential medium (Gibco BRL) supplemented as described above, with graded doses of antigen. Plates were incubated in an atmosphere of 90% O_2–10% CO_2, and supernatants were tested 5 to 7 days later for antibody by EIA on plates coated with OA and blocked with 1% bovine serum albumin (Sigma). Addition of supernatants was followed by addition of biotinylated goat anti-mouse IgA (Southern Biotechnology). Streptavidin peroxidase, followed by hydrogen peroxide and tetramethylbenzidine (Sigma), was used for detection.

RESULTS

Generation of oral tolerance to soluble nominal antigens. Mice were given 2 mg each of either OA or CA orally, and 1 week later they were challenged in the footpad with 10 μg of homologous antigen in CFA. T-cell stimulation assays were set up a week later with cells from draining lymph nodes, and the results are shown in Fig. 1. It can be seen that both proliferation (Fig. 1A and B) and cytokine secretion (Fig. 1C to F) are lower in cells from fed mice. To establish the phenotype of the responding cells, 10 μg of azide-free anti-CD4 or anti-CD8 (clones RM-5 and 53-6.7, respectively; PharMingen) per ml was added to the cultures (15, 47), and it can be seen that both proliferation (Fig. 2A) and cytokine secretion (Fig. 2B and C) are significantly reduced in the presence of anti-CD4, whereas they are largely unaffected by anti-CD8, suggesting that the cells responding to antigen in culture were CD4 T cells. To determine whether the systemic hyporesponsiveness to fed OA occurred in the face of a good mucosal IgA response, we looked for IgA-committed cells in Peyer's patches of fed mice in fragment cultures (21). Figure 3 shows that higher levels of anti-OA antibodies are, indeed, present in fragment cultures from mice rendered tolerant than in fragment cultures from unfed controls. Here, the lower levels of antibody seen at the higher antigen concentrations are probably due to neutralization of antibodies by antigen present in culture.

Effect of antigen dose in tolerance induction. Mice were fed orally with either 1 mg or 300, 100, 30, or 10 μg of CA and challenged in the footpad a week later with 10 μg of CA in CFA. Figure 4 shows that the degree of systemic hyporesponsiveness following feeding depends on the oral dose used. Cells from mice fed with the various doses but not challenged subcutaneously (s.c.) did not respond (data not shown). These data extend earlier reports which indicated that induction of oral tolerance may be linked to antigen dosage (11, 36). These data also demonstrate the potency of oral tolerance—a minute (10 μg) amount of soluble antigen given orally can down regulate the response to the same amount administered systemically in adjuvant.

Ability of microbial antigens to induce oral tolerance. In order to test whether oral exposure to soluble parasite antigens can affect subsequent immune responses to a systemic challenge with the parasite, we treated mice orally with sonicates of *E. coli*, *S. typhimurium*, *L. major*, or *S. aureus* (2 mg each) and challenged them in the footpad a week later with 10 μg of the homologous antigen in CFA. Figure 5 shows that, while *S. typhimurium* and *E. coli* sonicates do not induce oral tolerance, *L. major* and *S. aureus* sonicates do. Similar results were seen with oral doses ranging from 1 mg to 100 μg, and the response to *L. donovani* sonicate was similar to that seen with *L. major* sonicate (data not shown).

Inability of *E. coli* and *S. typhimurium* sonicates to affect bystander suppression of oral tolerance for OA. Since microbial sonicates are complex mixtures of epitopes and bioactive molecules, it is possible that the lack of demonstrable tolerance for *E. coli* or *S. typhimurium* sonicates may be related to the presence of molecules in these sonicates that can suppress tolerance in a non-antigen-specific manner. To test this, groups of mice were fed with a mixture of either OA and *S. typhimurium* sonicate or OA and *E. coli* sonicate and challenged in the footpad with the homologous mixtures, and T-cell proliferation in vitro in response to both sets of antigens was determined. Figure 6 shows the proliferation response to OA, and it can be seen that neither *E. coli* nor *S. typhimurium* antigens present during oral immunization and the subsequent s.c. challenge affect the generation of tolerance for OA. Proliferation responses to *E. coli* and *S. typhimurium* antigens were not affected by the presence of OA in these experiments (data not shown). Similar experiments were done with mixtures of *S. typhimurium* and *L. major* sonicates, and there was no abrogation of tolerance of *L. major* antigens (data not shown).

Effect of multiple oral doses on systemic hyporesponsiveness. The lack of demonstrable oral tolerance for the enteric commensal *E. coli* and for the cross-reactive *S. typhimurium* antigens raises the possibility that oral tolerance may be related to the frequency of exposure to a given antigen. Since mice are colonized at birth with *E. coli*, the systemic response scored in experiments done with adult mice reflects the effects of constant gastrointestinal exposure, rather than a single oral dose, on s.c. challenge. It is possible, therefore, that while the first oral exposure is in fact tolerogenic, subsequent oral exposure does not induce further tolerance. In order to examine this possibility, we looked at the effect of multiple oral doses of OA (1 mg each) on the systemic response to a subsequent s.c. challenge. Figure 7 shows that neither two oral doses 1 week apart (Fig. 7A), two oral doses 14 weeks apart (Fig. 7B), or three oral doses 1 week apart (Fig. 7C) increase systemic hyporesponsiveness beyond that achieved with a single oral dose. A similar pattern was also seen with two doses of *S. aureus* and *L. donovani* sonicate given a week apart (data not shown). Thus, multiple oral doses do not enhance systemic unresponsiveness beyond that seen with a single dose in this experimental system.

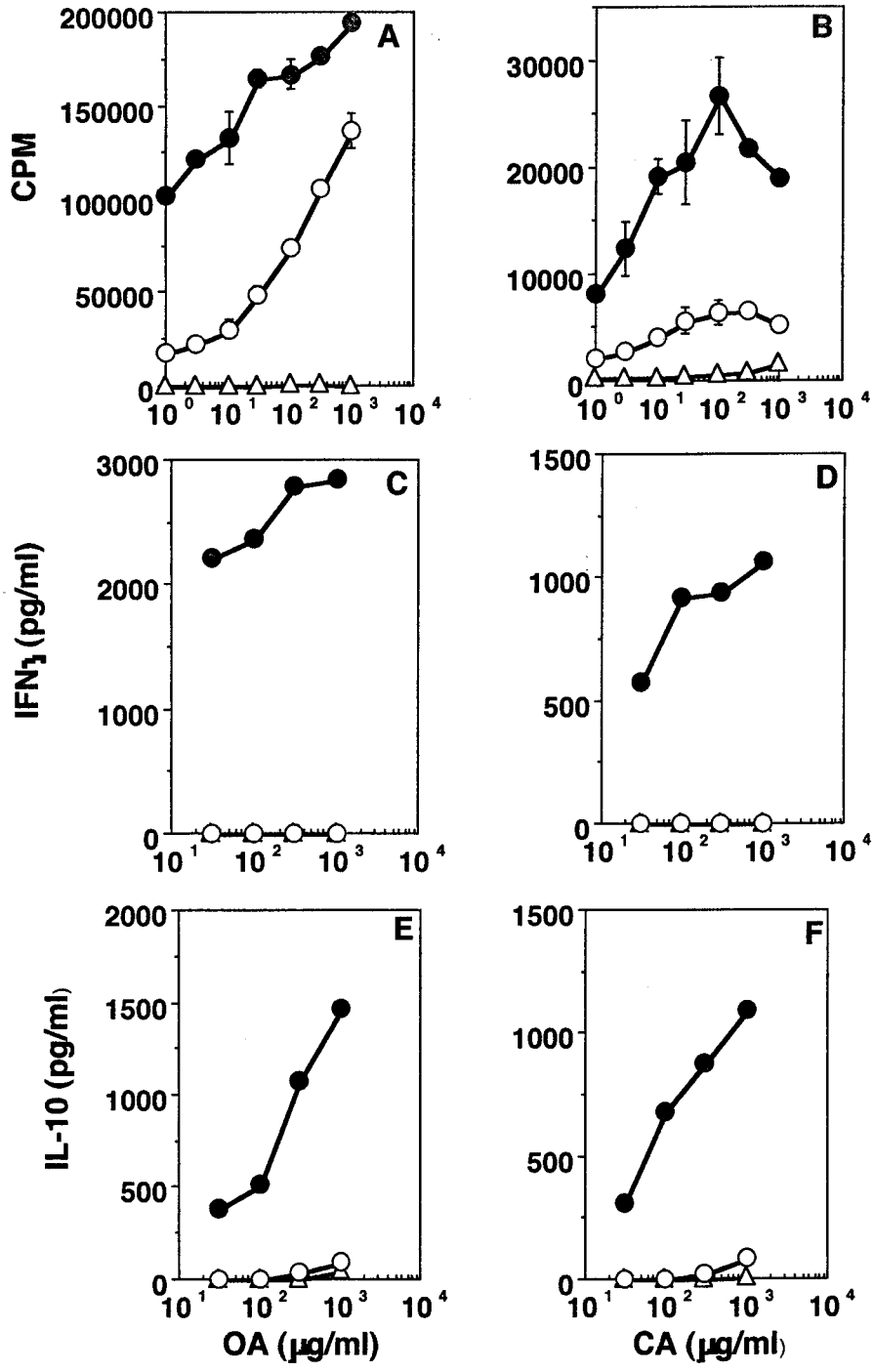

FIG. 1. Induction of oral tolerance for soluble nominal antigens. Proliferation (A and B), IFN-γ (C and D), and IL-10 (E and F) responses of lymph node cells from mice rendered tolerant (open circles), mice not rendered tolerant (filled circles), and control mice that were fed but not challenged s.c. (open triangles) are shown. Antigens used were OA (A, C, and E) and CA (B, D, and F). Results of one experiment, representative of four for each antigen, are shown.

Effect of orally administered antigens on subsequent infections. Since *S. aureus* sonicate induces demonstrable oral tolerance while *S. typhimurium* sonicate does not, we next assessed the effect of oral administration of these sonicates on the ability of the fed mice to clear a systemic challenge of live bacteria. Groups of mice were given *S. aureus* or *S. typhimurium* sonicate orally, challenged i.p. with live bacteria a week later, and sacrificed 5 to 6 days after challenge, and splenic bacterial load was determined by plating out individual spleen lysates on LB agar or SS agar, respectively. Figure 8A shows that mice fed with the tolerogenic *S. aureus* sonicate clear a homologous challenge infection as well as the unfed mice do and that bacterial clearance is not affected by multiple doses. On the other hand, mice fed with the nontolerogenic *S. typhimurium* sonicate actually clear a challenge infection better than unfed mice do, and the protection afforded by oral ad-

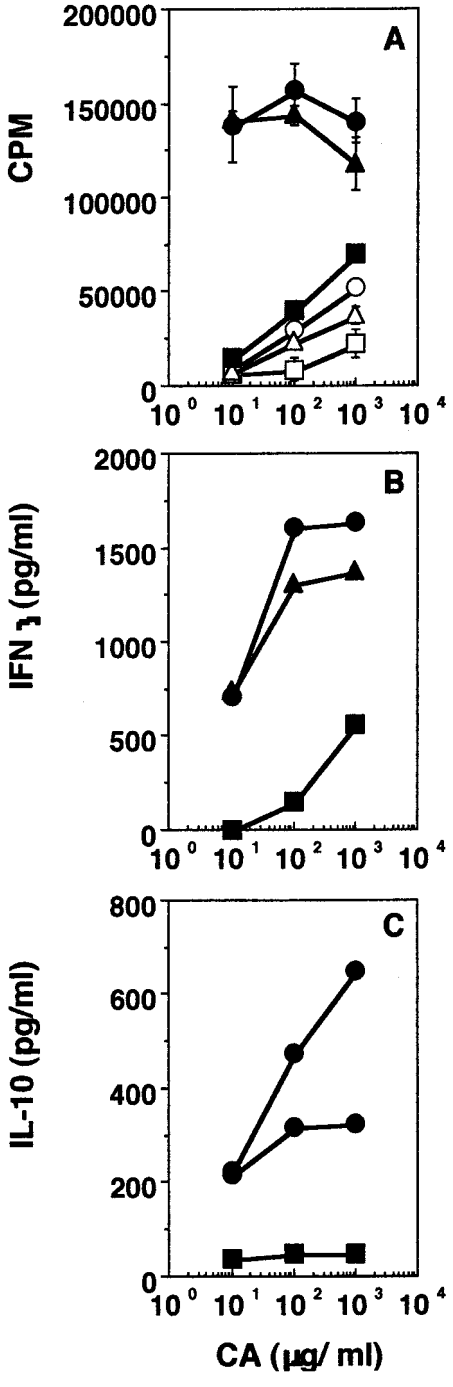

FIG. 2. Phenotype of cells responding in vitro. Proliferation (A), IFN-γ (B), and IL-10 (C) responses of lymph node cells from mice rendered tolerant (open symbols) and mice not rendered tolerant (closed symbols) cultured in the presence of anti-CD4 (squares) or anti-CD8 (triangles) are shown. No cytokines were detectable in supernatants of cultures from mice rendered tolerant. Results of one experiment, representative of two for each mouse group, are shown.

ministration and that afforded by i.p. administration of sonicate are similar (Fig. 8B). While a good anti-*Salmonella* antibody response was detected in sera of mice 1 week after i.p. immunization and immediately before s.c. challenge, no significant response was detected in sera of the fed mice (data not shown), indicating that antibodies are not responsible for the

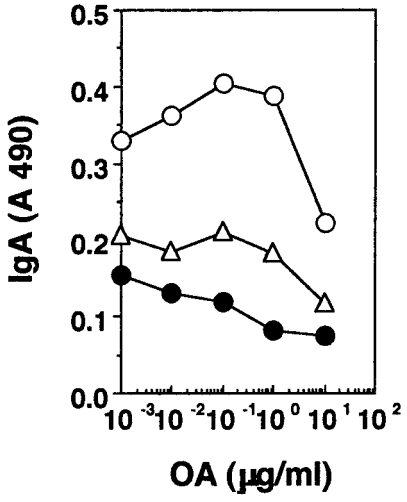

FIG. 3. Mucosal IgA responses are enhanced in oral tolerance. Peyer's patches from mice rendered tolerant (open circles), mice not rendered tolerant (filled circles), or control mice that were fed but not challenged s.c. (open triangles) were stimulated in fragment cultures with OA, and supernatants were assayed for anti-OA IgA. Results of one experiment, representative of two for each mouse group, are shown.

enhanced systemic clearance of bacteria that is seen with fed mice. Adoptive transfer experiments showed that purified T cells from fed mice could transfer protection to naive recipients while purified B cells were ineffective (Fig. 8C).

DISCUSSION

The intestine is home to a host of aerobic and anaerobic microorganisms that constitute the normal flora of the gut (37), and control of their number and diversity is required to prevent opportunistic infections and inflammatory bowel diseases. Individuals appear to be immunologically tolerant of their own intestinal flora but not of heterologous intestinal

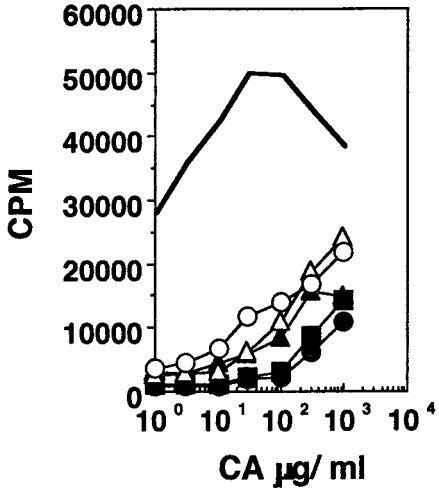

FIG. 4. Effect of antigen dose in the induction of oral tolerance. Proliferation responses of lymph node cells from mice not rendered tolerant (line with no symbols) or mice rendered tolerant with either 1 mg (filled circles), 300 μg (filled squares), 100 μg (filled triangles), 30 μg (open triangles), or 10 μg (open circles) of CA and challenged s.c. with 10 μg of CA in CFA are shown. Standard errors of the means were less than 10%. Results of one experiment, representative of four for each mouse group, are shown.

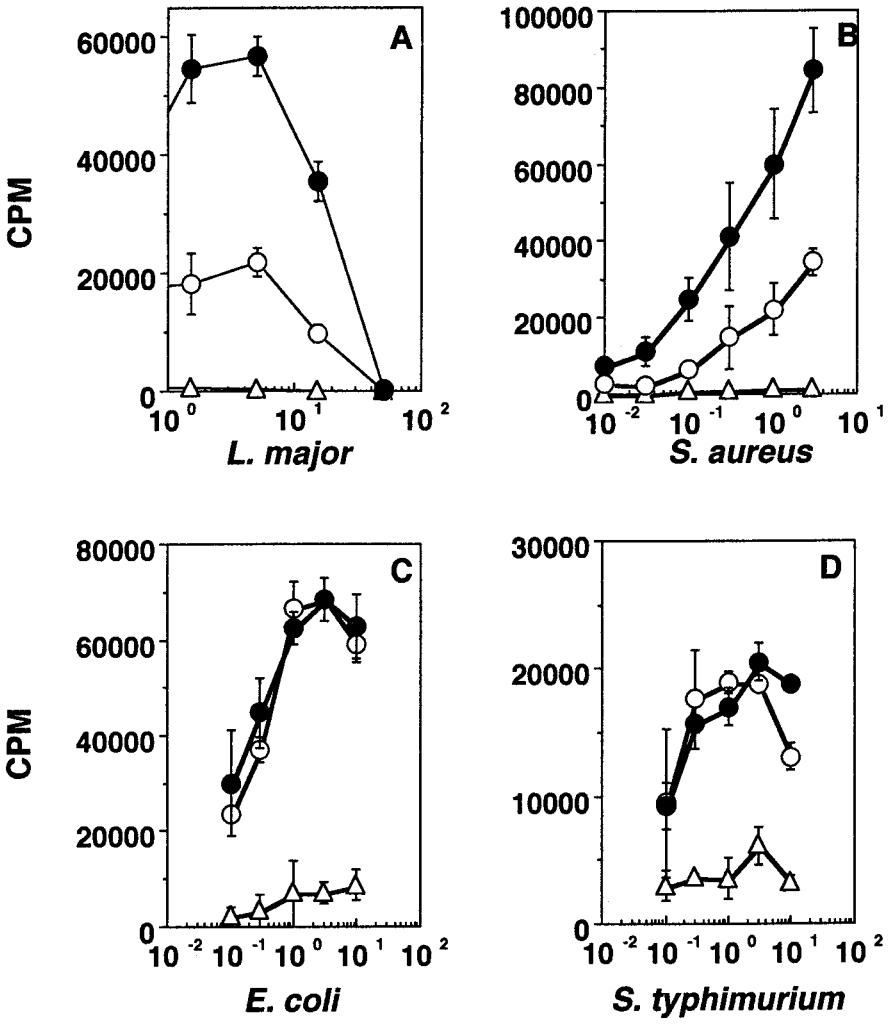

FIG. 5. Induction of oral tolerance for *L. major* (A), *S. aureus* (B), *E. coli* (C), and *S. typhimurium* (D) sonicates. Proliferation responses of mice rendered tolerant (open circles), mice not rendered tolerant (filled circles), and control unchallenged mice (open triangles) are shown. Units on the *x* axis are micrograms of the respective antigen per milliliter. Results of one experiment, representative of two (A and B) or three (C and D) for each mouse group, are shown.

flora (8), and tolerance of autologous flora has been shown to be disrupted in a model of experimental colitis (9). The mechanisms controlling tolerance of microbial antigens in this complex environment are unknown, but various mechanisms have been implicated in experimental models of oral tolerance of simple soluble and inert particulate antigens. These include clonal anergy (26, 42), serum factors (1, 18), suppressor T cells (20, 22, 23, 29, 34), and immunomodulation by γδ T cells (19, 24, 25, 27).

The presence of microorganisms and their products in the gut may influence systemic immunity in two distinct ways. On the one hand, some bacteria may express antigens resembling potential allergens, and they might, therefore, either cause or aggravate allergic reactions. In this context, it has been reported that rats colonized at birth with a recombinant *E. coli* strain producing OA make anti-OA IgE, but not antilipopolysaccharide or antifimbrial antibody (7), underlining the complex nature of responses to antigens in the gut. On the other hand, products of pathogenic organisms may induce oral tolerance, and this could conceivably lead to dampening of a subsequent protective immune response against the virulent organism. We tested antigens from various microorganisms, including normal gut flora and pathogens that invade by the enteric or systemic routes, in our experimental model of tolerance. We found that both leishmanial and staphyloccocal sonicates were tolerogenic in a T-cell stimulation readout assay (Fig. 5A and B), a pattern similar to that seen with OA or CA (Fig. 1A and B) and in keeping with the reported induction of systemic tolerance of orally administered staphylococcal enterotoxin B (28). Curiously, *E. coli* and *S. typhimurium* sonicates were not tolerogenic (Fig. 5C and D), raising the possibility that molecules that can suppress tolerance in a non-antigen-specific manner, as cholera toxin has been reported to do (10, 33, 38), may be present in the latter sonicates. However, this does not seem to be the case, as coadministration of these sonicates with either OA or leishmanial antigens does not abrogate tolerance of either (Fig. 5). Our results differ from those reported earlier (6) where *E. coli* heat-labile toxin was found to abrogate the induction of oral tolerance of unrelated antigens when administered together with them. This difference may be explained by the fact that the bacterial sonicates used in our study are made from heat-killed bacteria and therefore lack the adjuvanticity of the toxin. However, it has also been reported that cryptic determinants of an antigen may be nontolerogenic even when immunodominant determinants of the same antigen are tolerogenic (14), and we cannot con-

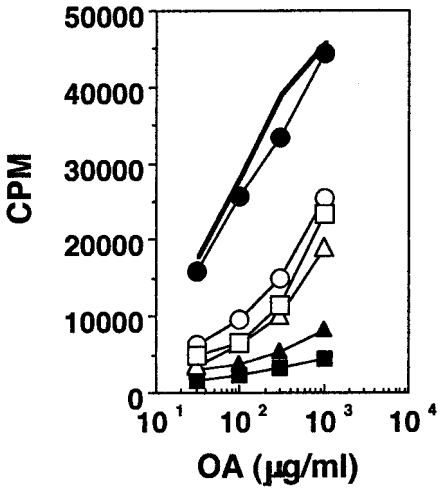

FIG. 6. Induction of oral tolerance for OA (open circles) is unaffected by the coadministration of *S. typhimurium* (open squares) or *E. coli* (open triangles) sonicates. Controls include responses of cells from mice fed with the respective mixtures but not challenged s.c. (filled squares and filled triangles) and from control mice not rendered tolerant and challenged s.c. with either OA (filled circles) or OA and *S. typhimurium* sonicate together (line with no symbols). Standard errors of the means were less than 10%. Results of one experiment, representative of three for each mouse group, are shown.

clusively rule out the possibility that certain mixtures of antigens will simply not induce tolerance because of a predominance of nontolerogenic determinants.

We next considered the possibility, envisaged earlier (3), that oral tolerance is related to the frequency of oral exposure to antigen. *E. coli* is a normal component of the murine gut flora, and the lack of oral tolerance of *E. coli* antigens (and of the cross-reactive *S. typhimurium* antigens) might indicate that, although initial oral exposure to these antigens is indeed tolerogenic, subsequent exposures may not enhance systemic hyporesponsiveness any further. Thus, the s.c. response that we see with both fed and unfed mice might well be an induced-tolerance response compared to the response in mice that were never exposed to the bacteria in their guts. Indeed, we found that multiple feeding with a tolerogenic antigen did not increase systemic hyporesponsiveness beyond that seen with a single dose (Fig. 7). In this context, it has been reported that oral tolerance is lacking in γδ knockout mice (19), and our prediction is that T-cell responses to *E. coli* sonicate following s.c. immunization will be higher in adult γδ knockout mice than in conventional mice.

The physiological consequences of oral tolerance for bacterial antigens may manifest not only as hyporesponsiveness of T cells from the mice rendered tolerant in vitro but also as an impaired ability of mice rendered tolerant to clear a subsequent systemic challenge with live bacteria. However, this appears not to be the case, and mice fed with the tolerogenic *S. aureus* sonicate clear a challenge infection as well as unfed mice do (Fig. 8A). Interestingly, *S. typhimurium* sonicate that is not tolerogenic by the oral route is actually immunogenic, and allows fed mice to clear a challenge infection better than unfed mice (Fig. 8B), and T cells, rather than B cells, are responsible for this enhanced clearance (Fig. 8C). These results extend our earlier data showing that live *S. typhimurium* given orally generates an excellent mucosal and systemic Th1 immune response (12). Our present data indicate that, although no direct T-cell readout of priming may be evident following oral administration of inert *S. typhimurium*, protective T-cell responses are, in fact, generated and can be read out as enhanced protection against challenge with virulent bacteria.

The oral administration of antigens has been shown to be effective in preventing or suppressing experimental autoim-

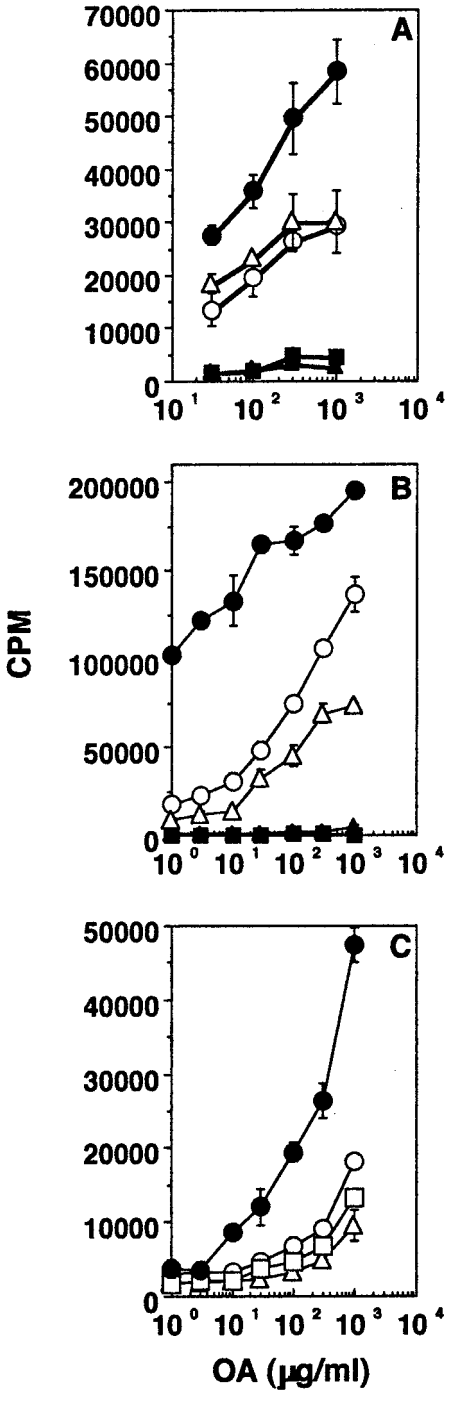

FIG. 7. Effect of multiple oral doses on tolerance. Responses of lymph node cells from mice not rendered tolerant (filled circles) or mice rendered tolerant with either a single dose of 1 mg (open circles), two doses of 1 mg each (open triangles), or three doses of 1 mg each (open squares) of OA are shown. Results of one experiment, representative of two for each group of mice, are shown. The oral doses were given 1 week apart for panels A and C and 14 weeks apart for panel B. Filled triangles and filled squares in panels A and B represent responses from control mice fed once or twice but not challenged s.c.

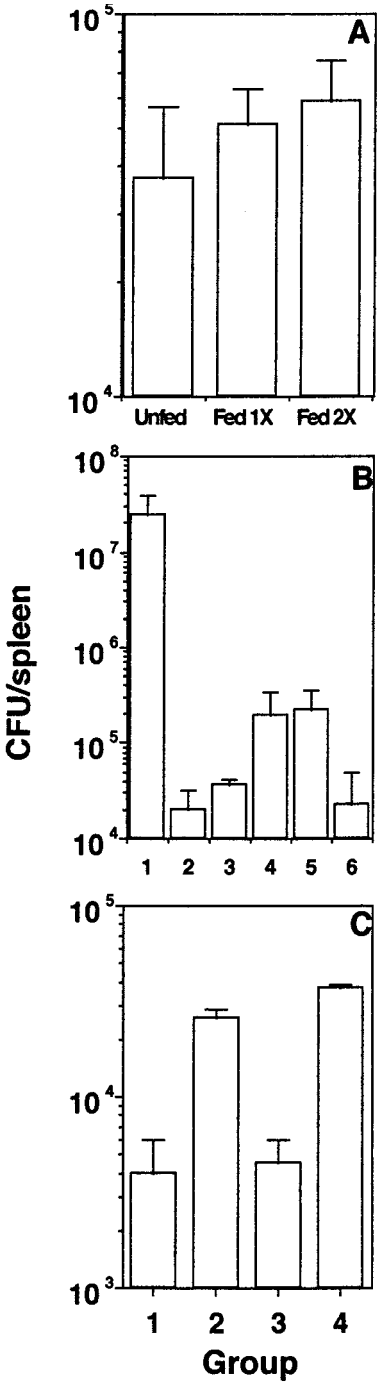

FIG. 8. Effect of orally administered antigens on subsequent homologous systemic infection. (A) Groups of mice were fed 2 mg of *S. aureus* sonicate either once or twice before i.p. challenge. (B) Mice were either unfed (bar 1); fed with 1, 2, or 3 mg of *S. typhimurium* sonicate (bars 2 to 4, respectively); or immunized with 10 or 100 μg of sonicate i.p. (bars 5 and 6, respectively) before i.p. challenge. (C) Purified splenic T cells (bar 1), splenic B cells (bar 2), or whole spleen cells (bar 3) from mice fed with 2 mg of *S. typhimurium* sonicate or whole spleen cells from unfed mice (bar 4) were adoptively transferred into naive recipients before i.p. challenge. Results of one experiment, representative of two for each group of mice, are shown.

mune encephalitis, collagen-induced arthritis, thyroiditis, and uveitis (5, 30, 32, 40, 43, 48), and the strategy is being considered for the clinical treatment of autoimmune diseases (31, 41, 45). However, a note of caution has been suggested by the report that oral administration of autoantigen may induce cytotoxic T cells that can lead to an induction of autoimmune diabetes (2). The present study shows that, even under conditions where systemic tolerance is observed, the effect may be entirely achievable with a single dose, and chronic or frequent oral administration may in fact immunize.

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