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Generation of Gamma Interferon Responses in Murine Peyer's Patches following Oral Immunization

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To date, oral immunizations have been shown to generate only Th2 responses in murine Peyer's patches (PP), raising the possibility that T cells present in PP may be capable of mounting only Th2 responses or that the microenvironment of PP does not favor the generation of Th1 cells. However, it is also possible that antigens that can generate Th1 responses have not yet been used for oral immunizations. This study shows that T cells in PP of mice immunized orally with live *Salmonella typhimurium* secrete large amounts of gamma interferon (IFN-γ) when they are stimulated with bacterial sonicate in vitro. Moreover, oral challenge of mice with live bacteria 4 months after immunization elicits a secondary IFN-γ response in PP and mesenteric lymph nodes. Parenteral immunization does not generate an IFN-γ T-cell response in PP, and parenteral challenge of orally immunized mice does not elicit a secondary response in PP. However, oral challenge of intraperitoneally immunized mice elicits a secondary IFN-γ response in PP and mesenteric lymph nodes, and intraperitoneal challenge of orally immunized mice elicits a secondary response in the spleen. The data suggest that memory T cells recirculate between mucosal and nonmucosal compartments and that they may be recruited to the site of antigenic challenge.

The immune system of the gastrointestinal tract can be divided into two separate but connected sites—an inductive site, comprising the Peyer's patches (PP) and mesenteric lymph nodes (MLN), where immune responses are initiated, and an effector site, which includes the lamina propria (LP) of the intestinal wall and the intraepithelial leukocyte compartment, where the cells exert their effect. Both immunoglobulin A (IgA)-secreting plasma cells and a large number of CD4 T cells are found in the LP, and it has been shown that Ig A-positive B cells and CD4 T cells from PP and MLN home to the LP (3, 6, 18, 19, 22).

Evidence to date indicates that CD4 cells in murine PP are predominantly of the Th2 type, secreting interleukin 4 (IL-4) and/or IL-5 and IL-10 in preference to gamma interferon (IFN-γ) when stimulated with specific antigen (Ag) (17, 30, 32–34). However, inert particulate Ags, such as sheep erythrocytes (33), and soluble Ags, such as tetanus toxoid (17, 34) or keyhole limpet hemocyanin (32), in the presence or absence of the mucosal adjuvant cholera toxin have been used for oral immunizations in these studies, and it is possible that such immunizations may not be optimal for the generation of Th1 responses. We know that excellent Th1 responses are generated against listerial, leishmanial, malarial, and other parasitic Ags (7, 12, 13, 25, 26, 28, 29). Thus, if the microenvironment of PP truly biases T cells towards the Th2 pathway, oral immunization with such an Ag should generate only a Th2 response in PP. On the other hand, if there is no such intrinsic bias, it should be possible to generate a Th1 response in PP. To address this issue, a clinical isolate of the enteric pathogen *Salmonella typhimurium* (Stm 754 [29]) was used as a model Ag for oral immunizations. It was chosen for several reasons. First, *S. typhimurium* is known to bind M cells of PP and to translocate into internal organs through PP (2, 4, 14), so that the organisms are likely to be immunogenic by the oral route. Second, this strain has been shown to generate a predominantly Th1 response in the spleen following intraperitoneal (i.p.) immunization with live organisms (29). Third, the strain has a high 50% lethal dose (LD_{50}) for BALB/c mice, and oral infection with 10^4 or 10^5 bacteria does not lead to their extensive replication in PP, a pattern of infection observed only when infection with the more virulent strains is attenuated by oral coadministration on anti-O antiserum (10). The low virulence of Stm 754 and the absence of necrosis in PP tissue make it possible to monitor T-cell responses over a long period of time.

This study monitors the kinetics of the anti-*Salmonella* IFN-γ response in T cells at mucosal and nonmucosal sites, and it compares the efficacies of one-dose and two-dose oral immunization schedules. It shows that although oral immunization is necessary for the initial generation of an IFN-γ response in PP, memory T cells generated in the spleen by parenteral immunization can be recalled in PP by challenging the mice orally with live bacteria. It also shows that IFN-γ-secreting CD4 T cells accumulate in the LP of immunized mice.

MATERIALS AND METHODS

Bacteria. A clinical isolate of *S. typhimurium* (Stm 754 [29]) was used in all experiments. Bacterial stocks were stored in glycerol broth at −70°C, and a fresh aliquot was plated out on Difco's salmonella-shigella agar (Fisher Scientific) for each immunization. Killed cells were obtained by treating bacteria in a boiling water bath for 45 min. The killed suspension was sonicated in phosphate-buffered saline (PBS) containing 10 mM phenylmethylsulfonyl fluoride (Sigma) as a protease inhibitor. The sonicate was then spun at 100,000 × *g* for 60 min to remove insoluble debris and to decrease lipopolysaccharide levels, and the supernatant was filtered and used as soluble Ag for in vitro assays.

Mice. Female BALB/c mice, 6 to 8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, Maine) and maintained under specific-pathogen-free conditions in the animal facility of the National Institute of Dental Research. Mice immunized with live bacteria were housed in microisolator cages in a separate isolator, and all procedures were carried out with biosafety level II precautions. The LD_{50} of the bacterial strain for these mice was determined to be 1.4×10^4 cells given i.p. and 9×10^6 cells given orally.

Immunizations. For i.p. immunization with live bacteria, mice were given 10^2

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cells i.p. in 0.2 ml of sterile PBS, followed by 10^3 cells 1 week later. In some experiments, a single dose of either 10^2 or 10^3 cells was used. For i.p. immunization with dead bacteria, they were given two doses of 10^8 cells each in PBS, 1 week apart. For oral immunization with live bacteria (OL), they were given either a single dose of 10^4 cells in 0.2 ml of 3.5% $NaHCO_3$ or two doses (10^4 followed by 10^5 cells, 10^4 followed by 10^6 cells, or 10^5 followed by 10^6 cells) in 3.5% $NaHCO_3$, 1 week apart. For oral immunization with dead bacteria, they were given two doses of 10^8 cells each in 3.5% $NaHCO_3$, 1 week apart. All oral doses were administered with a 20-gauge gavage needle attached to a tuberculin syringe. Biosafety regulations required clearance of infectious bacteria from mice before they were removed from their isolation chambers in the animal colony for cell culture work; thus, all mice were treated i.p. with four doses of ciprofloxacin (Miles Scientific) at 1 mg per mouse, 12 h apart (29), immediately before they were sacrificed for T-cell assays.

LP T cells. CD4 T cells from the LP of the small intestine were isolated by a modification of the method of Kramer and Cebra (15). Briefly, PP and mesenteries were removed from the small intestine, and the gut was slit open and cut into 3- to 5-cm-long pieces. The pieces were rinsed in several changes of Dulbecco modified Eagle medium (DMEM) containing 5% fetal bovine serum, gentamicin (50μ/ml), and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and cut further into 0.5-cm-long segments. These were then washed three to five times in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS; Biowhittaker) and three times in Ca^{2+}- and Mg^{2+}-free HBSS containing 0.05% EDTA. The EDTA was washed off with three to four rinses of medium, and the segments were digested three times for 30 min each at 37°C with a mixture of 10 U of type I collagenase (Worthington Biochemical Corp.) per ml, 0.5 μg of dispase (Boehringer Mannheim) per ml, 1.5 μg of type II DNase (Sigma) per ml, and 0.1 μg of soybean trypsin inhibitor (Sigma) per ml in DMEM. Cells released by enzyme digestion were passed through nylon wool columns twice and then spun on a 40 to 70% Percoll (Pharmacia) gradient at $500 \times g$ for 20 min at room temperature. Lymphocytes at the interface were collected and treated with a cocktail of anti-B220 (RA3-6B2; Pharmingen) and anti-CD8 (53-6.7; Pharmingen) for 45 min on ice. Cells were washed once and incubated for 30 min at 37°C in a 1/15 dilution of complement (Cedarlane Laboratories; obtained from Accurate Chemical and Scientific Corp. Westbury, N.Y.). Dead cells and residual epithelial cells were removed by spinning the cells on another Percoll gradient. The resulting suspension was stained with fluorescein isothiocyanate-conjugated anti-CD4 (RM4-5; Pharmingen) for 30 min on ice in PBS containing 1% bovine serum albumin (fraction V; Sigma) and 0.1% sodium azide (Sigma), washed three times, analyzed on a FACScan (Becton Dickinson), and found to be 92 to 95% $CD4^+$ (data not shown).

Ag-specific proliferation. Single-cell suspensions of spleen, PP, and MLN from naive or immunized mice were prepared by mechanical dispersion, and 3×10^5 cells per well were plated out in 96-well flat-bottomed plates (Costar) in 200 μl of DMEM supplemented with 10% fetal bovine serum (different lots contained 0.05 to 0.14 ng of endotoxin per ml; Biowhittaker), 2 mM L-glutamine (Biowhittaker), 50 μg of gentamicin (Gibco BRL) per ml, and 2×10^{-5} M β-mercaptoethanol (Sigma). The concentration of soluble bacterial sonicate Ag optimal for obtaining equivalent proliferative responses in all three tissue types was found to be 3 μg/ml, and although cultures were set up with 1, 3, and 10 μg of Ag per ml, only responses to the optimal concentration are shown. The method was modified slightly for LP assays, as Ag-presenting cells are lost during the isolation procedure. Thus, 2×10^5 LP T cells were plated out with 2×10^5 irradiated (2,000 R) syngeneic spleen cells or with various numbers of epithelial cells from the LP preparation (10^5 to 10^6 per well) in 200 μl of medium. All assays were set up in triplicate. Supernatants were collected at 48, 60, and 72 h, and the wells were pulsed with 1 μCi of [^{3}H]thymidine (Dupont, NEN) at 72 h. Cells were harvested onto glass fiber filters 16 h later, and [^{3}H]thymidine incorporation was assessed by liquid scintillation spectroscopy (Betaplate; LKB, Pharmacia).

Lymphokine assays. IFN-γ in culture supernatants was assayed by enzyme-linked immunosorbent assay (ELISA). Two micrograms of anti-IFN-γ (clone R4-6A2; Pharmingen) per milliliter was used as the capture antibody (Ab), and 0.5 μg of biotinylated anti-IFN-γ (clone XMG 1.2; Pharmingen) per ml was used as the detection Ab. Standard curves were set up with recombinant IFN-γ (Pharmingen). Streptavidin-peroxidase and ABTS substrate were purchased from Kirkegaard and Perry (Gaithersburg, Md.). The reaction was stopped by the addition of sodium dodecyl sulfate to 0.5%, and the A_{405} read on a computerized kinetic microplate reader (Molecular Devices, Dupont, NEN). The limit of detection was 10 pg/ml. A bioassay with the IL-4-sensitive cell line CT.4S was used to detect IL-4 in supernatants. CT.4S cells ready for assay were kindly supplied by W. Paul's laboratory (National Institute of Allergy and Infectious Diseases). Supernatants and recombinant IL-4 (Pharmingen) were plated out in a volume of 100 μl in 96-well plates, and 5×10^3 washed CT.4S cells were added to each well in 100 μl. After 24 h, the wells were pulsed with 1 μCi of [^{3}H]thymidine; cells were harvested 16 h later, and [^{3}H]thymidine incorporation was assessed as described above. Half-maximal stimulation of CT.4S cells required 30 to 35 pg of recombinant IL-4 per ml, and the limit of detection was 2 to 3 pg/ml.

Data plotting. For Fig. 2, 3, 4, and 6, pooled triplicate supernatants from spleen, PP, and MLN cultures of each mouse were assayed separately and the data were plotted as means ± standard errors (SE). The sample size was taken to be $n - 1$. For Fig. 5 and Table 1, for which tissues from two to five mice in each group were pooled for experiments, triplicates were assayed separately. For results shown in Fig. 1, tissues from three to five mice were plated out individually on salmonella-shigella agar and the data were plotted as mean CFU ± SE. The sample size was taken to be $n - 1$.

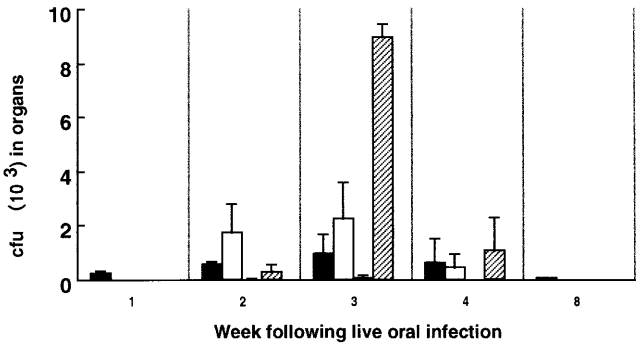


FIG. 1. Translocation of bacteria from the intestinal lumen into internal organs following oral immunization with 10^4 live Stm 754 cells. Bacterial counts are depicted as CFU in the following materials, from left to right: 3 ml of intestinal washout (■), whole MLN (□), 1 g of liver, and whole spleen (▨). Results for one experiment, representative of two, are shown. Three to five mice were used per group.

RESULTS

Translocation of bacteria from the intestinal lumen. Mice were immunized orally with 10^4 live cells of Stm 754, and bacterial counts in the intestinal lumen and internal organs were determined over time. Three to five mice were sacrificed at each time point, and each small intestine was washed out with 3 ml of PBS. Spleen, MLN, PP, lungs, liver (1 g), kidneys, and brain were harvested and ground individually in 2 ml of PBS. Washout and homogenates of each tissue (100 to 300 μl) were plated out on salmonella-shigella agar, and the number of viable bacteria was scored the next day as CFU. The limits of detection were 20 CFU for tissues and 30 CFU for intestinal washout. As shown in Fig. 1, bacteria could be recovered only from the intestinal lumen 1 week following immunization. Thereafter, detectable levels of bacteria were seen, successively, in the MLN and spleen, and they were present occasionally in the liver. No bacteria could be recovered from the lungs, brain, or kidneys, and they were recovered from the PP only very occasionally, and only when PP from several mice were pooled and plated out (data not shown).

Kinetics of IFN-γ response following one-dose oral immunization. At various times after mice were primed orally with a single dose of 10^4 live bacteria, spleen, PP, and MLN cells were stimulated in vitro with soluble Ag and 72 h later, culture supernatants were harvested and assayed for IFN-γ. The results are shown in Fig. 2. An IFN-γ response to primary immunization was detected in PP cells 1 week following immunization. The response peaked at 3 weeks and went down to baseline levels between 6 and 9 weeks. Only small amounts of IFN-γ were detected in MLN cultures, and the response was more transient in MLN than in PP. The primary response lasted longer in the spleen, dying out only by 9 to 13 weeks following oral immunization. In the absence of added sonicate Ag, IFN-γ levels were <10 pg/ml. Cells from unimmunized mice that were stimulated with Ag in culture also secreted <10 pg of IFN-γ per ml. Culture supernatants from all time points shown in Fig. 2 were tested for IL-4; levels ranged from 1 to 6 pg/ml (very close to the detection limit) whether the supernatants were harvested 48, 60, or 72 h after culture initiation, and there were no significant differences between levels seen in spleen, PP, and MLN cultures. When similar cultures were set

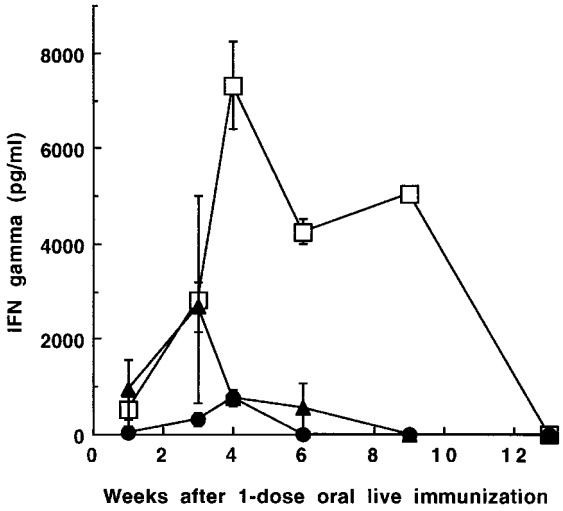


FIG. 2. Kinetics of IFN-γ response in spleen (□), PP (▲), and MLN (●) following one-dose OL priming. Results for one experiment, representative of two, are shown. Four to six mice were used per group.

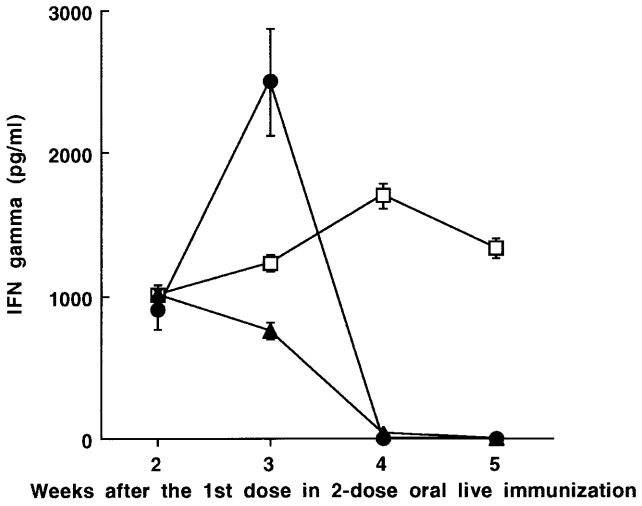


FIG. 3. Kinetics of IFN-γ response in spleen (□), PP (▲), and MLN (●) following two dose OL priming. Results for one experiment, representative of two, are shown. Five mice were used per group.

up with cells from mice immunized i.p. with live bacteria, there was no detectable IFN-γ in supernatants of PP and MLN cultures (<10 pg/ml; data not shown).

Kinetics of IFN-γ response following two-dose oral immunization. As shown above, the IFN-γ response following oral immunization was more sustained in the spleen than in the PP or MLN. Since bacteria administered orally seemed to shoot through PP without replicating at this site, and since bacterial loads were maintained at higher levels and for longer periods in the spleen than in the intestinal lumen, PP, or MLN (Fig. 1), it appeared possible that bacterial loads were linked to the IFN-γ response. Also, spleen cells from mice immunized i.p. with two doses of live Stm 754 (10^2 followed by 10^3 cells a week later) secreted more IFN-γ (2,996 ± 354 pg/ml) when stimulated with sonicate in culture 4 weeks after immunization than did spleen cells from mice immunized with a single dose of 10^2 (280 ± 48 pg/ml) or 10^3 (405 ± 56 pg/ml) cells. Thus, a two-dose oral immunization schedule was attempted. When mice were primed orally with 10^4 live bacteria, followed a week later with 10^5 bacteria, smaller amounts of IFN-γ were secreted overall, and there was no detectable response in PP or MLN 4 weeks after the first dose (Fig. 3) as opposed to >6 weeks following immunization with a single dose (Fig. 2), suggesting that the continuous presence of large numbers of bacteria in the intestinal lumen might dampen IFN-γ responses. This pattern was seen even when the doses used for oral priming were varied (Fig. 4). Mice were primed with either 10^4 and 10^5, 10^4 and 10^6, or 10^5 and 10^6 live bacteria 1 week apart, and spleen, PP, and MLN cells were stimulated with Ag in vitro 4 weeks after the first dose. Neither PP nor MLN cultures made any IFN-γ at this time, as was seen earlier (Fig. 3), and the negative data are not included in Fig. 4. However, spleen cells from mice primed with a single dose of live *S. typhimurium* orally secreted significantly larger amounts of IFN-γ when stimulated with Ag in culture than did cells from mice primed with any two-dose OL combination or i.p. immunization (Fig. 4). Very small amounts of IFN-γ were present in supernatants of spleen cell cultures following killed-cell immunization by the oral and parenteral routes, indicating that live bacteria are better than killed bacteria at priming for IFN-γ responses. In the absence of added Ag, IFN-γ levels in culture supernatants were <10 pg/ml.

Importance of CD4 T cells in the anti-*S. typhimurium* response. To establish that the anti-*S. typhimurium* response was mediated by CD4 T cells, 10 μg of azide-free anti-CD4 or anti-CD8 Ab (clones RM-5 and 53-6.7, respectively; Pharmingen) per ml was added (11, 31) to cultures of spleen, PP, and MLN cells obtained from mice primed orally with a single dose of 10^4 live bacteria 4 weeks earlier; the results are shown in Fig. 5. Ag-dependent proliferation (Fig. 5A), as well as secretion of both IFN-γ (Fig. 5B) and IL-4 (Fig. 5C), decreased significantly in the presence of anti-CD4, while the responses were largely unaffected in the presence of anti-CD8. An isotype-matched Ab (anti-B220, clone RA3-6B2; Pharmingen) was added to control cultures. Culture supernatants contained <10 pg of IFN-γ and <2 pg of IL-4 per ml if Ag was not added.

Generation of secondary IFN-γ responses in PP and MLN. Mice were immunized either with 10^4 live cells orally or with 10^2 live cells i.p. Seventeen weeks later, well after the primary response had died down, they were challenged with either 10^8 live bacteria orally or 2×10^5 live bacteria i.p. (approximately

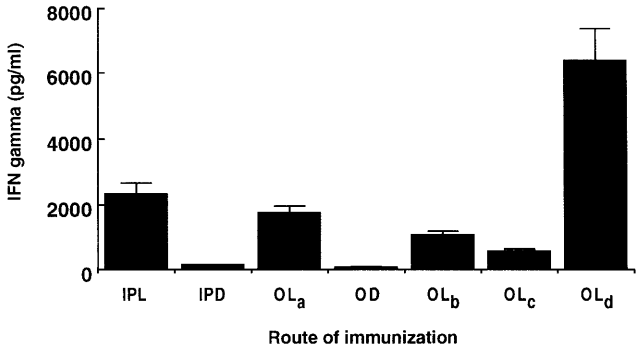
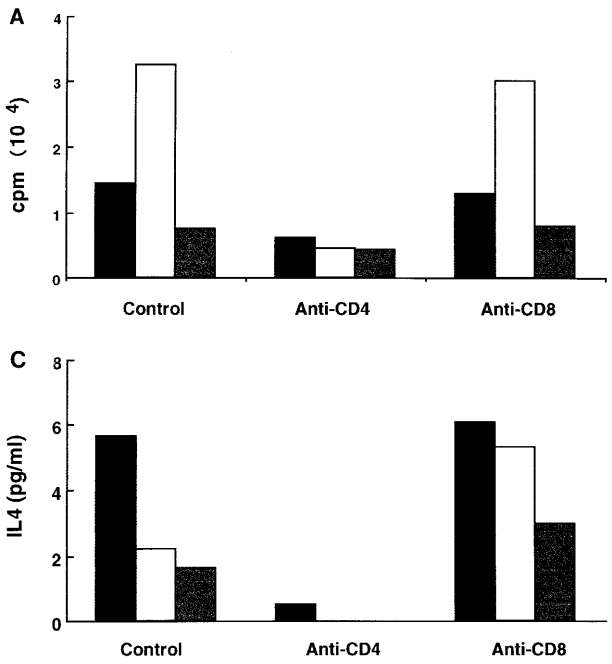


FIG. 4. IFN-γ responses in spleens of mice primed with two doses of bacteria 1 week apart. IPL, two-dose i.p. immunization with live bacteria; IPD, two-dose i.p. immunization with dead bacteria; OL_a; two-dose (10^4 and 10^5 cells) oral immunization with live bacteria; OD, two-dose oral immunization with dead bacteria; OL_b, two-dose (10^4 and 10^6 cells) oral immunization with live bacteria; OL_c, two-dose (10^5 and 10^6 cells) oral immunization with live bacteria; OL_d, one-dose (10^4 cells) oral immunization with live bacteria. Results for one experiment, representative of two, are shown. Four mice were used per group.



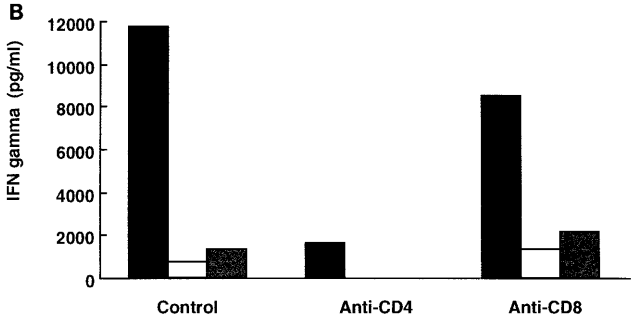


FIG. 5. Proliferation (A), IFN-γ responses (B), and IL-4 responses (C) of spleen (■), PP (□), and MLN (▩) cells from orally primed mice cultured in the presence of either anti-CD4 or anti-CD8 Ab. Results for one experiment, representative of two, are shown. Tissues from three to five mice were pooled. The SE for triplicates was <10% of the mean.

10 LD_{50} by the respective routes). One week later, spleen, PP, and MLN cells were harvested and cultured with soluble Ag, and the IFN-γ responses are shown in Fig. 6. When orally immunized mice were challenged orally, a secondary IFN-γ response was elicited in PP and MLN and there was a small response in spleen (A). On the other hand, when orally immunized mice were challenged i.p. (B), a secondary IFN-γ response was seen in spleen, and although there was a small response in MLN, there was no detectable response in PP. When i.p.-immunized mice were challenged i.p. (C), the secondary response was mainly in the spleen. Interestingly, when i.p.-immunized mice were challenged orally (D), IFN-γ responses could be elicited in both PP and MLN but not in the spleen. A possible explanation for the poor splenic response is that bacteria had not spread to the spleen 1 week after oral challenge. To address this issue, various tissues from this group of mice were plated out on salmonella-shigella agar, and it was observed that bacteria were present in the spleen, although in smaller numbers than in the MLN (950 ± 1,700 CFU as opposed to 8,240 ± 3,250 CFU). No IFN-γ response was seen when cells from unchallenged mice were stimulated with Ag in vitro (E), and oral challenge at 17 weeks with killed bacteria did not elicit any IFN-γ response (data not shown). Immunized mice survived in the long term and were sacrificed 10 weeks after challenge (there were four mice per group and two groups: orally immunized mice challenged orally and i.p.-immunized mice challenged i.p.). In contrast, all unimmunized controls died within 10 days following either i.p. or oral challenge (six and five mice per group, respectively).

Response of T cells in the LP. CD4 T cells from the LP of mice immunized 3, 6, and 9 weeks earlier (cells from two to three mice per group were pooled) were cultured with 2×10^5 irradiated syngeneic splenocytes or various numbers of epithelial cells obtained from the LP preparation (10^5 to 10^6 cells per well) and various doses of sonicate or killed Stm 754 cells. However, no response to Ag, either soluble or particulate, was seen in any group; the cells neither proliferated nor secreted IFN-γ or IL-4 (data not shown). Since conditions optimal for the triggering of LP T cells in vitro are not known, concanavalin A stimulation, known to work under less stringent conditions, was attempted with LP cells obtained from mice immunized either 4 or 9 weeks earlier. As shown in Table 1, LP cells from immunized mice secreted large amounts of IFN-γ (25 ng/ml) in response to concanavalin A stimulation, compared with cells from naive mice (0.2 ng/ml), and the response decreased over time, as shown by the 20-fold drop in the levels from 4 to 9 weeks following oral immunization. IL-4 responses at 4 weeks were similar (175 to 180 pg/ml) whether mice were primed orally or i.p., but by 9 weeks, the levels dropped to those seen in naive mice. Thus, T cells in the LP of immunized mice were predominantly of the Th1 type, as judged by polyclonal T-cell stimulation, and i.p. immunization was as effective at enhancing the IFN-γ response of CD4 T cells in the LP as oral immunization was.

DISCUSSION

Earlier studies have shown that T cells and IFN-γ are important in early defense against *Salmonella* infections. Trans-

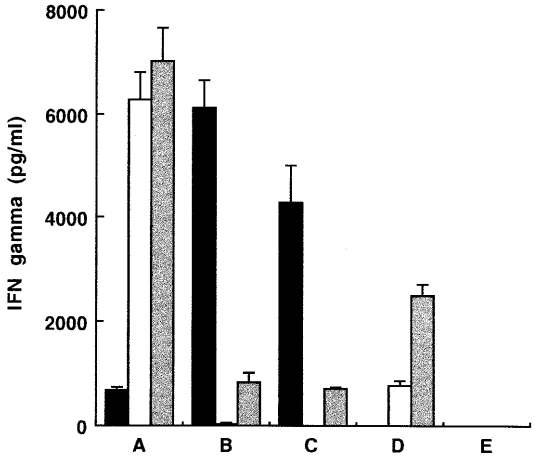


FIG. 6. Secondary IFN-γ responses in spleen (■), PP (□), and MLN (▩) of mice primed orally with 10^4 bacteria (A, B, and E) or i.p. with 10^2 bacteria (C and D). The mice were challenged orally (A and D) or i.p. (B and C) at 17 weeks with 10 oral or i.p. LD_{50}s of Stm 754. Control mice (E) were not challenged before cells were stimulated in culture. Results for one experiment, representative of two, are shown. Cells from two to three mice were pooled. The SE of triplicate supernatants was <10% of the mean.

TABLE 1. Response of LP CD4 cells to concanavalin A stimulation[a]

Type of immunization (wks elapsed)	IFN-γ (pg/ml)	IL-4 (pg/ml)	IFN-γ/IL-4 ratio
OL (4)	25,810	175	147
OL (9)	1,250	35	35.7
IPL[b] (4)	24,980	180	138
None	238	30	7.9

[a] Results for one experiment, representative of two, are shown. The SE of triplicates was <10% of the mean.
[b] IPL, i.p. immunization with live bacteria.

location of *S. typhimurium* from the intestinal lumen into internal tissues increases in mice that are depleted of CD4 or CD8 cells (8), human and mouse epithelial cells and fibroblasts pretreated with IFN-γ are resistant to infection by *S. typhimurium* (5), administration of exogenous IFN-γ to mice has at least a bacteriostatic effect (20), and neutralization of endogenously produced IFN-γ by the administration of specific Ab to mice increases mortality rates (24). Earlier studies (21, 23) have also shown that non-T cells from lymphoid organs of mice primed orally with *S. typhimurium* secrete IFN-γ when cultured with killed bacteria in vitro. This study shows that oral immunization of mice with live *S. typhimurium* generates CD4 T cells in PP and MLN that secrete IFN-γ when stimulated with *Salmonella* sonicate in vitro. On the other hand, oral immunization with killed bacteria, representative of inert Ags, generates no IFN-γ responses in PP or MLN. Thus, as has been shown for systemic Th1 responses (7, 12, 13, 25, 26, 28, 29), the generation of IFN-γ-dominant responses at mucosal sites also depends on the nature of the Ag used for immunization. Environmental signals are known to control the expression of genes that are necessary for the virulence of *S. typhimurium* (27), while the internalization of bacteria into nonphagocytic host cells and their survival within phagosomes may require the coordinate expression of several gene products (1, 9, 16). Thus, only live bacteria, by virtue of their adaptive capabilities, may succeed in presenting Ags appropriate for the generation of a Th1 response.

It is possible that the prolonged IFN-γ response in spleens of orally immunized mice, compared with responses in PP and MLN, is somehow linked to the higher bacterial load seen there following primary immunization and that Th1 cells from other sites accumulate in the spleen. Interestingly, one-dose oral immunization with live *S. typhimurium* generated a higher systemic IFN-γ response than the optimal two-dose i.p. immunization did (Fig. 4), suggesting that, at least in the case of responses directed against bacterial pathogens that invade through the gastrointestinal mucosa, oral immunization may be better than parenteral immunization at priming for a systemic Th1 response. It is stressed that the bacterial strain used in this study is relatively avirulent for BALB/c mice, and this, coupled with the low doses used for oral immunization, could explain the lack of bacterial replication and necrosis in PP and the prolonged IFN-γ responses seen.

An excellent secondary IFN-γ response was observed in PP and MLN of orally immunized mice when they were challenged orally with live *S. typhimurium* over 4 months after initial immunization and assayed a week later (Fig. 6). The secondary response in the MLN contrasts strikingly with the response to primary Ag encounter, which is of very low magnitude and very transient. It is possible that this pattern reflects quick recruitment of effector cells in the primary response to the spleen, where bacterial loads are highest, and of memory cells in the secondary response to the MLN to contain challenge bacteria entering through the PP and the intestinal epithelium.

Only a small secondary response was elicited in the spleens of orally immunized mice following oral challenge (Fig. 6). One explanation for this is that bacteria had not spread to the spleen 1 week after oral challenge; however, when tissues from such mice were plated out, bacteria were found in the spleen, although in smaller numbers than in the MLN. The possibility remains, however, that the magnitude of the secondary IFN-γ response is linked to bacterial loads. The second explanation is that mucosal and systemic memory Th1 cells are restricted to independent compartments, so that memory cells generated in PP cannot be recalled in the spleen. However, i.p. challenge of orally immunized mice elicited a secondary response mainly in the spleen (Fig. 6), while oral challenge of i.p.-immunized mice elicited a secondary response in PP and MLN and not in the spleen. Thus, it would appear that memory T cells are generated following both oral and i.p. immunizations and that they recirculate between mucosal and nonmucosal compartments. Such memory cells can be recalled both at mucosal sites (PP and MLN) and in the spleen, depending on whether the secondary challenge is given orally or i.p., in contrast to the pattern of the primary anti-*Salmonella* response, in which priming of T cells in PP and MLN occurs only if the immunization is oral.

It is not known whether effector CD4 cells primed in the PP accumulate in the LP. This question was addressed by looking at anti-*Salmonella* CD4 T-cell responses in the LP at various times after immunization, but no such responses could be demonstrated in the presence of either splenic Ag-presenting cells or intestinal epithelial cells. However, the cells responded well to concanavalin A stimulation, and cells from immunized mice produced significantly higher levels of IFN-γ than did cells from naive mice (Table 1). The route of immunization did not matter, and the levels of IFN-γ were similar in cultures of LP cells from mice immunized with live bacteria i.p. or orally. Since i.p. immunization elicited a systemic T-cell response in the complete absence of a mucosal T-cell response (Fig. 4 and data not shown), these data hint at the possible migration of effector IFN-γ-secreting CD4 cells to the LP both from mucosal inductive sites and from the spleen. However, there is no direct evidence for this.

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