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# Differential Production of Interleukin-12 mRNA by Murine Macrophages in Response to Viable or Killed *Salmonella* spp.

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**The use of attenuated *Salmonella* spp. as live oral vaccines and as vaccine carriers for foreign antigens has been extensively studied. We have shown that appropriately prepared nonviable organisms are as effective as viable organisms in eliciting humoral immune responses against a foreign antigen delivered by these vectors. It is not clear how strain viability affects the development of a cell-mediated immune response. In the present study, we demonstrate that BALB/c mice orally immunized with viable attenuated *Salmonella* spp. were protected against subsequent challenge while animals immunized with killed organisms were not. Protection was correlated with increased production of interleukin-12 (IL-12) p40 mRNA in the Peyer's patches within hours of oral administration. Peritoneal macrophages from lipopolysaccharide (LPS)-responsive and LPS-unresponsive mice were also examined for production of IL-12 p40 mRNA following exposure to the viable or killed attenuated *Salmonella* carrier. There was dramatic upregulation of IL-12 p40 mRNA following exposure of macrophages to either viable or killed organisms. By 4 h postexposure, viable organisms had induced a 27-fold increase in IL-12 p40 mRNA levels while killed organisms had induced a 9-fold increase in IL-12 p40 mRNA levels. This was observed in macrophages isolated from both LPS-responsive and unresponsive mice. The higher levels of IL-12 induced by viable *Salmonella* spp. may result in the development of a Th1 response and cell-mediated immunity, while the lower levels of IL-12 induced by killed *Salmonella* spp. may not be sufficient to promote a Th1 response.**

*Salmonella* spp. are facultative intracellular bacteria that initially infect the mucosal surfaces of the gut and are able to survive within macrophages. These organisms are taken up by microfold cells (M cells), which overlie Peyer's patches in the small intestine (17, 21). *Salmonella* spp. can be transported through the M cells to the Peyer's patches, where macrophages phagocytose the invading organisms. Some of these organisms will be processed by the macrophages and bacterial antigens presented to T and B cells also present within the Peyer's patches. The primed B cells then migrate to the mesenteric lymph nodes, where they undergo differentiation, enter the general circulation via the thoracic duct, and subsequently populate the lamina propria, where clonal expansion and terminal differentiation into plasma cells occur (3). However, some *Salmonella* spp. are able to survive and multiply within macrophages and therefore are resistant to humoral immunity. Macrophages can transport these bacteria via the same route to the general circulation, resulting ultimately in the development of enteric fever. In this scenario, cell-mediated immunity (especially T-cell help) is important for protection against typhoid fever.

Helper T-cell responses can be divided into two classifications, Th1 and Th2, which differentially regulate the type of immune response and are defined by the profile of cytokines secreted (19, 20). Th1 cells secrete gamma interferon, interleukin-2 (IL-2), and tumor necrosis factor beta, which provide help for cell-mediated immunity. Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13, which support the humoral arm of the immune response. Factors which promote the development of Th1 and Th2 responses are of great interest, since the production of such factors could essentially dictate the type of immune response which develops. Recent evidence indicates that IL-12 is one such factor and that it plays a crucial role in the development of cell-mediated immunity. This heterodimer is composed of two subunits, designated p35 and p40, which together promote the development of Th1 cells from precursors (i.e., Th0 cells) (14). This cytokine plays a central role in protection against a variety of bacterial and parasitic diseases on the basis of this ability to promote the development of Th1 cells (13).

In humans, immunologic protection against typhoid fever, caused by *Salmonella* spp., can be achieved by parenteral immunization with acetone-killed whole cells (1) or purified capsular polysaccharide (22). Protection can be achieved by oral immunization with live attenuated vaccines such as *Salmonella typhi* Ty21a (10, 11, 16, 27). However, killed oral typhoid vaccines are not effective at inducing protective anti-typhoid immunity (7, 8). The reason for this difference in the ability of orally administered viable or killed organisms to elicit a protective response is not clear. A reasonable hypothesis is that viable organisms are able to induce some Th1-mediated cellular immunity which is not induced by orally administered killed organisms, possibly related to production of IL-12.

The purpose of this study was to use a murine model of salmonellosis to investigate the role of IL-12 in the protection derived from oral immunization with viable organisms. In the present study, we investigate the possibility that direct interaction between *Salmonella* spp. and murine macrophages results in the production of IL-12.

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## MATERIALS AND METHODS

**Animals.** Female BALB/c and C3H/HeN mice were purchased from Charles River, and C3H/HeJ mice were purchased from Jackson Laboratory. All mice were 8 to 10 weeks of age. Mice were housed in filter-topped cages containing sterile bedding and fed sterile food and water. They were not treated with antibiotics.

**Bacterial strains.** *S. dublin* SL1438 is a nonreverting, aromatic-dependent, histidine-requiring mutant (kindly provided by Bruce Stocker, Stanford University School of Medicine). Overnight cultures of strain SL1438 were diluted in Luria broth supplemented with 1 μg of dihydroxybenzoic acid per ml and incubated at 37°C until mid-log phase. The bacteria were harvested by centrifugation and resuspended in sterile normal saline. To produce nonviable SL1438, bacteria were resuspended in 10 ml of 70% ethanol and incubated at 4°C overnight. The bacterial suspension was harvested and resuspended in sterile normal saline (5, 6). Posttreatment viability was examined by plating serial dilutions on Luria broth agar supplemented with dihydroxybenzoic acid. *S. dublin* SL1363 is the wild-type parent (S4454) of *S. dublin* SL1438 (24).

**Oral immunization and challenge.** Groups of BALB/c mice were immunized orally on days 0 and 7 with doses containing $10^{10}$ CFU each of either viable or ethanol-killed SL1438. Inocula for immunizations were prepared from log-phase cultures, resuspended in sterile normal saline to a final volume containing $2 \times 10^{10}$ CFU/ml, and administered intragastrically in 0.5-ml doses via a feeding tube. Mice were challenged orally on day 14 with doses containing $10^7$ SL1363 cells prepared from log-phase cultures and resuspended in sterile normal saline.

**Isolation and stimulation of macrophages.** Murine peritoneal macrophages were elicited from naive animals by injecting 0.25 ml of incomplete Freund's adjuvant into the peritoneal cavity of each mouse. Three days later, mice were sacrificed by $CO_2$ asphyxiation and peritoneal cells were harvested by lavage with RPMI 1640 (containing L-glutamine) supplemented with 1% heat-inactivated fetal bovine serum (GIBCO BRL, Gaithersburg, Md.). The cells were pelleted by centrifugation and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (complete medium). The cells (3 ml) were dispensed into 25-$cm^2$ tissue culture flasks, and macrophages were allowed to adhere for 30 min in a humidified 5% $CO_2$ incubator at 37°C. Nonadherent cells were removed, and adherent cells were washed with 4 ml of RPMI 1640. The adherent cells were then incubated in 4 ml of complete medium for 90 min. The yield of adherent cells was approximately $10^7$ cells per flask, and the purity of macrophages was greater than 95% Mac-$1^+$ as determined by fluorescence-activated cell sorting.

Macrophages were incubated with 10 μg of *Escherichia coli* lipopolysaccharide (LPS) (Difco, Detroit, Mich.) per ml, complete medium, or viable or ethanol-killed SL1438 at a multiplicity of infection of 1 for 60 min. The macrophages were then washed three times with phosphate-buffered saline to remove extracellular bacteria and further incubated in complete medium containing 50 μg of gentamicin per ml to kill any remaining extracellular bacteria. At the indicated time points, total RNA was isolated from macrophages.

**RNA isolation and cDNA synthesis.** At either 4 or 24 h postinoculation, macrophages were lysed with a QIAshredder (QIAGEN, Chatsworth, Calif.) and total RNA was isolated as described in the QIAGEN RNeasy kit insert. Total RNA was ethanol precipitated, washed with 75% ethanol, and resuspended in 15 μl of diethylpyrocarbonate-treated double-distilled water. The concentration of total RNA was determined by the Invitrogen DNA Dipstick method.

cDNA was synthesized as follows. A 10-μg portion of total RNA was added to 4 μl of 5× Moloney murine leukemia virus reverse transcriptase (RT) buffer (Promega, Madison, Wis.), 1 μl of deoxynucleoside triphosphate mix (dNTPs; final concentration, 0.5 mM each), 1 μl of random primers (2.5 mM; GIBCO BRL), and 1 μl of Moloney murine leukemia virus RT (RNase H minus [200 U/ml]; Promega). The mixture was incubated at room temperature for 30 min and then at 37°C for 90 min. The transcriptase was inactivated at 95°C for 5 min, and the cDNA was ethanol precipitated and washed with 75% ethanol.

**Oral immunization and removal of Peyer's patches.** Groups of BALB/c mice were immunized orally as above with $10^{10}$ CFU each of either viable or ethanol-killed SL1438. Six hours following oral immunization, the mice were sacrificed. Six to eight Peyer's patches from the entire length of the small intestine were removed from each animal for isolation of total RNA by homogenization in Tri-Reagent (Molecular Research Center, Inc., Cincinnati, Ohio) as described by the manufacturer. cDNA was synthesized as described above.

**RT-PCR analysis.** PCR primer pairs specific for the gene encoding murine IL-12 p40 which amplify a 266-bp fragment were synthesized as described previously (4). cDNA prepared as described above was amplified in 0.6-ml tubes as follows. cDNA and double-distilled water were heated at 75°C in a heat block. A master mix containing 1 μl each of sense and antisense primers (final concentration, 1 ng), 10 μl of 10× *Taq* DNA polymerase buffer, 8 μl of $MgCl_2$ (2 mM), 2 μl each of dNTPs (200 mM each), and 1 μl of *Taq* DNA polymerase (5 U; Promega) was added (total volume, 100 μl) and then layered with mineral oil. PCR was performed in a RoboCycler (Stratagene, La Jolla, Calif.) with an initial window of 3 cycles of 45 s of denaturation at 94°C, 75 s of annealing at 58°C, and 105 s of extension at 72°C followed by 29 cycles of 35 s at 94°C, 45 s at 58°C, and 75 s at 72°C. The reaction products were visualized by electrophoresis of 20 μl of the reaction mixture on a 2% agarose gel containing ethidium bromide. The identity of the amplified band was confirmed by dideoxy sequencing as described previously (4). cDNA was also amplified for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) as a housekeeping gene to control for the efficiency of reverse transcription and amount of input cDNA.

**QC-RT-PCR analysis.** To quantify the amount of RNA in samples, quantitative competitive (QC)-RT-PCR was performed as described previously (4). The IL-12 p40 competitor DNA is 78 bp smaller than the 266-bp IL-12 p40 fragment amplified by RT-PCR and is amplified by the same primer pair. To produce such a competitor, RT-PCR was used to amplify RNA isolated from LPS-activated murine macrophages with PCR primers specific for murine IL-12 p40. To produce a smaller competitor fragment, an internal segment was removed by cleavage with a unique restriction enzyme (*Nla*III). End segments were then isolated and ligated, resulting in the construction of an IL-12 p40 competitor which had 78 bp deleted. Serial dilutions (1:3) of IL-12 p40 competitor DNA starting at 30 pg per reaction were added to individual tubes, each containing the same amount of cDNA from a particular sample, and amplified by PCR. PCR was carried out as described above and analyzed by electrophoresis of 20 μl of the reaction mixture on a 2% agarose gel containing ethidium bromide. Amplification was quantified by visualization. As the amount of competitor decreased, a point of equivalence at which there were equal amounts of sample cDNA and competitor was reached. The point of equivalence was compared for different samples. For some reactions, PCR was carried out in the presence of $^{33}P$-labeled positive-strand primer. Amplification was quantified by visualization and radioactive counts.

## RESULTS

**Survival of BALB/c mice immunized with viable attenuated or killed *Salmonella* spp.** To investigate in an experimental animal model whether viability of the vaccine strain is important for protection against challenge with wild-type *Salmonella* strains, groups of BALB/c mice were orally immunized with $10^{10}$ viable or killed cells of *S. dublin* SL1438 (a nonreverting, aromatic-dependent, histidine-requiring mutant) and subsequently challenged with $10^7$ cells of wild-type parent *S. dublin* SL1363 (approximately 100 50% lethal doses). Groups of mice were orally immunized on days 0 and 7 and then challenged with SL1363 on day 14. By day 6 postchallenge, all mice immunized with killed SL1438 were moribund with ruffled fur whereas all mice immunized with viable SL1438 were healthy. By day 9 postchallenge, 6 of 10 mice immunized with killed SL1438 were dead and the remainder were near death and were sacrificed. In contrast, all mice immunized with viable SL1438 remained healthy. Therefore, in a system analogous to human vaccination against typhoid fever, mice immunized with viable SL1438 were protected against challenge with wild-type SL1363 whereas those immunized with killed SL1438 were not.

**Differential production of IL-12 p40 mRNA by macrophages from BALB/c mice.** To begin the investigation of the cellular basis for the observed difference in immunologic protection against challenge with SL1363, IL-12 p40 mRNA production by macrophages from BALB/c mice was examined following exposure to either viable or killed *Salmonella* spp. Elicited peritoneal macrophages were infected in vitro with either viable or killed SL1438, and at 4 h postinfection total RNA was extracted and reverse transcribed and the levels of IL-12 p40 mRNA were assessed by PCR. As shown in Fig. 1, macrophages infected with viable SL1438 produced qualitatively larger amounts of IL-12 p40 mRNA than did macrophages infected with killed SL1438. As a control, macrophages were treated with either LPS or complete medium. Products of PCR amplification performed for G3PDH revealed similar efficiencies of reverse transcription and equal amounts of input cDNA between samples.

Bioactive IL-12 is secreted as a heterodimer consisting of p35 and p40 subunits. Therefore, a prerequisite for secretion of this cytokine at mucosal sites is the production of mRNA for both subunits. Using RT-PCR, we evaluated the production of IL-12 p35 mRNA by macrophages infected with either viable or killed SL1438, using murine IL-12 p35 specific primers as previously described (4). While it was clear that IL-12 p40

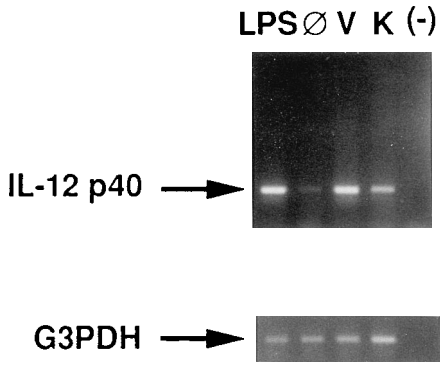


FIG. 1. Induction of IL-12 p40 mRNA by macrophages from BALB/c mice 4 h following exposure to viable attenuated or killed *Salmonella* spp. Peritoneal macrophages from BALB/c mice were incubated with LPS (lane LPS), complete medium (lane Ø), or viable (lane V) or ethanol-killed (lane K) SL1438 at a multiplicity of infection of 1 for 60 min. The macrophages were washed three times with PBS to remove extracellular bacteria and further incubated in complete medium containing gentamicin to kill any remaining extracellular bacteria. At 4 h, total RNA was isolated from macrophages and reversed transcribed, and cDNA from each sample was subjected to PCR for determination of IL-12 p40 mRNA levels. cDNA from each sample was also subjected to PCR amplification for expression of the housekeeping gene, G3PDH.

mRNA production was induced by infection of the macrophages with either viable or killed *Salmonella* spp., this was not the case for IL-12 p35 mRNA, which was constitutively produced by these cells (data not shown). This in vitro finding correlates with our previous demonstration of constitutive IL-12 p35 mRNA production in Peyer's patches and mesenteric lymph nodes from BALB/c mice orally immunized with viable attenuated *Salmonella* spp. (4).

**Differential production of IL-12 p40 mRNA by macrophages from C3H mice 4 h postinfection.** Since LPS is able to induce the expression of IL-12 from macrophages, it was necessary to determine whether the production of IL-12 p40 mRNA was either (i) an LPS-related event or (ii) truly a function of strain viability. Mice from the C3H lineage were used to address these two possibilities. C3H/HeN ($Lps^n$) and C3H/HeJ ($Lps^d$) are isogenic except for the *Lps* allele (28, 30). C3H/HeJ mice are hyporesponsive to the mitogenic and toxic effects of LPS and therefore differ from C3H/HeN mice in their responsiveness to low concentrations of LPS. This difference in responsiveness to LPS could be due to a difference in macrophage activation, even though macrophages from both strains of mice are able to phagocytose *Salmonella* spp. normally (12, 23, 25, 26, 29). In addition, macrophages from these two strains of mice differ in secretion of and responsiveness to various cytokines (2, 9, 18).

Elicited peritoneal macrophages from either C3H/HeN (Fig. 2A) or C3H/HeJ (Fig. 2B) mice were infected in vitro with either viable or killed SL1438, and at 4 h postinfection, total RNA was extracted and reverse transcribed and the levels of IL-12 p40 mRNA were assessed by PCR. Macrophages from both strains of mice infected with viable SL1438 produced qualitatively larger amounts of IL-12 p40 mRNA than did macrophages infected with killed SL1438. Products of the PCR amplification performed for G3PDH revealed similar efficiencies of reverse transcription and equal amounts of input cDNA between samples. As shown in Fig. 2, macrophages from both strains of mice produced IL-12 p40 mRNA in response to high concentrations of LPS (10 μg/ml). At lower concentrations of LPS, there was differential production of IL-12 p40 mRNA by macrophages from these two strains of mice. Macrophages from C3H/HeJ mice did not produce detectable levels of IL-12 p40 mRNA when exposed to 10 ng of LPS per ml, while macrophages from C3H/HeN mice did (data not shown). The higher levels of LPS were used in these experiments to function as a positive control for the PCR amplification. Since macrophages from both LPS-responsive and LPS-unresponsive animals produced IL-12 p40 mRNA in response to *Salmonella* spp. at a multiplicity of infection of 1, it is unlikely that the observed response is solely an LPS-mediated event related to the LPS content of these gram-negative bacteria. Moreover, macrophages exposed to viable SL1438 produced qualitatively larger amounts of IL-12 p40 mRNA than did macrophages exposed to killed SL1438, irrespective of the LPS genotype. This indicates that the difference in production of IL-12 p40 mRNA is most probably a function of strain viability.

**Quantification of *Salmonella*-induced IL-12 p40 mRNA production by macrophages from C3H mice by QC-RT-PCR.** To quantify the differences observed in IL-12 p40 mRNA between macrophages infected with viable or killed SL1438, QC-RT-

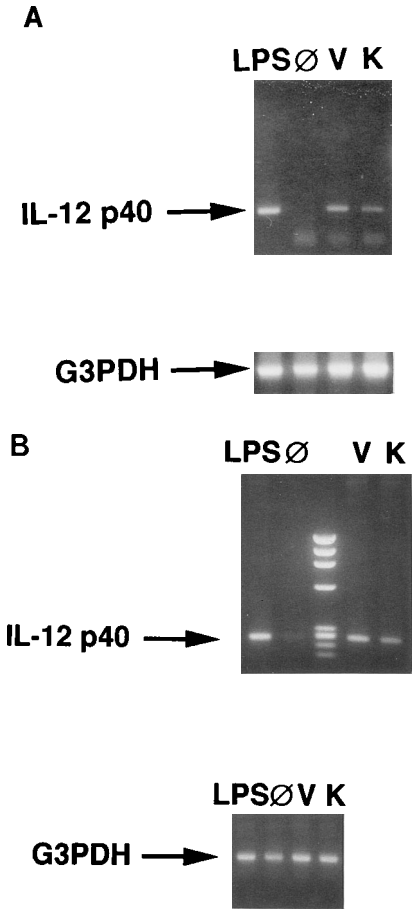


FIG. 2. Induction of IL-12 p40 mRNA by macrophages from C3H mice 4 h following exposure to viable attenuated or killed *Salmonella* spp. Peritoneal macrophages from C3H/HeN (A) or C3H/HeJ (B) mice were incubated with LPS (lanes LPS), complete medium (lanes Ø), or viable (lanes V) or ethanol-killed (lanes K) SL1438 at a multiplicity of infection of 1 for 60 min. The macrophages were washed three times with PBS to remove extracellular bacteria and further incubated in complete medium containing gentamicin to kill any remaining extracellular bacteria. At 4 h, total RNA was isolated from macrophages and reversed transcribed, and cDNA from each sample was subjected to PCR for determination of IL-12 p40 mRNA levels. cDNA from each sample was also subjected to PCR amplification for expression of the housekeeping gene, G3PDH.

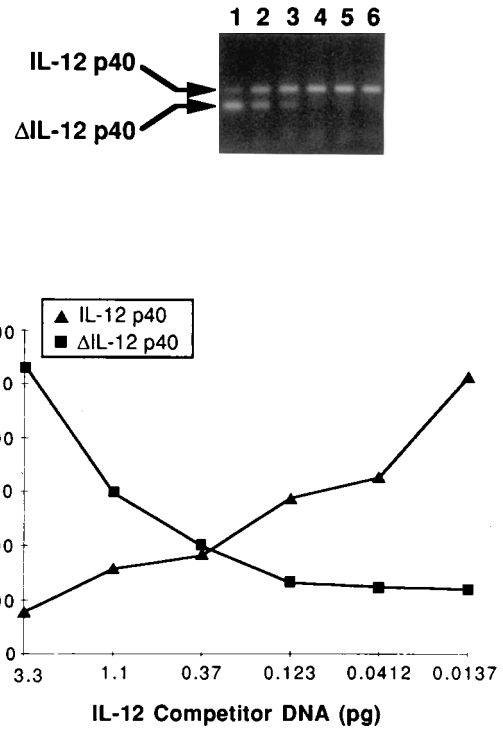


FIG. 3. QC-RT-PCR quantification of *Salmonella*-induced IL-12 p40 mRNA production by macrophages. cDNA samples from macrophages from C3H/HeJ mice infected with viable SL1438 were analyzed by QC-RT-PCR to quantify IL-12 p40 mRNA production. Serial dilutions (1:3) of IL-12 p40 competitor DNA were added to individual tubes, each containing the same amount of sample cDNA, and amplified by PCR in the presence of $^{33}$P-radiolabeled primers. The locations of the 266-bp sample IL-12 p40 mRNA fragment and the 188-bp competitor (ΔIL-12 p40) are shown, and a point of equivalence can be determined.

PCR was performed. A competitor, 78 bp smaller than the 266-bp IL-12 p40 fragment amplified by RT-PCR, was constructed (see Materials and Methods). As shown in Fig. 3, when limiting dilutions of competitor DNA were amplified by PCR in the presence of a constant amount of sample cDNA, it was possible to precisely determine the relative amounts of IL-12 p40 cDNA present in a particular sample. As the amount of competitor DNA was decreased, a point of equivalence representing equal amounts of competitor DNA and IL-12 p40 cDNA present in the experimental sample was reached. From such competition, it was possible to quantify relative differences between IL-12 p40 mRNA production in different samples.

As an example, QC-RT-PCR was used to quantify IL-12 p40 mRNA production in macrophages from C3H/HeJ mice 4 h after infection of the macrophages with viable SL1438. Amplification reactions were performed with radiolabeled primers specific for murine IL-12 p40. Limiting dilutions of competitor DNA were amplified in the presence of a constant amount of IL-12 p40 cDNA. As shown in Fig. 3, a 266-bp IL-12 p40 fragment readily competed in a concentration-dependent manner for amplification with the competitor in the macrophages infected with viable SL1438. Since QC-RT-PCR was performed in the presence of radiolabeled primers, it was possible to quantify the amount of amplified IL-12 p40 cDNA by scintillation counting. The amplified fragments were excised, radioactivity was counted, and the results were plotted against the concentration of competitor DNA added. For macrophages infected with viable SL1438, the point of equivalence was 0.37 pg. By visual inspection, the point of equivalence was lane 2, an indication of the greater sensitivity obtained with radiolabeled primers in this system. This offset between visual inspection and radiolabeled primers was consistent, allowing differences in IL-12 mRNA production to be determined by visualizing ethidium bromide-stained gels.

To quantify the difference in IL-12 p40 mRNA production between macrophages from different strains of C3H mice infected with either viable or killed SL1438, serial threefold dilutions of IL-12 p40 competitor DNA were added to individual tubes, each containing the same amount of cDNA from a particular sample, and PCR was performed. Macrophages from both C3H/HeN (Fig. 4A) and C3H/HeJ (Fig. 4B) mice infected with viable SL1438 had a point of equivalence in lane 3, and macrophages infected with killed SL1438 had a point of equivalence threefold lower (lane 4). Compared with control macrophages (data not shown), macrophages from both C3H/HeN and C3H/HeJ mice infected with viable SL1438 produced approximately 27-fold more IL-12 p40 mRNA (Table 1). Macrophages from both strains of mice infected with killed SL1438 produced approximately ninefold more IL-12 p40 mRNA than did control macrophages. Since the relative production of

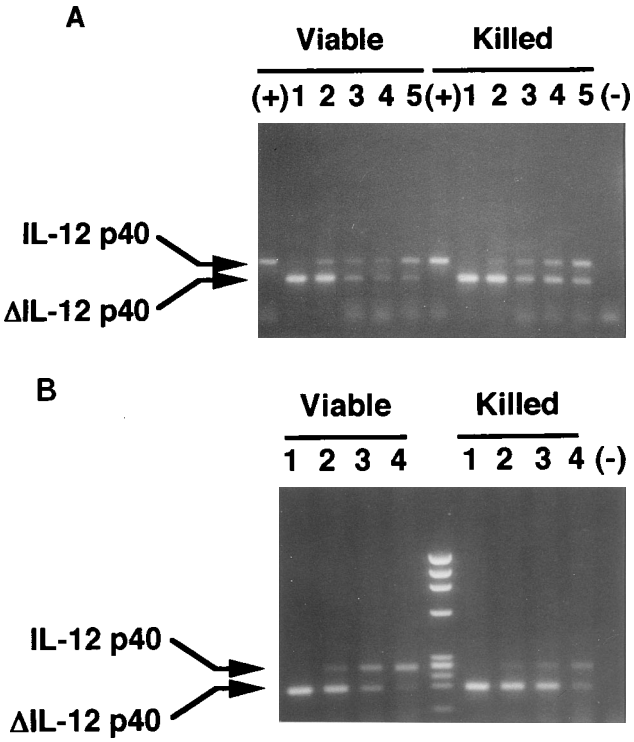


FIG. 4. QC-RT-PCR for the production of IL-12 p40 mRNA by macrophages from C3H mice 4 h following exposure to viable attenuated or killed *Salmonella* spp. cDNA samples from C3H/HeN (A) or C3H/HeJ (B) mice remaining after RT-PCR had been performed for the experiment in Fig. 2 were also analyzed by QC-RT-PCR to quantify IL-12 p40 mRNA production. Serial dilutions (1:3) of IL-12 p40 competitor DNA starting at 30 pg per reaction (lane 1) were added to individual tubes each containing the same amount of cDNA from a particular sample and PCR amplified. The locations of the 266-bp sample IL-12 p40 mRNA fragment and the 188-bp competitor (ΔIL-12 p40) are shown, and a point of equivalence can be determined. Macrophages from both C3H/HeN (A) and C3H/HeJ (B) mice infected with viable SL1438 had a point of equivalence in lane 3, and macrophages infected with killed SL1438 had a point of equivalence approximately threefold lower in lane 4. In addition, a negative control is shown (−) in which DNA was omitted from the PCR, as well as a sample without competitor (+).

TABLE 1. Increases in IL-12 p40 mRNA production by macrophages from C3H mice exposed to viable attenuated or killed *Salmonella* spp. as determined by QC-RT-PCR[a]

| Time post-exposure (h) | Strain | Treatment | Relative increase in IL-12 p40 mRNA level |
|---|---|---|---|
| 4 | C3H/HeN | Medium | 1 |
| | | Viable | 27 |
| | | Killed | 9 |
| | C3H/HeJ | Medium | 1 |
| | | Viable | 27 |
| | | Killed | 9 |
| 24 | C3H/HeN | Medium | 1 |
| | | Viable | 3 |
| | | Killed | <3 |
| | C3H/HeJ | Medium | 1 |
| | | Viable | <3 |
| | | Killed | <3 |

[a] Peritoneal macrophages were exposed to either complete medium, viable attenuated SL1438, or killed SL1438 at a multiplicity of infection of 1 for 60 min. The macrophages were washed three times with PBS to remove extracellular bacteria and further incubated in complete medium containing gentamicin to kill any remaining extracellular bacteria. At 4 or 24 h, total RNA was isolated from macrophages and reversed transcribed, and cDNA from each sample was subjected to QC-RT-PCR. Amplification was quantified by visualization. As the amount of competitor decreased, a point of equivalence was reached in which there were equal amounts of sample cDNA and competitor. The point of equivalence was compared between different samples, with IL-12 p40 mRNA from macrophages exposed to complete medium arbitrarily set at 1.

IL-12 p40 mRNA by both groups of macrophages was the same, it can be concluded that the differential production seen as a result of infection with viable or killed SL1438 is also not due to the presence of LPS on these gram-negative organisms.

**Quantification of IL-12 p40 mRNA produced by macrophages from C3H mice 24 h postinfection.** To determine whether the observed increase in IL-12 p40 mRNA is sustained over time, macrophages from C3H/HeN or C3H/HeJ mice were infected in vitro with either viable or killed SL1438 and at 24 h postinfection, total RNA was extracted and reverse transcribed and the levels of IL-12 p40 were assessed by QC-RT-PCR. Macrophages from C3H/HeN and C3H/HeJ mice infected with viable or killed SL1438 produced quantitatively similar amounts of IL-12 p40 mRNA (Table 1). Compared with control macrophages, there was an approximately threefold increase in levels of IL-12 p40 mRNA. Therefore, the dramatic and rapid upregulation of IL-12 p40 mRNA observed at 4 h postexposure is a transient event.

**Differential production of IL-12 p40 mRNA in Peyer's patches from BALB/c mice 6 h postimmunization.** Macrophages from both BALB/c and C3H mice infected in vitro with viable *Salmonella* spp. produced quantitatively larger amounts of IL-12 p40 mRNA than did macrophages infected with killed *Salmonella* spp. This difference in the ability of viable and killed *Salmonella* spp. to induce production of IL-12 p40 mRNA may be related to the observed difference in immunologic protection against challenge. To determine if there was an in vivo correlate of this differential IL-12 p40 mRNA production, Peyer's patches from BALB/c mice orally immunized with either viable or killed SL1438 were examined. Groups of mice were orally immunized with either viable or killed SL1438 and sacrificed 6 h postimmunization. Total RNA was extracted from the Peyer's patches and reverse transcribed, and the levels of IL-12 p40 mRNA were assessed by PCR. As shown in Fig. 5, Peyer's patches from mice immunized with viable SL1438 produced qualitatively larger amounts of IL-12 p40 mRNA than did Peyer's patches from mice immunized with killed SL1438. Products of the PCR amplification performed for G3PDH revealed similar efficiencies of reverse transcription and equal amounts of input cDNA between samples. As previously observed (4), IL-12 p35 mRNA was constitutively produced by Peyer's patches from these mice (data not shown).

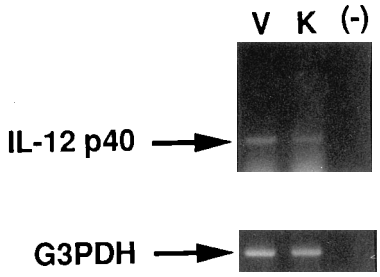


FIG. 5. Induction of IL-12 p40 mRNA in Peyer's patches from BALB/c mice 6 h following exposure to viable attenuated or killed *Salmonella* spp. Groups of mice were orally immunized with $10^{10}$ CFU of either viable or ethanol-killed SL1438 and sacrificed 6 h postimmunization. Total RNA was extracted from the Peyer's patches and reverse transcribed, and the levels of IL-12 p40 mRNA were assessed by PCR. cDNA from each sample was also subjected to PCR amplification for expression of the housekeeping gene, G3PDH.

The difference in IL-12 p40 mRNA production was quantified by QC-RT-PCR. As shown in Fig. 6, there was a threefold difference between the levels of IL-12 p40 mRNA production in Peyer's patches from mice immunized with viable and killed *Salmonella* spp. This is the same as the difference seen with macrophages in vitro.

## DISCUSSION

Recently, we reported the results of a study in which mice orally immunized with *S. dublin* EL23, a nonreverting, aromatic-dependent, histidine-requiring mutant transformed with a plasmid which carries a gene that codes for production of the B subunit of the heat-labile toxin (LT-B) of enterotoxigenic *E. coli*, were analyzed for their ability to initiate production of IL-12 mRNAs at mucosal sites (4). At 6 or 20 h following oral inoculation, the Peyer's patches and mesenteric lymph nodes

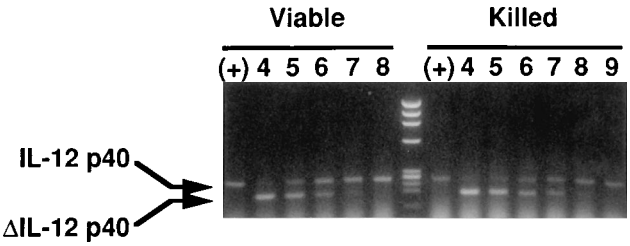


FIG. 6. QC-RT-PCR for the production of IL-12 p40 mRNA in Peyer's patches from BALB/c mice 6 h following exposure to viable attenuated or killed *Salmonella* spp. cDNA samples from BALB/c mice remaining after RT-PCR had been performed for the experiment in Fig. 5 were also analyzed by QC-RT-PCR to quantify IL-12 p40 mRNA production. Serial dilutions (1:3) of IL-12 p40 competitor DNA starting at 1.1 pg per reaction (lanes 4) were added to individual tubes each containing the same amount of cDNA from a particular sample and PCR amplified. The locations of the 266-bp sample IL-12 p40 mRNA fragment and the 188-bp competitor (ΔIL-12 p40) are shown, and a point of equivalence can be determined. There is a threefold difference between the levels of IL-12 p40 mRNA production in Peyer's patches from mice immunized with viable and killed *Salmonella* spp.

were removed and poly(A)$^+$ mRNA was prepared from each tissue. Constitutive production of an mRNA encoding the p35 subunit of IL-12 was observed in control as well as immunized mice. Conversely, production of an mRNA encoding the p40 subunit of IL-12 was not detected in control animals but was dramatically upregulated in orally inoculated mice. By using QC-RT-PCR, differences in the magnitude of IL-12 p40 mRNA production were quantified. At 6 h after oral inoculation with the *Salmonella* construct, mice had 12.1- and 8.4-fold increases in IL-12 p40 mRNA levels in the Peyer's patches and mesenteric lymph nodes, respectively, compared with control mice. By 20 h, the pattern of increased mRNA production was reversed, showing 2.5- and 17.6-fold increases in the Peyer's patches and mesenteric lymph nodes, respectively. One likely possibility is that the increased production of IL-12 p40 mRNA in the Peyer's patches and mesenteric lymph nodes results from interactions between *Salmonella* spp. and macrophages in these tissues.

In the study reported here, we focused on the ability of viable or killed *S. dublin* to elicit production of IL-12 p40 mRNA in Peyer's patches and in macrophages from LPS-responsive and LPS-unresponsive mice. While the data presented are representative, RT-PCR for IL-12 p40 mRNA has been performed on elicited peritoneal macrophages a total of 18 times and QC-RT-PCR has been performed a total of 6 times. Taken together, the following conclusions can be reached. Exposure of macrophages to either viable or killed organisms resulted in a dramatic and rapid upregulation of IL-12 p40 mRNA when compared with that in control macrophages. By 4 h postexposure, viable organisms had induced a 27-fold increase in IL-12 p40 mRNA levels while killed organisms had induced a 9-fold increase. This was observed in macrophages isolated from both LPS-responsive and LPS-unresponsive mice. By 24 h postexposure, the levels of IL-12 p40 mRNA had decreased to less than threefold above control level in macrophages from both strains of mice. The observed increase in IL-12 p40 mRNA levels is unlikely to be solely LPS mediated, for the following reasons: (i) exposure of murine macrophages to *Listeria monocytogenes* (a gram-positive facultative intracellular pathogen) results in production of IL-12 (14), and (ii) the IL-12 p40 mRNA level increased in macrophages from both C3H/HeN (*Lps*$^n$) and C3H/HeJ (*Lps*$^d$) mice. As noted previously, C3H/HeJ mice are hyporesponsive to the mitogenic and toxic effects of LPS and therefore differ from C3H/HeN mice in their responsiveness to low concentrations of LPS. This difference in responsiveness to LPS could possibly be due to a difference in macrophage activation, even though macrophages from both strains of mice are able to phagocytose *Salmonella* spp. normally (12, 23, 25, 26, 29). In addition, macrophages from these two strains of mice differ in secretion of and responsiveness to various cytokines (2, 9, 18).

These in vitro data were supported by results of in vivo studies in which BALB/c mice were immunized orally with viable or killed SL1438 and Peyer's patches were examined 6 h later for the production of IL-12 p40 mRNA. The difference in IL-12 p40 mRNA production was quantified by QC-RT-PCR. There was a threefold difference in the levels of IL-12 p40 mRNA production in Peyer's patches from mice immunized with viable and killed *Salmonella* spp. This is the same as the difference seen with macrophages in vitro.

The temporal shift in production of IL-12 p40 mRNA levels observed both in vivo (from Peyer's patches at 6 h postinoculation [reported above and in reference 4] to mesenteric lymph nodes at 20 h post-inoculation) and in vitro (elevated expression in macrophages at 4 h postexposure and near-baseline levels by 24 h postexposure) is consistent with our understanding of the pathogenesis of these organisms. Taken together, these results suggest that when *Salmonella* spp. contact macrophages in the Peyer's patches, an immediate upregulation of IL-12 expression results. These organisms subsequently contact macrophages in the mesenteric lymph nodes as they follow their natural progression in the disease.

We have recently demonstrated an in vivo correlate of these findings by depletion of IL-12 in unimmunized BALB/c mice by using an anti-IL-12 monoclonal antibody prior to oral challenge with wild-type *Salmonella* spp. (15). Mice pretreated with anti-IL-12 antibody had increased salmonellosis and reduced survival times compared with mice receiving control antibody. Furthermore, administration of exogenous murine recombinant IL-12 dramatically increased survival times of mice challenged orally with wild-type *Salmonella* spp. These results demonstrate that endogenous and exogenous IL-12 significantly augment the mucosal immune response against intracellular pathogens such as *Salmonella* spp.

Coupled with our previous findings, the present study begins to explain at a molecular level how orally administered viable *Salmonella* spp. might stimulate cell-mediated immunity to a greater extent than orally administered killed organisms. Specifically, the higher levels of IL-12 induced by viable *Salmonella* spp. may result in the development of a Th1 response and cell-mediated immunity. In contrast, the lower levels of IL-12 induced by killed *Salmonella* spp. may not be sufficient to promote a Th1 response, resulting in failure to protect against challenge with virulent wild-type organisms. These findings provide insight into a possible mechanism for anti-typhoid immunity induced by oral immunization with viable *Salmonella* spp.

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