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Expression of the Murine Interleukin-4 Gene in an Attenuated *aroA* Strain of *Salmonella typhimurium*: Persistence and Immune Response in BALB/c Mice and Susceptibility to Macrophage Killing

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Cytokines are potentially useful in vaccination as adjuvants or modulators of the type of response induced. The work below describes the expression of a cloned cytokine gene for murine interleukin-4 (mIL-4) by a live vaccine vector, an attenuated *aroA* strain (SL7207) of *Salmonella typhimurium*, in a murine model system. SL7207 was used as a carrier for two different high-level expression vectors. Both resulting strains, designated SL7207(pOmpAmIL-4) and SL7207(pKKmIL-4), expressed the cloned gene product as monitored by both immunological and biological assays. However, SL7207(pOmpAmIL-4) produced mIL-4 at higher levels and was more stable in vitro than SL7207(pKKmIL-4). When SL7207(pOmpAmIL-4) was used as a live vaccine in BALB/c mice, this strain grew and survived at higher levels than the parental attenuated strain or empty plasmid-carrying strain in spleens, livers, and intestines. This difference in growth and survival did not appear to be caused by alterations in specific lymphocyte-mediated anti-*Salmonella* immune responses such as delayed-type hypersensitivity or serum antibody as measured by enzyme-linked immunosorbent assay; such alterations have been induced by IL-4 administration in other in vivo systems, and the lack of effect here may reflect the fact that IL-4 is not secreted from the bacteria in large quantities, most of the cytokine being in the cytoplasmic-membrane-bound fraction. Conversely, the ability of mouse macrophages to kill the bacteria in vitro was inhibited by bacterial production of mIL-4. This reduction in macrophage killing activity suggests that bacterial production of mIL-4 may be detrimental to host defense against *Salmonella* infection and may explain the enhanced bacterial growth and survival in vivo.

Attenuated strains of *Salmonella typhimurium* recently have received much attention for their potential use as live vaccines against virulent *Salmonella* infections and also as live vaccine vectors carrying cloned genes of protective protein antigens of other pathogenic agents. Examples of the latter include various proteins from *Escherichia coli* (6, 11–13, 32, 44), streptococcal M protein (54), dengue virus envelope antigen (16), *Plasmodium* sporozoite antigen (56), a peptide of the β subunit of cholera toxin (51), surface protein (gp63) of *Leishmania major* (62), fragment C of tetanus toxin (20), influenza A virus nucleoprotein (60), and hepatitis B virus antigens (57). Studies in animal models have demonstrated that immunization with these heterologous vaccines is an effective and safe way to induce both mucosal and serum antibodies against both *Salmonella* and foreign antigens. *aroA* mutants have a defect in aromatic amino acid synthesis caused by a deletion in the gene (30). They are dependent on the mammalian host for these amino acids and also require *para*-aminobenzoic acid (PABA), which is not present in mammals. Thus, while the bacteria will colonize a mammalian host, their potential for growth is severely limited, particularly by their supplies of PABA. Their virulence is therefore greatly reduced; for example, the lethal dose for an intravenous (i.v.) injection into mice is lowered from a few organisms for the wild-type strain to the order of 10^5 to 10^6 (30).

The immune system can respond to different types of bacterial, viral, and parasitic infections with a variety of effector mechanisms, which have varying degrees of effectiveness against each pathogen. For instance, while circulating antibodies may be effective against extracellular bacteria and act to neutralize exotoxins (21, 34), some viruses will be eliminated only by a cytotoxic T-cell response (59), whereas intracellular bacteria or parasites within phagocytes might require a response which would activate macrophages to kill the intracellular pathogen (33, 42, 43). A major part of the regulation of effector function appears to be carried out by T cells. T-helper-cell responses have been divided recently into two types, Th_1 and Th_2, which appear to regulate the type of immune response and can be distinguished on the basis of cytokines secreted by those cells (48). Th_1 cells secrete gamma interferon (IFN-γ), interleukin-2 (IL-2), and lymphotoxin, whereas Th_2 cells secrete IL-4, IL-5, and IL-10 (48, 49). Both types of cells secrete other cytokines such as IL-3 and granulocyte-macrophage colony-stimulating factor (49). Th_1 cells are involved in providing help for macrophage activation (23–25) and delayed-type hypersensitivity (DTH) responses (10), whereas Th_2 cells are thought

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to be more important in providing help for antibody responses (5, 14, 36, 61).

When vaccinating against a particular organism, it is of obvious importance to induce the appropriate type of immune response to provide protection against that pathogen. Cytokines have been used in vaccination as immunological adjuvants in a number of animal models and in some clinical trials. They have enhanced the protection induced by killed or recombinant vaccines and in some cases have clearly altered the type of immune response induced rather than simply the magnitude (for a review, see reference 28). In addition to effects in vaccination, the administration of cytokines during an active infection can sometimes alter the immune response induced. In the *Leishmania* infection model which has been used for much of the study on dichotomy in T-helper-cell responses, in vivo administration of IFN-γ can push the response toward protective Th_1 immunity, while IL-4 can mediate the opposite effect, leading toward a nonprotective Th_2 response (9, 15). Use of a live vaccine vector represents both vaccination and infection. Thus, these collective observations constitute the rationale for the proposal that expression of appropriate cytokines by a live vaccine vector may be an effective means of either enhancing protective immune responses or perhaps diverting a mixture of host immune responses in the direction most appropriate for pathogen neutralization.

In this report, we investigate the consequences of expressing the cytokine murine IL-4 (mIL-4) in an attenuated *S. typhimurium* strain subsequently introduced into normal BALB/c mice. Our interest in IL-4 derived primarily from observations that this cytokine is the only mediator to date capable of directing helper-T-cell precursors into a Th_2 response in vitro (40, 58) and of skewing immune responses toward Th_2 in vivo (9, 15). As mentioned above, Th_2 cells are potent regulators of antibody production (50) and therefore may be the most desirable effector T cell to provide protective immunity against pathogens such as extracellular bacteria. Since *Salmonella* infection normally induces a Th_1 response (62), it provides a valuable system for exploring the Th switching capacity of IL-4 in vivo. Importantly, IL-4 exhibits numerous other immunity-modulating properties both in vitro and in vivo, including the switching of antibody responses to immunoglobulin G subtype 1 (IgG1) and IgE isotypes (50) and the deactivation of macrophages (38, 41). We examine here the consequence of mIL-4 expression by attenuated *S. typhimurium* on specific immune responses to these microorganisms. Our data indicate a significant local effect on macrophage function, which may explain enhanced in vivo growth of the bacteria, but no modification of systemic immunity. The latter probably reflects the lack of active secretion of mIL-4 by the bacteria under the conditions of this study.

MATERIALS AND METHODS

Mice. Six-week-old female BALB/c mice innately susceptible to *Salmonella* spp. were purchased from Simonsen Laboratories (Gilroy, Calif.). Purina Mouse Chow (Purina, St. Louis, Mo.) and fresh water were available ad libitum. All mice were acclimatized for at least 1 week before use.

Bacterial strains, plasmids, and media. *S. typhimurium* LB5000 (r^- m^+) has been described previously (7). The attenuated strain SL7207, used throughout this study, was produced and generously provided by B. Stocker, Stanford University, Stanford, Calif. SL7207 was derived from the same virulent *S. typhimurium* ancestor as SL3261 (17, 30, 31) by introduction of an extensive deletion in the *aroA* gene in the aromatic amino acid biosynthetic pathway. The deletion renders the bacteria auxotrophic for two compounds not present in mammalian tissue, PABA and 2,3-dihydroxybenzoate. This auxotrophy results in attenuation of SL3261, the 50% lethal dose (LD_{50}) by the intraperitoneal route increasing to $>3 \times 10^6$ compared with 10 to 20 bacteria for the virulent parent (57). SL7207 appears to be as nonvirulent as SL3261 and to be as effective as a live vaccine for the protection of BALB/c mice against virulent challenge (57a).

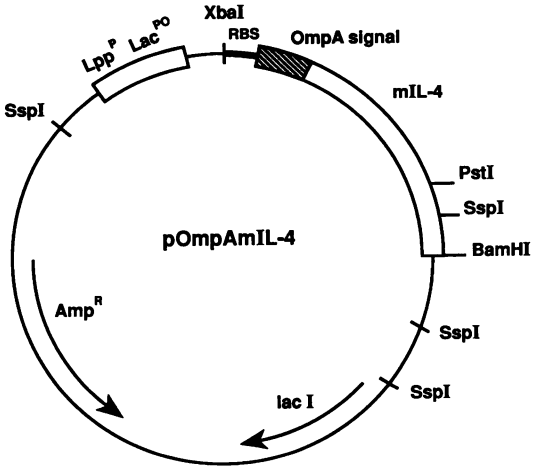

FIG. 1. Schematic representation of secretion plasmid pOmpAmIL-4. Construction of this plasmid is described in Materials and Methods. Abbreviations: Amp^r, ampicillin resistance; lac I, the *lacI* gene; Lpp^P, lipoprotein promoter; Lac^{PO}, *lac* promoter-operator; RBS, ribosome-binding site. OmpA signal (hatched box), outer membrane signal sequence coding the signal peptide of the OmpA protein.

All plasmids were maintained in *E. coli* K-12, using the host strain JM105 (63) or MM294 (4). Plasmid pOmpAmIL-4 (Fig. 1) was obtained from R. A. Kastelein, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, Calif. This plasmid carries a 0.65-kb cDNA fragment coding for mIL-4 cloned into the high-level expression vector pIN-III-OmpA-2 (26). pOmpAmIL-4 encodes a fusion protein which consists of a signal sequence (21 amino acids) from outer membrane protein (OMP) OmpA of *E. coli* (26) and the mature polypeptide sequence (120 amino acids) of mIL-4 (53). The signal sequence directs transport of the protein across the cytoplasmic membrane (26, 45). Transcription of this fusion protein is driven by the constitutive lipoprotein promoter (lpp^P) of *E. coli* and the *lac* promoter and operator (lac^{PO}) (45). Plasmid pKKmIL-4 was constructed from the expression vector pKK223-3 (Pharmacia, Piscataway, N.J.), which contains a strong *tac* promoter. pKK223-3 was digested with *Pst*I (the *Pst*I site was chosen because the cloned mIL-4 gene contains its own ribosome-binding site upstream of its ATG codon) and then blunt ended with DNA polymerase. The mIL-4 fragment and the vector pOmpA were isolated from an *Xba*I-*Bam*HI digest of pOmpAmIL-4 (Fig. 1), and both fragments were gel purified and treated with DNA polymerase to create blunt ends. The empty vector was self-ligated to create the deletion construct known as pOmp. The blunt-ended vector pKK223-3 and mIL-4 insert were mixed and ligated. After transformation of *E. coli* MM294 by standard procedures (27), ampicillin-resistant transformants were selected and digested with *Pst*I and

*Hin*dIII to determine the correction orientation of the insert and then tested for production of mIL-4 by enzyme-linked immunosorbent assay (ELISA) (described below). One such ampicillin-resistant, mIL-4-producing isolate, designated pKKmIL-4, was selected for further study. All three plasmids, pOmpAmIL-4, pOmp, and pKKmIL-4, were retransformed into *S. typhimurium* SL7207 via the restriction modification strain LB5000 ($r^- m^+$), using published methods (3). Both SL7207 pOmpAmIL-4 and pKKmIL-4 were used in in vitro stability and expression analyses. Genetically manipulated strains were routinely tested for serologic characteristics with *Salmonella* anti-H and anti-O diagnostic sera (O-factor 4) purchased from Sigma Chemical Co., St. Louis, Mo. Enzymes for plasmid manipulations were purchased from New England BioLabs (Beverly, Mass.) and used as recommended by the manufacturer. Geneclean (Bio 101, Inc., La Jolla, Calif.) was used for DNA purification as outlined by the manufacturer.

All bacteria were cultured in L broth or on L-agar plates unless otherwise stated. Minimal medium consists of 2% Noble agar (Difco Laboratories, Detroit, Mich.) containing M9 salts, glucose, and Ca-Mg salts as described previously (52). To grow *aroA hisG* strains on minimal medium, the medium was supplemented with the aromatic amino acids tryptophan, histidine, tryosine, and phenylalanine at a final concentration of 40 μg/ml each and 2,3-dihydroxy-benzoate, PABA, and *para*-hydroxybenzoic acid at 10 μg/ml each (all supplements from Sigma).

Cytokines. Recombinant mIL-4 (*E. coli*) was kindly provided by W. Dang and S. Menon, DNAX. Recombinant mouse IFN-γ was obtained from Schering Research, Bloomfield, N.J.; and recombinant mouse tumor necrosis factor alpha (TNF-α) was kindly provided by A. Zlotnik, DNAX.

Measurement of plasmid stability in vitro. Initially, plasmid-containing *S. typhimurium* cells were streaked onto L-agar plates containing 50 μg of ampicillin per ml from a frozen stock and grown overnight at 37°C. The next day, a single loopful of bacteria was resuspended in 5 ml of phosphate-buffered saline (PBS), and another 1 μl of each culture was inoculated into tubes containing 5 ml of L broth without antibiotic and regrown to stationary phase for 24 h. On the next day, 1 μl of each culture was used to inoculate the cultures for the following day, and the remaining L-broth culture was serially diluted 10^8-fold in 10 ml of PBS without antibiotic, plated on nonselective medium, and scored 24 h later. The in vitro plasmid segregation from SL7207 was confirmed by transferring 100 colonies from nonselective medium to medium containing 50 μg of ampicillin per ml or no ampicillin; the culture was scored 24 h later to distinguish between plasmid-containing and plasmid-free *S. typhimurium*. This protocol was followed throughout the 10 days of the experiment. Each 24-h cycle represents approximately 14 generations of growth for a total of about 140 generations per strain. Assays were performed five times, with similar results.

Gene expression in *S. typhimurium*. While the construct pOmpAmIL-4 includes a copy of the *lacI* gene (Fig. 1), the *lac* operon is not present in the chromosome of *S. typhimurium*. Therefore, the production of mIL-4 by strains carrying the plasmid was tested in the presence or absence of the inducer IPTG (isopropylthio-β-D-galactosidase; Sigma). Cultures of M9 medium (10 ml containing 50 μg of ampicillin per ml supplemented with histidine and aromatic metabolites) were inoculated with SL7207(pOmpAmIL-4) (from an overnight culture) to a starting optical density of 0.05 (at 600 nm). At an optical density of 0.4, the cultures were induced by the addition of IPTG to a final concentration of 0.2 mM. After 2 more h of growth, cells were harvested by centrifugation. Periplasmic proteins were prepared by osmotic shock as described by Koshland and Botstein (37). After osmotic shock, the remaining unfractionated pellet was saved as the cytoplasmic-membrane-bound fraction and resuspended in PBS–0.5% Triton X-100. All extracts were snap frozen and stored at −80°C. Constitutive gene expression of SL7207 (pOmpAmIL-4) and SL7207(pKKmIL-4) was measured as described above except that cultures were grown in L broth and no IPTG was added.

IL-4 ELISA. To assay mIL-4, microtiter plates (Dynatech Laboratories, Chantilly, Va.) were coated with the 100 μl of anti-IL-4 monoclonal antibody (BVD4-1D11) diluted to 1 μg/ml in PBS, and the plates were incubated for 1 h at 37°C. After coating, the plates were washed three times for 3 min each time in PBS with 0.05% Tween 20 (PBS-T). Triplicate 100-μl 1:1 dilutions of supernatant, periplasmic, and cytoplasmic-membrane-bound fractions in PBS–1% bovine serum albumin (PBS-B) and serial dilutions in PBS-B of 1 μg of an mIL-4 standard per ml were added, and the plates were incubated for 2 h at room temperature and washed as described above. A 75-μl portion of NIP-conjugated monoclonal antibody (BVD6-24-G-NIP) diluted 1/1,000 in PBS-T–1% bovine serum albumin (PBS-TB), was added, and plates were incubated for 1 h at room temperature. Plates were washed as described above, and 75 μl of horseradish peroxidase–anti-NIP antibody per ml diluted 1/3,000 in PBS-TB was added; the plates were then incubated for 1 h at room temperature. Plates were washed and incubated as described above, and 100 μl of a substrate solution containing 0.1 M citric acid (5 parts), 8% sodium phosphate (3 parts), 1 mg of ABTS (2,2′-azino-bis-3-ethylbenz-thiazoline-6 sulfonic acid; Sigma) per ml in distilled H_2O (8 parts), and 0.5 μl of H_2O_2 (Sigma) per ml was added. After 10 min, the absorbancies were measured in a Dynatech Auto Reader MR580 at 405 nm. A mIL-4 standard as described above was used in each assay. Anti-IL-4 antibodies and anti-NIP were kindly provided by J. Silver and J. Abrams, DNAX.

IL-4 bioassay. The bioassay for mIL-4 was performed as described previously for IL-2 (47, 48). HT-2 cells were plated at 2,500 cells per well in 96-well tissue culture plates, and dilutions of an *E. coli* mIL-4 standard (DNAX) or test solutions were added to the wells. At 48 h, proliferation of the HT-2 cells was assessed by colorimetric assay, using the dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide; Sigma].

Oral and i.v. administration of *S. typhimurium* into BALB/c mice. Inocula for oral administration were prepared from frozen stocks; cells were grown at 37°C to mid-log phase (optical density at 600 nm = 0.5) in 1.5 liters of L broth (containing PABA and 2,3-dihydroxybenzoate at 10 μg/ml) supplemented with 50 μg of ampicillin per ml for plasmid-bearing strains or without ampicillin for strain SL7207. Cells were chilled and harvested by centrifugation, and the pellet was washed in PBS and then resuspended in 2 ml of PBS to give about 2×10^{11} cells per ml. With a pipette tip, groups of mice were fed with 0.05 ml (10^{10} cells). The inoculum dose was calculated by plating serial dilutions onto L-agar plates with or without ampicillin. For i.v. administration, cells were grown, harvested, and resuspended as described above. Cells were diluted to approximately 1.5×10^7 cells per ml in PBS. Groups of mice were inoculated i.v. in a tail vein with 3×10^6 organisms in 0.2 ml of PBS. The inoculum dose was again verified as for oral administration. Experi-

ments were performed three to four times, with similar results.

In vivo colonization and persistence. Groups of female BALB/c mice were inoculated orally or i.v. with bacteria as described above. On days 1, 3, 5, 10, 12 or 14, 21, and 48 postinoculation, animals were sacrificed, and spleen, liver, and 15 cm of the small intestine were removed aseptically, homogenized in 10 ml of PBS, and serially diluted in PBS. Viable counts were performed on these homogenates with MacConkey or salmonella-shigella agar as the growth medium with or without ampicillin (50 μg/ml), as required, to assess the in vivo stability of the plasmid. Genetically manipulated strains were routinely tested for serologic characteristics with *Salmonella* anti-H and anti-O diagnostic sera (O-factor 4). Data are expressed as geometric means ± 1 standard error of the mean, with each experimental point representing at least 10 mice.

LD_{50}. To determine LD_{50}s for strains SL7207 and SL7207(pOmpAmIL-4), mice were inoculated i.v. as described above with various doses of bacteria and monitored over a 60-day period.

DTH. At 30 days post-i.v. infection with 3×10^6 CFU of SL7207 or SL7207(pOmpAmIL-4), the DTH response was assessed. Groups of mice were injected in the left footpad with 50 μl of PBS containing 10^6 heat-killed SL7207 (65°C, 2 h). The footpad thickness (mean difference in thickness, in 0.1-mm units, between injected and noninjected footpads) was measured after 24 and 48 h with a dial-gauge caliper.

Antigens. Lyophilized, phenol-water-extracted *S. typhimurium* lipopolysaccharide (LPS; Difco) was reconstituted with and diluted in distilled H_2O at 10 mg/ml. Flagellar antigen from SL7207 was prepared as described before (22) at a final concentration of 1.6 mg/ml in PBS. Preparations of SL7207 OMPs were made as described previously (29) at a final concentration of 0.5 mg/ml in PBS. Whole-cell antigens were prepared as follows. Log-phase cells of SL7207 were harvested by centrifugation, washed in PBS and fixed in 2% formaldehyde, rewashed with PBS, centrifuged, resuspended in PBS at a final concentration of 2.5×10^{11} cells per ml, and then snap frozen.

Measurement of antibody response. To determine any possible effects of *Salmonella* mIL-4 expression on total antibody, or specific isotype responses to the bacteria, sera were obtained from 10 to 15 individual BALB/c mice by tail bleeding at 15, 30, and 60 days after i.v. infection with 3×10^6 CFU of the different strains. These sera were titrated by ELISA against the four different antigen preparations described above. ELISAs were performed as described above for the IL-4 ELISA, with plates being coated overnight with 2×10^8 killed cells per ml, or 5 μg of LPS per ml, a 1/1,600 dilution of a purified flagellar preparation, or a 1/500 dilution of OMP. Following washing and blocking, serial dilutions of the mouse sera were added to the plates and the plates were incubated for 2 h at room temperature. Biotinylated anti-immunoglobulin conjugates were all obtained from Pharmingen (San Diego, Calif.) and used at predetermined optimal concentrations in the assay. These included anti-mouse immunoglobulin and anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, anti-IgM, and anti-IgA. The anti-isotype antibodies used have been shown previously to be specific for the correct isotypes (31a). After a 1-h incubation with the biotin conjugates and washing, the plates were incubated with a streptavidin-horseradish peroxidase conjugate (Pierce Chemical, Rockford, Ill.) for 1 h at room temperature, and finally developed with ABTS substrate (Sigma). Titers were defined as the reciprocals of the serum dilution giving an optical density of 0.05 (18, 19).

Determination of macrophage *Salmonella*-killing activity. The ability of macrophages to kill mIL-4-expressing and nonexpressing strains was determined as described previously (35). Resident peritoneal macrophages were harvested from normal BALB/c mice in RPMI (J.R.H. BioSciences, Lenexa, Kans.)–20% normal mouse serum. Monolayers of peritoneal macrophages were plated in triplicate in 24-well tissue culture plates at 10^6 cells per well and allowed to adhere for 3 h at 37°C. After 3 h, nonadherent cells were washed off and medium was replaced. Adherent cells were incubated overnight at 37°C with 1,000 U of IFN-γ, 100 U of TNF-α, or 100 U of recombinant IL-4 (rIL-4) per ml. Approximately 10^7 CFU of SL7207(pOmp) or SL7207(pOmp AmIL-4) bacteria were added in PBS–20% normal mouse serum, 0.5 ml per well. After 30 min, three SL7207(pOmp) and three SL7207(pOmpAmIL-4) wells, untreated, were washed several times with PBS to remove extracellular bacteria, lysed with 0.05% Triton X-100, and then plated to L-agar plates containing ampicillin (50 μg/ml) to provide a baseline number of CFU that had infected the macrophages. Cells were washed hourly to remove extracellular bacteria, and the remaining cells were washed well and lysed at 7 h. The percent killing was calculated as follows: 100− [(CFU after 7 h × 100)/CFU at baseline].

Statistics. All experiments were performed two to five times, and the significance of the differences observed was assessed by regression analysis, Mann-Whitney U test, or unpaired Student's *t* test, using the computer program StatView (Abacus Concepts, Berkeley, Calif.). Differences were considered significant when P values of <0.05 were obtained.

RESULTS

Stability and expression of two IL-4 gene-containing plasmids. In this experiment, the stability and level of in vitro expression of two plasmids (pOmpAmIL-4 and pKKmIL-4) which include the mIL-4 gene were examined. In the absence of antibiotic selection, the strain bearing the high-copy-number plasmid pKKmIL-4 showed significant plasmid loss, with more than 85% of the recovered isolates being ampicillin sensitive after approximately 14 generations (24 h), and was completely lost by the third subculture. In contrast, cells bearing pOmpAmIL-4 lost their plasmid more slowly. After approximately 140 generations (10 days), 22% of cells remained ampicillin resistant (data not shown). During each 24-h interval of the experiment, 10 selected ampicillin-resistant colonies were picked, and the level of in vitro mIL-4 produced was measured by ELISA. All tested ampicillin-resistant colonies throughout the 10 days of the experiment made mIL-4 (data not shown). These findings demonstrate that plasmid pOmpA is a more stable vector for the mIL-4 gene than plasmid pKK223-2 in the absence of antibiotic selection.

Expression of the mIL-4 gene was tested in strain SL7207(pOmpAmIL-4) under noninducing or inducing (presence of IPTG) conditions and under noninducing conditions for SL7207(pKKmIL-4). Three separate experiments, each assayed with triplicate cultures, gave similar results. Under noninducing conditions, SL7207(pKKmIL-4) had very low but detectable levels (1 ng per 10^9 cells) of mIL-4 in the periplasmic fractions and higher levels (>300 ng per 10^9 cells) in the unfractionated cytoplasmic or membrane-bound fractions. SL7207(pOmpAmIL-4) had 3- to 10-fold more

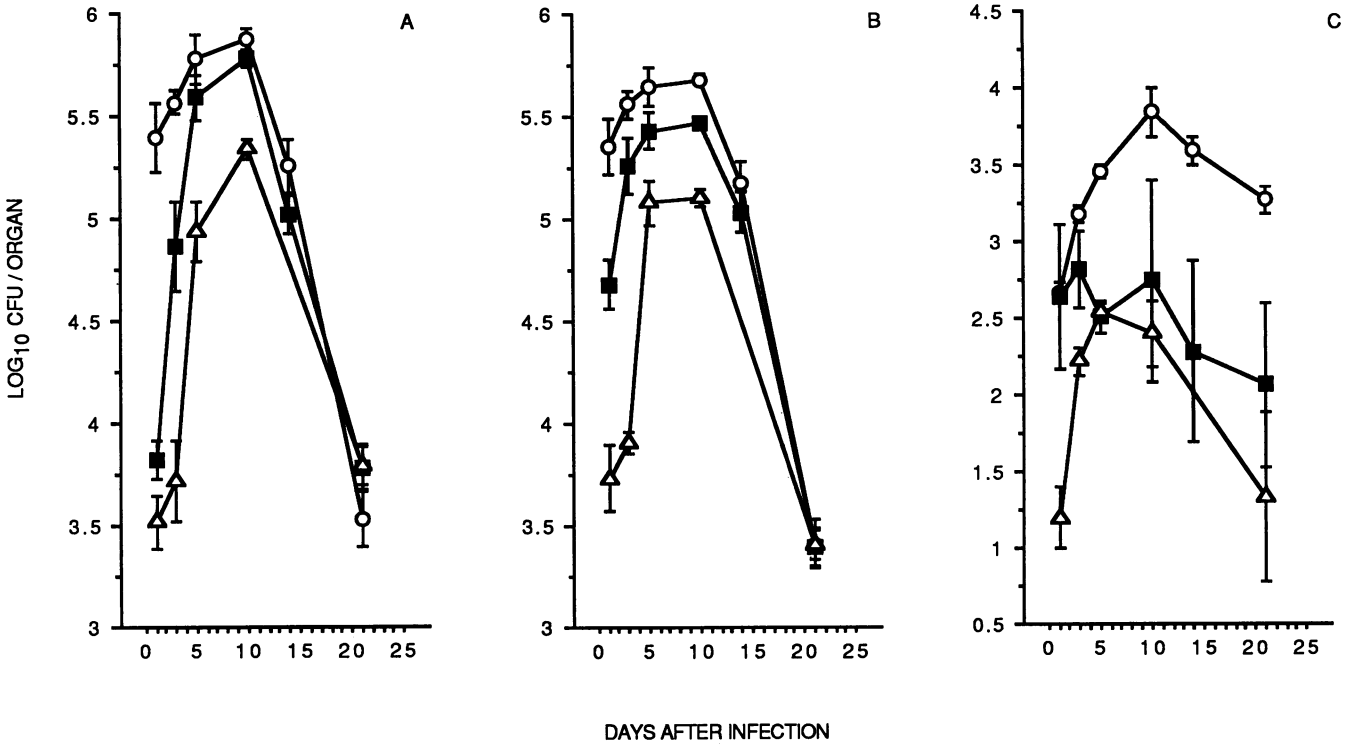

FIG. 2. Comparison of the number of CFU isolated from spleens (A), livers (B), and intestines (C) of BALB/c mice after i.v. infection with 3×10^6 SL7207 (■), SL7207(pOmp) (△), or SL7207(pOmpAmIL-4) (○). Each point represents the geometric mean of three to four experiments. Vertical bars = 1 standard error of the geometric mean. $n = 20$ (days 1, 3, and 21); $n = 30$ (days 5, 10, and 14).

mIL-4 than SL7207(pKKmIL-4) in the pellet containing the cytoplasmic-membrane-bound fractions (>1,100 ng) and periplasmic factions (>10 ng). Supernatants of each culture were examined for the presence of mIL-4 in the growth medium; little to no secreted mIL-4 was detected for SL7207(pKKmIL-4) (<0.1 ng), whereas SL7207(pOmp AmIL-4) had higher levels (>10 ng). As expected, when SL7207(pOmpAmIL-4) was cultured under inducing conditions, it had significantly higher levels of mIL-4 in the culture medium (approximately 3-fold more [>7 ng]) and higher levels in both periplasmic and unfractionated cytoplasmic-membrane-bound fractions (approximately 10-fold more). However, this overproduction of protein appeared to become toxic to cells which were swollen and no longer rod shaped when viewed under a microscope.

In vitro biological assay on HT-2 cells indicated that the IL-4 produced and detected by ELISA was also biologically active, with approximately 10 ng of IL-4 obtained from the periplasmic fraction of 10^9 cells. On the basis of these results and the plasmid stability data presented above, only SL7207(pOmpAmIL-4) grown under noninducing conditions was studied further.

***S. typhimurium* strains expressing mIL-4 grow and persist at higher levels in vivo.** Before we examined the effect of mIL-4 expression on specific immune responses to SL7207, it was important to evaluate the impact of this cytokine expression on bacterial growth and colonization. As shown in Fig. 2A and B, 24 h after i.v. administration of 3×10^6 bacteria, parental strain SL7207 was detectable at moderate levels in spleens and liver (10^3 to 10^4 bacteria per organ). In contrast, strain SL7207(pOmpAmIL-4) was detectable at higher levels (10^4 to 10^5 bacteria per organ) at the same sites, showing an average 10- to 50-fold increase in numbers compared with either parental strain SL7207 or SL7207 carrying plasmid pOmp, which lacks the mIL-4 gene (Fig. 2A and B), and the differences were statistically significant ($P < 0.001$) in both spleens and livers. Between days 1 and 10, the number of CFU of all three strains increased in both the spleens and livers but never exceeded the number inoculated. By day 5, pronounced splenomegaly was observed for all three strains tested. After day 10, the counts fell rapidly in both spleens and livers and were negative for all three strains tested in all mice by day 48 (data not shown). Similar results were obtained when bacterial counts in equivalent intestinal samples were compared, although more variability at this site was noted (Fig. 2C). The differences between mIL-4-expressing and nonexpressing bacteria were more marked in the intestines than in liver and spleen.

One of the advantages of attenuated *S. typhimurium* as a vaccine vector is its suitability for oral administration. We therefore compared the effectiveness of IL-4-secreting SL7207 versus parental SL7207 upon oral administration to mice. Groups of BALB/c mice were given oral doses of 10^{10} parental SL7207 or strain SL7207(pOmpAmIL-4). Oral administration of these microorganisms led to very low levels of colonization of spleens and livers. Between days 1 and 5, both strains were detectable at low levels in both organs (<100 bacteria per organ). After day 5, the counts decreased rapidly, so that by days 14 and 21 both organs were clear of *S. typhimurium* (data not shown). However, by day 1 posttreatment, both strains were detectable in intestines at 10^2 to $10^{3.5}$ per sample (Fig. 3). In the SL7207(pOmpAmIL-4) group, the number of bacteria increased until day 10 to $10^{3.7}$ CFU and then remained relatively constant at moderate

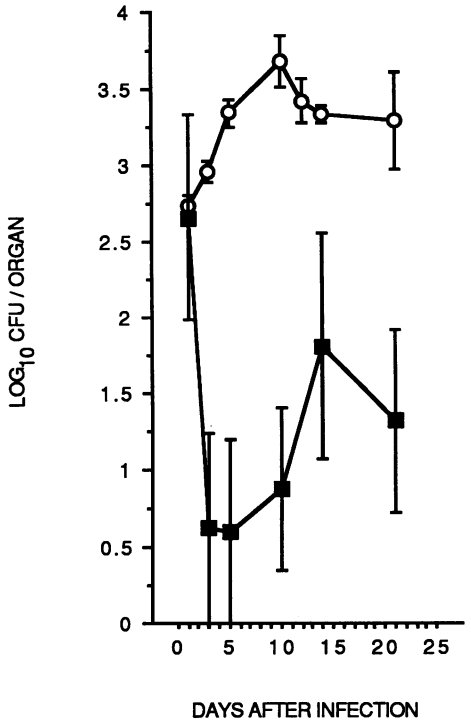

FIG. 3. Comparison of the number of CFU isolated from intestines of BALB/c mice after oral infection with 10^{10} SL7207 (■) or SL7207(pOmpAmIL-4) (○). Each point represents the geometric mean of three to four experiments. Vertical bars = 1 standard error of the geometric mean. n = 20 (days 1, 3, and 21); n = 30 (days 5, 10, and 14).

levels (>3,000 CFU) through day 21. In contrast, bacterial counts in the SL7207-vaccinated group declined after day 1 and never averaged more than 100 CFU per sample. By day 48, all mice were cleared of both strains (data not shown).

Plasmid stability in vivo. To assess the stability of pOmp AmIL-4 in vivo, viable counts were performed on liver, spleen, and intestine homogenates on MacConkey agar (containing PABA and 2,3-dihydroxybenzoate at 10 μg/ml) with ampicillin as the growth medium. At day 10 (following i.v. treatment), greater than 90% of the colonies recovered from all three organs were ampicillin resistant, with greater than 80% of the colonies remaining ampicillin resistant by day 21 (data not shown). At days 1, 5, 10, and 21, five or more ampicillin-resistant colonies were selected and tested with anti-O-4–anti-H antisera. All colonies agglutinated and all colonies expressed IL-4, as assessed by ELISA (data not shown).

The LD_{50} is reduced by mIL-4 expression. It was important to determine whether the enhanced in vivo growth of SL7207(pOmpAmIL-4) was associated with altered virulence of this strain compared with the parental strain SL7207. We therefore examined the effects of injecting both strains i.v. into unprimed BALB/c mice. In one experiment in which groups of 20 mice were given serial 10-fold dilutions of bacteria starting at 3×10^8, the LD_{50}s calculated by the method of Reed and Muench (55) were 1.34×10^7 for SL7207(pOmpAmIL-4) and 3×10^7 for the parental strain SL7207. In a second experiment in which groups of five mice were given serial twofold dilutions of either strain from 2×10^8 to 3.25×10^6, the calculated LD_{50}s (i.v.) were 1.76×10^7 for the IL-4-bearing strain and 3.5×10^7 for the parental strain. These data indicate that the IL-4-bearing strain had an LD_{50} approximately half that of the parental strain, which might reflect the increased growth of this microorganism in vivo. This small and explainable difference in LD_{50}s would argue against any profound protective or toxic effect of bacterial IL-4 expression on the mice.

DTH responses to attenuated *S. typhimurium* are unaffected by mIL-4 expression. To explore the possibility that production of IL-4 by the bacteria might affect T-helper-cell responses, DTH responses to killed *S. typhimurium* were assessed at 30 days post-i.v. infection with 3×10^6 CFU of SL7207(pOmpAmIL-4) or parental strain SL7207. The parental strain was used in these studies because its growth is closer to that of the mIL4 strain than is the growth of SL7207(pOmp), thus reducing the chance of simple differences in bacterial burden affecting immune responses. At 24 h after footpad injection of 10^6 CFU of heated-killed SL7207, the mean difference in thickness between injected and noninjected footpads was 2.33 mm (n = 15) for the mice given SL7207(pOmpAmIL-4) as live vaccine. For the mice vaccinated with parental strain SL7207, this difference was 2.02 mm (n = 15), whereas the mean swelling induced by a PBS injection was only 0.3 mm. Thus, both strains induced a DTH response, but there was no significant difference between the two groups. Similar results were obtained after 48 h.

Serum antibody responses to attenuated *S. typhimurium* are unaffected by mIL-4 expression. Serum samples from mice infected i.v. with 3×10^6 CFU of the parental strain or SL7207(pOmpAmIL-4) were collected 15, 30, and 60 days postinfection and assayed for antibodies specific for several *Salmonella* antigens. There were no significant differences in the anti-*Salmonella* antibody responses observed in mice infected with either SL7207(pOmpAmIL-4) or SL7207 (Ta-

TABLE 1. Specific anti-*Salmonella* serum IgG and IgG1 titers in BALB/c mice immunized with live SL7207 or SL7207(pOmpAmIL-4)

Antigen	ELISA titer (log_{10}) on given day posttreatment[a]									
	IgG						IgG1			
	SL7207			SL7207 (pOmpAmIL-4)			SL7207		SL7207 (pOmpAmIL-4)	
	15	30	60	15	30	60	30	60	30	60
Whole cells	2.68	4.32	5.27	2.82	4.44	5.07	4.05	3.98	3.72	3.86
LPS	2.95	5.01	4.83	3.02	4.82	4.82	4.62	4.58	4.29	4.49
Flagella	3.34	4.19	4.16	3.36	3.92	3.81	4.19	3.74	3.92	3.15
OMP	3.56	5.24	5.06	3.72	5.30	5.25	4.80	4.65	4.55	4.53

[a] Data points represent means of sera from 10 to 15 individual mice 15, 30, and 60 days post-i.v. treatment with either SL7207 or SL7207(pOmpAmIL-4).

TABLE 2. Specific anti-*Salmonella* serum IgG2a, IgG2b, IgG3, IgM, and IgA response in BALB/c mice immunized with live SL7207 or SL7207(pOmpAmIL-4)

Antibody class or isotype[b]	ELISA titer (log_{10}) on given day posttreatment[a]			
	SL7207		SL7207 (pOmpAmIL-4)	
	30	60	30	60
IgG2a	4.03	3.88	3.17	3.99
IgG2b	3.71	3.36	3.60	3.32
IgG3	4.31	3.84	3.90	3.74
IgM	3.44	3.04	3.28	3.07
IgA	3.07	3.03	3.07	3.17

[a] Data points represent means of sera from 10 to 15 individual mice 15, 30, and 60 days post-i.v. treatment with either SL7207 or SL7207(pOmpAmIL-4).
[b] Whole cells were used as antigen.

ble 1). This conclusion was apparent when either whole serum IgG levels or specific immunoglobulin isotype responses to a panel of *Salmonella* antigens were compared (Tables 1 and 2).

***Salmonella* killing ability of macrophages is decreased by mIL-4 expression.** As the expression of mIL-4 by *S. typhimurium* clearly led to increased numbers of live bacteria in spleens, livers, and intestines, without any detectable effect on cell-mediated or humoral immune responses to the bacteria, it was of interest to determine the possible mechanism behind the higher counts. Thus, the ability of BALB/c macrophages to kill *S. typhimurium* in vitro was assessed.

Macrophages from the peritonea of normal BALB/c mice were collected as described in Materials and Methods and then incubated with either SL7207(pOmp) or SL7207(pOmpAmIL4). Intracellular bacterial counts performed after a 7-h culture and compared with a baseline of 30 min indicated reduced killing of SL7207(pOmpAmIL4) in comparison to SL7207(pOmp) (Fig. 4). This apparent protection from macrophage killing occurred even with the addition of the recombinant macrophage-activating cytokines IFN-γ and TNF-α either alone or in combination (Fig. 4). These data indicate that the expression of mIL-4 by *S. typhimurium* is able to confer some protection against macrophage microbicidal activity. This effect was mimicked by the addition of exogenous rIL-4, which was able to reduce killing of SL7207(pOmp) to only 2% ($P < 0.025$), whereas killing of SL7207(pOmpAmIL-4) was unaffected by the addition of exogenous IL-4; i.e., it was 13% with or without exogenous IL-4.

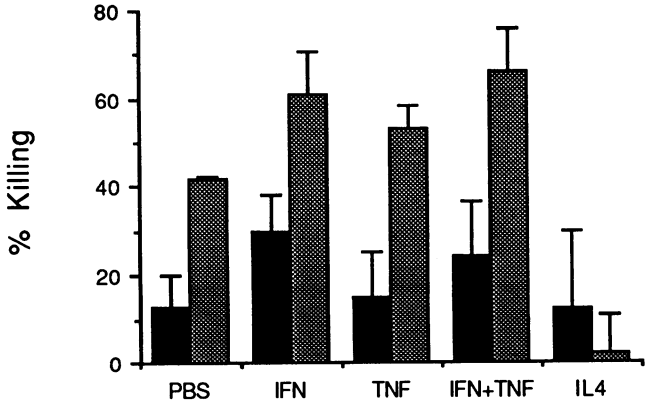

FIG. 4. Effects of recombinant IFN-γ (rIFN-γ), recombinant TNF-α (rTNF-α), and rIL-4 on macrophage killing activity. Monolayers of peritoneal macrophages from untreated BALB/c mice were incubated with 10^2 U of rTNF-α, 10^3 U of rIFN-γ, 10^2 U of rIL-4, and 10^2 U and 10^3 U of rTNF-α plus rIFN-γ, respectively, for 24 h; their effect on percentage of SL7207(pOmpAmIL-4) (■) or SL7207(pOmp) (▩) not surviving 7 h of incubation was determined, and their *Salmonella*-killing activities were compared as described in Materials and Methods. Results are expressed as mean percent killing. Vertical bars = 1 standard error of the mean.

DISCUSSION

The data presented here demonstrate that an attenuated *aroA* (deletion) strain of *S. typhimurium*, SL7207, is a useful vehicle for expression of the mIL-4 gene in vitro and in vivo. Initially, we compared the stability and levels of expression of mIL-4 in vitro with two different recombinant plasmids, each containing the gene for mIL-4 under a different promoter. Our data clearly indicate that both the stability of the plasmids and the levels of expression of the mIL-4 gene were quite different. In contrast to the plasmid-bearing strain SL7207(pOmpAmIL-4), which was clearly more stable throughout the 10 days of the experiment, the strain bearing pKKmIL-4 lost its plasmid very rapidly (>85%, within 24 h) in the absence of antibiotic selection and was completely lost after 30 generations. SL7207(pOmpAmIL-4) also produced more mIL-4 and was therefore selected for all subsequent work.

The effects of mIL-4 expression on bacterial colonization and persistence in vivo were then examined. Bacterial counts were assessed after i.v. and oral infection, with the striking finding that higher numbers of bacteria were obtained in the liver, spleen (i.v. route only), and intestine (both routes) following infection with the mIL-4-bearing strain than after infection with either the parental strain or the strain carrying the empty plasmid. While carriage of the empty plasmid appeared to decrease the in vivo growth of the bacteria to some extent, this detrimental effect was more than overcome by inserting the mIL-4 gene into the plasmid. The results of LD_{50} assays correlated with the increased growth of the mIL-4 strain, the lethal dose being halved by mIL-4 expression. The difference in colony counts between SL7207(pOmpAmIL-4) and the control strains was very large in the intestinal samples, giving differences in counts of CFU in intestine of up to 100- to 1,000-fold higher whether inoculation was by the oral or the i.v. route. It would appear that the expression of mIL-4 confers some advantage on the bacteria with respect to multiplication or survival in mouse tissue or both. This advantage may explain the extraordinary in vivo stability of the plasmid and is not mimicked by in vitro growth of the mIL-4-expressing bacteria, which is identical to that of controls.

As increased in vivo growth of the mIL-4-bearing strain could have been due to effects of bacterial mIL-4 on specific immune responses to *S. typhimurium*, we compared the immune responses to SL7207(pOmpAmIL-4) with those to the parental strain. To avoid coincidental effects on immunity caused by higher bacterial burdens and not directly by mIL-4, we compared the mIL-4-bearing strain with the parent, as growth of the parent was closer to growth of the mIL-4 strain than was that of the empty plasmid strain. An examination of DTH responses showed that both the parental and the mIL-4 strain induced measurable DTH responses to SL7207 but that there was no significant difference between the strains. In the event of a major shift towards

Th_2-type responses, DTH might be expected to decrease. An extensive analysis of anti-*Salmonella* antibody responses was then undertaken. Serum samples were taken at several times after live vaccine injection and were assayed for total specific antibody responses against four different antigens, as well as for isotype-specific anti-*Salmonella* responses. No difference was found between SL7207(pOmpAmIL-4) and SL7207. Again, in the event of a large shift towards Th_2 responses, it might have been expected that IgG1 titers would increase while IgG2a titers decreased. In addition to the above data, we have performed preliminary challenge experiments with wild-type bacteria and have found no statistically significant change in the amount of protection conferred by the mIL-4-bearing strain (data not shown).

Thus, our findings suggest that intrinsic expression of mIL-4, while aiding bacterial growth or survival, had no apparent effect on specific anti-*Salmonella* immune responses. However, a possible explanation for this difference was revealed when we examined the ability of murine peritoneal macrophages to kill the SL7207(pOmpAmIL-4) and SL7207(pOmp) strains in vitro, when clear differences emerged. In every circumstance tested, macrophages were less able to kill the mIL-4-bearing strain than SL7207 (pOmp). This was true whether the cells were left without exogenous cytokines or were treated with the recombinant macrophage-activating cytokines IFN-γ and TNF-α. This inhibition of killing was mimicked by the addition of rIL-4 to the cultures, but interestingly, exogenous rIL-4 did not affect the killing of the mIL-4-bearing strain, perhaps because it was already maximally protected.

It may appear anomalous that the expression of mIL-4 by the bacteria is not affecting specific immunological responses that have been influenced by IL-4 in other studies (9, 15, 40, 58). It should be remembered, however, that the cytokine is not secreted by the bacteria; thus, one possible explanation is that mIL-4 is only released in sufficient quantities on bacterial lysis and that the effect is either intracellular in the phagocyte, or very local, and insufficient quantities are available to affect lymphocytes. We are currently constructing a plasmid that will lead to secretion of the cytokine in the hope that greater effects on specific immune responses might be seen. Inhibition of macrophage killing by IL-4 has been observed previously in several systems (38, 41), and, in addition, in vivo administration of IL-4 to mice infected with *L. monocytogenes* resulted in increased bacterial growth (33). This may be particularly relevant as there appear to be at least some common mechanisms of defense against *Salmonella* and *Listeria* spp., which both infect macrophages. In the first month or so of an *aroA Salmonella* infection, the host is cross-protected against *Listeria* spp., and this cross-protection is thought to be mediated by activated macrophages (52). There are a large number of possible mechanisms by which IL-4 might inhibit killing, and we are currently attempting to determine exactly which killing mechanisms are being inhibited. Experiments on macrophage killing indicated no difference in phagocytosis of the two strains (data not shown).

While the lack of any effect of IL-4 on specific immune responses is slightly disappointing in relation to the potential of such vectors as live vaccines, it is quite possible that the large increase in intestinal colonization by the mIL-4-expressing strain might lead to more efficient mucosal immunization, and we are currently in the process of examining the effects of vaccination with these strains on protection against oral challenge with a virulent strain, SL1344, and on the mucosal IgA response. If the mIL-4 strain is markedly better in either of these two assays, it could be of great potential benefit in vaccination. It is also important to determine why the increase in colonization of intestines is so large in relation to other tissues. Further analysis of bacterial growth in the intestines, which would distinguish among bacteria in the lumen, the intestinal circulation, lamina propria, or Peyer's patches, is currently under way.

In addition to bacterial killing, macrophages are also able to have a suppressive effect in *Salmonella* infection (2, 39). Following infection with *aroA* strains, there is suppression of both antibody responses to injected antigens and in vitro responses of spleen cells to B- and T-cell mitogens (2). The suppression of in vitro mitogen and other responses can be reversed by IL-4 (1), and thus it will be interesting to see whether infection with the mIL-4-carrying *Salmonella* strain will lead to lower immunosuppression, which would probably be desirable. These studies are currently under way.

Liew and coworkers recently have described the use of *S. typhimurium* to express human IL-1β in vivo (8). Mice infected with these bacteria produced a significant antibody response against the cytokine and were better protected against lethal gamma irradiation. The authors emphasized the point that expression of therapeutic cytokines by *S. typhimurium* might be an effective method of delivery directly to the lymphoid organs; thus, the usefulness of such expression vectors might not be limited to vaccination. This is also true of bacteria expressing mIL-4, and since mIL-4 has been shown to protect against septic shock (unpublished observation), which is presumably due to an effect on the macrophage, we are currently assessing the effect of infection with these bacteria on their resistance to LPS-mediated stock.

Cytokines expressed by *S. typhimurium* can clearly have biological effects in the host, and we are currently examining different constructs designed to express other cytokines and cytokine combinations.

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