Studies of Immunity and Bacterial Invasiveness in Mice Given a Recombinant *Salmonella* Vector Encoding Murine Interleukin-6

SARAH J. DUNSTAN,¹ ALISTAIR J. RAMSAY,² AND RICHARD A. STRUGNELL^{1*}

Department of Microbiology, University of Melbourne, Parkville, Victoria 3052,¹ and The John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory,² Australia

Received 18 January 1995/Returned for modification 13 March 1995/Accepted 28 March 1996

Interleukin-6 (IL-6) was expressed in *Salmonella typhimurium* in an attempt to increase the mucosal immune response against the bacterium. Murine IL-6 was PCR amplified from cDNA, cloned, sequenced, and found to be functionally active when expressed in *S. typhimurium* BRD509, the Δ aroA Δ aroD vaccine strain. Expression of murine IL-6 did not appear to adversely affect the growth of salmonellae, as the construct was retained in the absence of antibiotic selection and the growth rate was unaffected compared with that of the parent strain in vitro. However, IL-6 expression led to a significant reduction in bacterial invasiveness in vitro and in vivo. Splenocytes and small intestinal lamina propria lymphocytes were isolated from mice orally immunized with BRD509 expressing IL-6 (pKK233-2/IL-6), and the number of antibody-secreting cells was determined by the ELISPOT technique. No differences were observed between mice immunized with BRD509(pKK233-2/IL-6) and those immunized with BRD509(pKK233-2) with respect to the antibody subclass-specific responses elicited despite the markedly reduced invasiveness of the former. Serum antibody responses were also examined by a kinetic enzyme-linked immunosorbent assay (ELISA), and equivalent levels of antibody response were detected in mice given BRD509(pKK233-2/IL-6) and those given BRD509(pKK233-2). The humoral immune response against bacterial lipopolysaccharides was also examined in transgenic IL-6-deficient mice given oral inocula of BRD509. Equivalent numbers of antibody-secreting cells (ELISPOTs) were observed in the spleens and laminae propriae of both IL-6-deficient (–/–) mice and control (+/+) mice harboring an intact IL-6 gene, whereas small, yet significant differences in the serum immunoglobulin A ELISA titers were observed. These data suggest that the immunoglobulin A response against *Salmonella* lipopolysaccharides is largely IL-6 independent.

Invasive salmonellae are responsible for enteric fevers in humans and several animal species. The predominant cause of human enteric fever is *Salmonella typhi* (reviewed in reference 21), the agent of typhoid fever, which is spread via the fecal oral route. Once ingested, *S. typhi* invades from the small bowel into the reticuloendothelial system, wherein the bacterium replicates. Live attenuated vaccines afford greater protection against virulent *Salmonella* infections than do traditional whole-cell killed vaccines, and the best-studied rationally attenuated vaccines are those based on salmonellae carrying mutations in the pre-chorismate pathway (14, 15). Chorismate is an essential intermediate for de novo synthesis of aromatic compounds including aromatic amino acids. Pre-chorismate, or *aro*, mutants have been investigated extensively as vaccines in the murine model of typhoid fever, in which oral infection with *Salmonella typhimurium* is used, and as vaccine vectors for systemic and mucosal immunization (12, 14, 22, 29). In these studies, *Salmonella aro* mutants have been shown to be excellent oral vaccines and also to have considerable potential as vectors for the presentation of heterologous vaccine antigens to the immune system.

Studies of mice have revealed that a variety of responses are induced against recombinant antigens expressed by *S. typhimurium aro* mutants, including cytotoxic T cells (33), delayed-type hypersensitivity, and systemic antibody responses (12, 19, 28). The use of these constructs to elicit mucosal immune

responses has been less successful however, and the requirements for high levels of mucosal recognition of encoded antigens clearly need further investigation. One means by which mucosal responses may be enhanced is through the coexpression of cytokines, which have been shown, in other vector systems, to increase the immunoreactivity of encoded vaccine antigens. We have demonstrated that Th2-type cytokines can significantly boost mucosal immunoreactivity to heterologous vaccine antigens when coexpressed in recombinant poxvirus vectors (24–26). Given the increased interest in mucosal vaccination as a way of preventing many serious infections, notably in the light of the current human immunodeficiency virus epidemic (8), we attempted to boost the immunogenicity of recombinant salmonellae by encoding the cytokine interleukin-6 (IL-6). IL-6 is a multifunctional cytokine, originally identified for its ability to induce B-cell terminal differentiation (23), which markedly enhances the production of secretory immunoglobulin A (IgA) by cultured mucosal B cells (4). In vivo, we have recently shown that murine IL-6 plays a crucial role in the development of antibody responses at mucosae (24) and that when encoded in recombinant virus vectors can significantly boost both systemic and mucosal antibody responses (24, 26).

Here we report that *S. typhimurium* expressing IL-6 does not induce increased humoral antibody responses against bacterial cell surface antigens. Our studies in IL-6-deficient (–/–) mice further suggest that antibody responses against lipopolysaccharides (LPS) occur largely independently of this factor.

MATERIALS AND METHODS

Bacteria. *S. typhimurium* SL1344 (14), BRD509 which is an Δ aroA Δ aroD mutant of SL1344 (33), and *S. typhimurium* LB5010 (5) were the generous gifts of G. Dougan, Imperial College London.

* Corresponding author. Mailing address: Department of Microbiology, The University of Melbourne, Parkville, Victoria, Australia 3052. Phone: 61-3-3445712. Fax: 61-3-3471540. Electronic mail address: Richard.Strugnell@muwayf.unimelb.edu.au.

Mice. BALB/c mice were obtained from The University of Melbourne Department of Microbiology animal facility, whereas IL-6-deficient ($-/-$) mice and control counterpart ($+/+$) mice (17) were obtained from Manfred Kopf (Max-Planck Institute for Immunobiology, Freiburg, Germany) and bred at the John Curtin School of Medical Research, Australian National University, Canberra, Australia.

Construction of *S. typhimurium* expressing murine IL-6. The murine IL-6 gene was obtained from the plasmid pcD-mIL-6 (7) by PCR amplification with the oligonucleotides 5'-GGCATAAGCTTCTAGGTTTGCCGAGTAGATCTC (oligonucleotide IL-6A) and 5'-CCAGAAGCTTTCATGAAATTCCTCTCTGCAA GAG (oligonucleotide IL-6B). The PCR product was digested with *Hind*III and ligated into pBSSK II. The gene was sequenced before subcloning into expression vector pKK233-2 (2) with *Bsp*HI and *Hind*III restriction sites incorporated into oligonucleotides IL-6A and IL-6B. In pKK233-2, the IL-6 gene is under the control of the *trc* promoter and the ribosome binding site is supplied by the vector. Inducible expression is achieved in *Escherichia coli* by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG), whereas in *S. typhimurium* constitutive expression is obtained because of the absence of the *lacI* repressor. Clonings were conducted in *E. coli* TG-1 (Boehringer Mannheim). After construction, pKK233-2/IL-6 was transformed into restriction-negative/modification-positive *S. typhimurium* LB5010 and then transduced with bacteriophage P22 (*int* mutant [integration-deficient mutant]) into the aromatic mutant of *S. typhimurium* SL1344, BRD509.

Western immunoblot of IL-6 and bioassay of IL-6 activity. Western immunoblots were performed by the method of Towbin et al. (31) with the rat monoclonal antibody 20F3, which binds to murine IL-6 (13). The antibody was diluted 1/500 in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 0.05% bovine serum albumin. Bound antibody was detected with horseradish peroxidase-conjugated anti-rat antibody (Dakopatts). The IL-6 bioassay was performed with the IL-6-sensitive hybridoma B9 (1).

Infection of mice and monitoring of immune responses. Mice were infected orally with approximately 10^{10} bacteria in 200 μ l with a gastric lavage needle 30 min after administration of 100 μ l of 10% sodium bicarbonate. At designated intervals after immunization, the mice were killed and splenectomized, and the numbers of bacteria present in the spleens were determined by viable counting. Mice were then boosted, as in the primary immunization, with 10^{10} bacteria orally. The titers of antibodies present in mouse sera were determined by standard enzyme-linked immunosorbent assay (ELISA) or a kinetic ELISA (18, 32) with rabbit anti-mouse immunoglobulins (affinity isolated, horseradish peroxidase conjugated; Silenus). For ELISAs, 96-well plates (Nunc) were coated with LPS at a concentration of 10 μ g of LPS per ml in PBS. The number of antibody-secreting cells present in tissues was determined by the ELISPOT technique (30). Single cell suspensions were isolated with DNase and collagenase (lungs) as described elsewhere (25) or by passage of organs (spleens) through small sieves. For gut ELISPOTs, a mechanical method was followed to prepare lamina propria lymphocytes (LPL) after their removal from the Peyer's patches and intra-epithelial lymphocytes (11). Isolated cells were incubated overnight in 96-well plates previously coated at 5 μ g per well with *S. typhimurium* LPS purified by the method of Westphal and Jann (34). After incubation, the cells were removed, and the ELISPOTs were identified with isotype-specific conjugated antibodies (ICN) and BCIP (5-bromo-4-chloro-3-indolylphosphate) as the substrate.

In vitro cell invasion assays. The ability of recombinant salmonellae to invade tissue culture cells in vitro was determined by the method of Robins-Browne and Bennett-Wood (27), with the following formula: (number of bacteria found after gentamicin treatment/total number of bacteria associated with cells) $\times$ 100.

RESULTS

Construction and stability of *S. typhimurium* expressing recombinant murine IL-6. The gene encoding murine IL-6 was obtained for procaryotic expression by PCR amplification from the plasmid pcD-mIL-6, which contained the cDNA for murine IL-6. The PCR product was digested with *Hind*III and ligated into pBSSK⁺ digested with *Hind*III. Clones containing the PCR product were isolated, and one clone, pBSSK⁺/IL-6, was sequenced to ensure that no amplification errors had occurred. The gene encoding murine IL-6 was digested from pBSSK⁺/IL-6 with the enzymes *Bsp*HI and *Hind*III and ligated into the *Nco*I and *Hind*III sites of pKK233-2, yielding pKK233-2/IL-6. This strategy ensured that the sequence of the cloned murine IL-6 gene was identical to that of the cDNA. The plasmid pKK233-2/IL-6 was then transformed into *S. typhimurium* LB5010 and transduced from this mutant *S. typhimurium*, which makes rough, chemotype cLPS (RcLPS), into the Δ aroA Δ aroD mutant of *S. typhimurium* SL1344, known as BRD509, by *int* mutant P22 bacteriophage-mediated transduction.

The stability of the *S. typhimurium* carrying IL-6 was deter-

FIG. 1. Western blot of recombinant IL-6. Salmonellae expressing IL-6 were subjected to SDS-polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose. The nitrocellulose was reacted with rat monoclonal antibody 20F3, and the reaction was developed with horseradish peroxidase-conjugated anti-rat antibody and 4-chloro-1-naphthol. Lanes: A, molecular mass markers (with sizes [in kilodaltons] indicated on the left); B, recombinant IL-6; C, BRD509(pKK233-2); D, SL1344(pKK233-2); E, BRD509(pKK233-2/IL-6); F, SL1344(pKK233-2/IL-6).

mined by growth in vitro without antibiotics (data not shown). BRD509 maintained pKK233-2 and pKK233-2/IL-6 in the absence of antibiotic selection for approximately 100 generations. The growth rates of BRD509(pKK233-2/IL-6) and BRD509 (pKK233-2) were also compared in vitro for a period of 12 h and were found to be equivalent (data not shown). These findings suggest that the expression of recombinant IL-6 by salmonellae does not adversely affect the ability of the bacterium to replicate.

Analysis of cytokine expression. IL-6 expression was initially determined by Western immunoblotting. Whole-cell protein preparations of BRD509(pKK233-2) or BRD509(pKK233-2/IL-6) were electrophoresed in sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gels, and the proteins were transferred to nitrocellulose. The anti-IL-6 monoclonal antibody 20F3 bound to a protein with a molecular mass of 25 kDa expressed in BRD509(pKK233-2/IL-6) which was not present in BRD509(pKK233-2) (Fig. 1). The gene product was approximately 1.5 kDa smaller in size than the purified recombinant murine IL-6, which migrated at 26.5 kDa. The purified recombinant IL-6 was a *lacZ* α -peptide in-frame fusion in which the α -peptide contributed approximately 1.5 kDa. Recombinant IL-6 was not detected in SDS-polyacrylamide gels stained with Coomassie blue (data not shown).

Lysates of BRD509(pKK233-2) and BRD509(pKK233-2/IL-6) were also prepared and incubated with the IL-6-sensitive hybridoma B9. IL-6-mediated proliferation of B9 cells was blocked by rabbit polyclonal antisera against murine IL-6. Growth of the cells was determined by uptake of 3 H-labelled thymidine. Lysates of BRD509(pKK233-2/IL-6) induced proliferation of B9 cells whereas no proliferation was observed following incubation with lysates of BRD509(pKK233-2) (data not shown). Supernatants of *Salmonella* cultures were also tested, and supernatants from BRD509(pKK233-2/IL-6) were found to stimulate proliferative activity to a level that was approximately 10% of that stimulated by lysates, suggesting that some IL-6 was released from the bacteria during growth.

Anti-salmonella immune responses and bacterial growth in BALB/c mice immunized with BRD509 expressing IL-6. BALB/c mice were immunized orally with 10^{10} BRD509(pKK233-2/IL-6) or BRD509(pKK233-2) cells. At day 10, 10 mice from each group were killed, and the numbers of bacteria present in the spleens of the animals were determined by viable counting in media with and without ampicillin (Fig. 2). Mice infected with BRD509(pKK233-2/IL-6) harbored fewer bacte-

FIG. 2. Colonization of spleens of infected mice at day 10. Groups of 10 mice were infected orally with either BRD509(pKK233-2) (circles) or BRD509(pKK233-2/IL-6) (triangles). Ten days later, the animals were killed, and the number of bacteria present in the spleen of each animal was determined by plating onto Luria-Bertani (LB) agar alone (open symbols) or containing ampicillin (closed symbols). G mean, geometric mean calculated for each group; P value, geometric mean for the group compared with BRD509(pKK233-2) on LB agar by one-way analysis of variance; ND, not done.

ria in their spleens (geometric mean, 68) in contrast to animals infected with BRD509(pKK233-2) (geometric mean, 346). These values were significantly different ($P < 0.05$).

Next, we used an ELISPOT assay to determine the systemic

immune responses in mice orally infected with either construct and boosted with 10^{10} CFU of the same organism 28 days later. The spleens were examined for cells secreting anti-LPS antibodies either 6 or 10 days after the day 28 boost, and responses were compared with those of unboosted animals which received only a single dose of bacteria (Fig. 3). There were no significant differences in the subclass-specific responses of mice which received BRD509(pKK233-2/IL-6) compared with those of mice which received BRD509(pKK233-2) despite the marked decrease in the number of organisms recovered from mice given the former. In addition, all animals which received a second dose of salmonellae had increased ELISPOT responses compared with the responses of animals which received only a single dose. No ELISPOT activity was detected in the lungs of any animals after primary or secondary infection with BRD509 (data not shown).

In a separate experiment, animals were killed 10 days after the day 28 boost immunization, and small intestinal LPL were isolated for measurement of local mucosal ELISPOTs (Table 1). High numbers of LPL-secreting IgA antibodies specific for *S. typhimurium* LPS were found in similar amounts in mice immunized with BRD509(pKK233-2/IL-6) and BRD509(pKK233-2).

Serum antibody responses were examined by a kinetic ELISA against LPS extracted from BRD509 (Fig. 4). Mice were bled at day 10 after primary infection and again 10 days

FIG. 3. Salmonella LPS-specific ELISPOTs in spleens of infected mice. Splenocytes were prepared from animals which were infected and then boosted (on day 28) with BRD509 containing either pKK233-2 or pKK233-2/IL-6. Each result is the mean for two animals, and the error bars show the range of responses. ELISPOTs were developed with antibodies specific for murine IgG, IgM, or IgA to determine the isotype. The ELISPOT responses obtained were compared with those found in unboosted animals and were assayed in animals 6 (day 34) or 10 (day 38) days after the second (boost) infection with BRD509 carrying either pKK233-2 or pKK233-2/IL-6. ASCs, antibody-secreting cells.

TABLE 1. LPL ELISPOT responses in mice infected with recombinant salmonellae^a

Animal no.	No. of LPS-specific antibody-secreting cells/10 ⁶ LPLs after infection with indicated recombinant strain			
	IgA		IgM	
	BRD509 (pKK233-2)	BRD509 (pKK233-2/IL-6)	BRD509 (pKK233-2)	BRD509 (pKK233-2/IL-6)
1	1.6 × 10 ⁴	3.0 × 10 ³	3.0 × 10 ²	0
2	1.5 × 10 ⁴	2.6 × 10 ⁴	1.7 × 10 ²	3.0 × 10 ²
3	1.3 × 10 ⁴	1.4 × 10 ⁴	6.0 × 10 ²	0
4	1.4 × 10 ⁴	2.2 × 10 ⁴	0	4.0 × 10 ²
Mean	1.4 × 10 ⁴	1.6 × 10 ⁴	2.7 × 10 ²	1.7 × 10 ²

^a Preliminary experiments were conducted to determine the kinetics of the ELISPOT response in salmonella-infected mice (1a). These experiments revealed that LPL populations were ELISPOT negative until day 20 postinfection and were maximal 5 to 10 days after boost immunization. LPLs were prepared from the small intestines of mice 10 days after a boost infection with recombinant salmonellae. The lymphocytes were analyzed for *salmonella* LPS-specific antibody-secreting cells in an ELISPOT assay.

after booster (day 38). Antibody responses of similar magnitudes were detected in mice given BRD509(pKK233-2/IL-6) and those given BRD509(pKK233-2). An increase in ELISA activity against LPS was seen after the initial immunization when the total antibody response was determined; however, this increase was not detected by the IgA ELISA.

Invasion of cells in vitro by *S. typhimurium* expressing IL-6. The absence of salmonellae in the spleens of mice infected with BRD509(pKK233-2/IL-6) suggested that this factor might interfere with the invasion of *S. typhimurium* through the gastrointestinal epithelium. To examine cell invasion by recombinant salmonellae, Hep-2 cells were infected with *S. typhimurium* SL1344 in vitro, and invasive organisms were recovered after treatment with gentamicin. Preliminary studies showed the virulent SL1344 to be more invasive for Hep-2 cells than the *S. typhimurium* Δ aroA Δ aroD mutant of SL1344, known as BRD509, so SL1344 was transduced with P22 carrying pKK233-2/IL-6. The expression of IL-6 by SL1344 was equivalent to that seen in BRD509 (Fig. 1). Cell monolayers were infected with *S. typhimurium* SL1344(pKK233-2) or SL1344(pKK233-2/IL-6) for either 30 or 120 min prior to treatment with gentamicin, and the percentage of organisms which invaded cells was determined (Fig. 5). The numbers of bacteria

FIG. 5. In vitro invasion assay of salmonellae expressing IL-6. Wild-type *S. typhimurium* SL1344 was studied for its ability to invade Hep-2 cells after 30 min and 2 h of incubation to allow for attachment. Attachment was not grossly affected by expression of IL-6 (data not shown). The percentages of attached bacteria which subsequently invaded cells are shown.

associated with the monolayers at each time point were also determined, and the percentages of these bacteria inside Hep-2 cells were calculated by the number of invading organisms divided by the total number of associated organisms (multiplied by 100). Gentamicin-protected (i.e., viable intracellular) SL1344(pKK233-2/IL-6) organisms were recovered in very low numbers from Hep-2 cells compared with SL1344(pKK233-2). Reduced numbers of viable bacteria were found inside Hep-2 cells infected with SL1344(pKK233-2/IL-6) following invasion for 30 and 120 min prior to gentamicin treatment. The reduced invasiveness of *S. typhimurium* expressing IL-6 was observed with six different transductants of SL1344 and BRD509 (data not shown).

Anti-salmonella immune responses in IL-6-deficient transgenic mice immunized with BRD509. IL-6-deficient (-/-) mice and their counterpart wild-type (+/+) controls were orally immunized with 10¹⁰ CFU of BRD509. The numbers of salmonellae recovered from the spleens of three control mice

FIG. 4. Serum antibody responses in mice infected with BRD509(pKK233-2) (◆) or BRD509(pKK233-2/IL-6) (○). Serum samples were collected prior to infection (□), at day 10 after primary infection, and at day 38, 10 days after boost infection. The amount of LPS-specific antibody present in the sera was determined by a kinetic ELISA with sera diluted 1/50. Individual results are shown for the total serum antibody and IgA responses. mOD, milli-optical density value.

FIG. 6. LPS-specific splenocyte and LPL ELISPOTs from IL-6-deficient and control mice immunized with BRD509. Splenocytes and LPL from the small intestines of IL-6-deficient and control mice were isolated 10 days after a boost infection with salmonellae. The lymphocytes were analyzed for *Salmonella* LPS-specific antibody-secreting cells (ASCs) in an ELISPOT assay. ELISPOTs were developed with antibodies specific for murine IgG (G), IgM (M), or IgA (A) to determine the isotype. Bars represent the individual number of ASCs detected from four IL-6-deficient (–/–) mice and three wild-type (+/+) mice.

and three IL-6-deficient mice on day 10 were found to be equivalent (data not shown). On day 28 the remainder of the mice were given a secondary oral immunization with 10¹⁰ CFU of BRD509. Ten days following the boost, the mice were killed and the splenocytes and LPLs were isolated. The numbers of LPS-specific antibody-secreting splenocytes and LPLs as determined by ELISPOT were comparable in both IL-6-deficient (–/–) and wild-type (+/+) mice, with both groups of mice producing LPS-specific antibody-secreting cells, a high proportion of which produced IgA (Fig. 6).

The mice were bled on days 10, 28, and 38, and ELISAs were performed with the sera. The serum antibody titer was designated as the dilution of specific antibody that gave a level fivefold above the basal level of preimmune sera. A consistent, small but significant decrease ($P < 0.05$) was detected in the IgA antibody titers generated by IL-6-deficient (–/–) mice against LPS compared with those generated by wild-type (+/+) mice (Fig. 7).

DISCUSSION

There is considerable interest in the use of recombinant mucosal pathogens such as attenuated salmonellae as vectors

FIG. 7. IgA antibody raised against LPS by IL-6 knockout and control mice immunized with BRD509. Serum samples were collected prior to infection and on days 10, 28, and 38, and 10 days after boost infection. The amount of LPS-specific antibody present in the sera was determined by ELISA with the serum antibody titer designated as the dilution of specific antibody that gives a value fivefold above the basal level of preimmune sera. The results for individual IL-6 (–/–) mice (◆) and IL-6 (+/+) mice (□) are shown. ↑, oral immunization.

for inducing mucosal immune responses (9, 20). While there are a number of reports outlining good systemic and, occasionally, mucosal immune responses against antigens expressed by salmonellae (9, 12, 19, 29), data from studies of the interaction between salmonella and the mucosal immune system are, by contrast, limited. Our study examines the ability of an aromatic mutant of *S. typhimurium* to induce *S. typhimurium* LPS-specific antibody-secreting cells in the lamina propria, an important effector site for mucosal immune responses, and the ability of bacterial IL-6 expression to influence this response. Our study shows that *S. typhimurium* can elicit potent autologous immune responses and as such holds promise as a vaccine vector. We demonstrate that salmonella-specific B cells are present in the lamina propria of the small intestine at a high concentration after oral administration of the bacterium. Bacterial expression of IL-6, which was stable in vitro but much less so in vivo, did not increase the level of IgA-specific anti-LPS antibody-secreting cells. To date, there are no reports of successful attempts to boost mucosal immune responses against salmonella vaccine vectors through coexpression of cytokines, although other studies have detailed the expression of inflammatory, e.g., IL-1 β (6), or regulatory, e.g., IL-4 (10), cytokines by recombinant *S. typhimurium*. In neither case were antigen-specific responses increased.

BRD509(pKK233-2/IL-6) failed to colonize the spleens of mice by 14 days after oral infection and displayed extremely limited invasiveness in vitro, unlike bacteria which contained only the vector or bacteria which had segregated the expression plasmid containing the IL-6. Nevertheless, strong specific serum antibody responses were recorded, and high numbers of cells secreting anti-LPS antibodies were found in the spleens and small intestinal mucosae of mice fed BRD509(pKK233-2/IL-6). The unexpected finding was that expression of IL-6 appeared to markedly reduce the invasiveness or intracellular survival of recombinant salmonellae in vivo, determined by viable counts of bacteria isolated from spleen cells. It is not known whether the bacteria failed to invade M cells or were cleared in the Peyer's patches prior to reaching the spleen. IL-6 is recognized as a potent activator of the acute-phase response,

and IL-6 expression by bacteria may enhance phagocytic clearance of the bacteria at this site of primary invasion. No similar decrease in virulence was recorded in *S. typhimurium* vectors encoding other cytokines, including IL-1 β (6) or IL-4 (10). Rather, the latter displayed increased virulence in mice, probably because of an inhibitory effect of IL-4 on antibacterial macrophage activity (10).

Previous studies with recombinant salmonella vectors have shown that overexpression of foreign antigens can reduce the residual virulence of *aro* mutants; however, such toxic expression is usually manifest as construct instability in which segregation occurs in vitro and in vivo. In our study, segregation was observed only when selection pressure was placed on the salmonella population, such as in tissue culture invasion assays or during infection in vivo. While the precise role of IL-6 in limiting invasiveness or intracellular survival of salmonellae has yet to be defined, it is intriguing that this immunostimulatory cytokine may also reduce bacterial virulence. There is now clear evidence that IL-6 plays an important role in the development of both systemic and mucosal antibody responses in vivo (17, 24) and can enhance such responses up to 10-fold when expressed as a component of recombinant poxvirus vectors (24, 26). In the latter, the encoded protein is secreted from cells infected by the virus; however, it is important to note that most of the IL-6 produced by recombinant salmonellae in the present study was not secreted from the bacterial cell. Indeed, culture supernatants showed only 10% of the bioactivity of bacterial lysates. It was likely, therefore, that most of the vector-driven IL-6 produced in vivo was not released until lysis of the bacterial cell, including lysis of ingested bacteria occurring within macrophages. This may not have been the optimal expression system for IL-6, and salmonellae specifically engineered to secrete encoded IL-6 may elicit antibody responses of greater magnitude; we are preparing constructs to test this hypothesis.

In light of the finding that salmonellae expressing IL-6 do not induce increased serum Ig or gut antibody-secreting cell responses, the role of IL-6 in the development of salmonella-specific responses was investigated in IL-6-deficient mice. The antibody responses to salmonellae (BRD509) elicited in IL-6-deficient ($-/-$) mice were compared with those in control wild-type ($+/+$) mice. The numbers of LPS-specific antibody-secreting cells isolated from the spleens and laminae propriae of both IL-6-deficient ($-/-$) and wild-type ($+/+$) mice were equivalent, whereas the IgA antibody titer generated against LPS showed a slight, yet significant decrease in IL-6-deficient ($-/-$) animals. This marginal difference could explain why we are unable to see a boosting effect on IgA levels when IL-6 is encoded by salmonellae. In addition, bacterial LPS is a recognized T-cell-independent antigen and, as such, may not require T-cell-derived IL-6 for isotype switching. Indeed, peritoneal cavity B-cell precursors (B1 cells), which populate the gut and are thought to be the major cell subsets responsive to bacterial cell surface antigens such as LPS, do not appear to require IL-6 for IgA production (3). This factor is apparently important for IgA production by conventional mucosal B cells (24), which respond to T-cell-dependent antigens, and we have studies underway to examine the antibody responses generated in IL-6 ($-/-$) mice to a known T-cell-dependent antigen expressed by salmonellae.

The results from studies of poxviruses have shown that immune responses against these viruses and the antigens that they coexpress can be grossly affected by cytokine expression. The advantages that such systems have are that the antigen and cytokine are colocalized to the infected cell and that the cytokine is secreted to reach its target effector cell. Bacterial ex-

pression of cytokines is more problematic, apart from obvious potential differences in glycosylation, folding, and secretion of the eucaryotic cytokine by a procaryote. Bacteria such as salmonellae and mycobacteria reside within cells, particularly within the phagosome of professional phagocytes such as macrophages, and it is likely that cytokines expressed or secreted into this environment may not be optimally effective in modulating immune responses. We are undertaking further studies to determine the most efficient way of using cytokine expression as a means of enhancing the immunogenicity of recombinant bacterial vaccine vectors, including the construction of recombinants engineered to secrete the encoded factor.

ACKNOWLEDGMENTS

Murine recombinant IL-6 was generously donated by Richard Simpson of the Ludwig Institute (Royal Melbourne Hospital), pcD-mIL-6 was donated by Frank Lee (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, Calif.), and monoclonal antibody 20F3 was donated by John Abrams (DNAX). IL-6-deficient ($-/-$) and appropriate control mice were originally provided by Manfred Kopf. Expert help in the performance of ELISPOT analysis was rendered by Jim Vadolas, and *Salmonella typhimurium* BRD509 LPS was purified by Cameron Simmons. Roy Robins-Browne's advice and Vikki Bennett-Wood's assistance in conducting invasion assays were most gratefully received.

This work was supported by The National Health and Medical Research Council of Australia (R.A.S.), The Commonwealth AIDS Research Grants Committee (A.J.R.), and the WHO/UNDP Trans-disease Vaccinology Program (A.J.R.). R.A.S. and A.J.R. are, respectively, members of the Cooperative Research Centre for Vaccine Technology and the Cooperative Research Centre for the Biological Control of Vertebrate Pest Populations.

REFERENCES

1. Aarden, L. A., E. R. De Groot, O. L. Schaap, and P. M. Lansdorp. 1987. Production of hybridoma growth factor by human monocytes. *Eur. J. Immunol.* **17**:1411-1416.
- 1a. Allen, J. S. Unpublished data.
2. Amann, E., and J. Brosius. 1985. 'ATG vectors' for regulated high-level expression of cloned genes in *Escherichia coli*. *Gene* **40**:183-190.
3. Beagley, K. W., S. Bao, A. J. Ramsay, J. H. Eldridge, and A. J. Husband. 1995. IgA production by peritoneal cavity B cells is IL-6 independent: implications for intestinal IgA responses. *Eur. J. Immunol.* **25**:2123-2126.
4. Beagley, K. W., J. H. Eldridge, F. Lee, H. Kiyono, M. P. Everson, W. J. Koopman, T. Hirano, T. Kishimoto, and J. R. McGhee. 1989. Interleukins and IgA synthesis. Human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells. *J. Exp. Med.* **169**:2133-2148.
5. Bullas, L. R., and J. I. Ryu. 1983. *Salmonella typhimurium* LT2 strains which are $r^{-}m^{+}$ for all three chromosomally located systems of DNA restriction and modification. *J. Bacteriol.* **156**:471-474.
6. Carrier, M. J., S. N. Chatfield, G. Dougan, U. T. A. Nowicka, D. O'Callaghan, J. E. Beesley, S. Milano, E. Cillari, and F. Y. Liew. 1992. Expression of human IL-1 β in *Salmonella typhimurium*. A model system for the delivery of recombinant therapeutic proteins in vivo. *J. Immunol.* **148**:1176-1181.
7. Chiu, C.-P., C. Moulds, R. L. Coffman, D. Rennick, and F. Lee. 1988. Multiple biological activities are expressed by a mouse interleukin-6 cDNA clone isolated from bone marrow stromal cells. *Proc. Natl. Acad. Sci. USA* **85**:7099-7103.
8. Cohen, J. 1993. AIDS research: the mood is uncertain. *Science* **260**:1254-1255.
9. Curtiss, R., R. Goldschmitt, R. Pastian, M. Lyons, S. M. Michalek, and J. Mestecky. 1986. Cloning virulence determinants from *Streptococcus mutans* and the use of recombinant clones to construct bivalent oral vaccine strains to confer protective immunity against *S. mutans*-induced dental caries, p. 173-180. In S. Hamada, S. M. Michalek, H. Kiyono, L. Menaker, and J. K. McGhee (ed.), *Molecular microbiology and immunology of Streptococcus mutans*. Elsevier, Amsterdam.
10. Denich, K., P. Borlin, P. D. O'Hanley, M. Howard, and A. W. Heath. 1993. 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. *Infect. Immun.* **61**:4818-4827.
11. Dunkley, M. L., and A. J. Husband. 1989. Role of antigen in migration patterns of T cell subsets arising from gut-associated lymphoid tissue. *Reg. Immunol.* **2**:213-224.
12. Fairweather, N. F., S. N. Chatfield, A. J. Makoff, R. A. Strugnell, J. Bester,

- D. J. Maskell, and G. Dougan. 1990. Oral vaccination of mice against tetanus by use of a live attenuated *Salmonella* carrier. *Infect. Immun.* **58**:1323–1326.
13. Fletcher Starnes, H., M. K. Pearce, A. Tewari, J. H. Yim, J. Zou, and J. S. Abrams. 1990. Anti-IL-6 monoclonal antibodies protect against lethal *Escherichia coli* infection and lethal tumor necrosis factor- α challenge in mice. *J. Immunol.* **145**:4185–4191.
 14. Hoiseth, S. K., and B. A. D. Stocker. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature (London)* **291**:238–239.
 15. Jones, P. W., G. Dougan, C. Hayward, N. Mackensie, P. Collins, and S. N. Chatfield. 1991. Oral vaccination of calves against experimental Salmonellosis using a double *aro* mutant of *Salmonella typhimurium*. *Vaccine* **9**:29–34.
 16. Kaufmann, S. H. E., and C. H. Ladel. 1994. Application of knockout mice to the experimental analysis of infections with bacteria and protozoa. *Trends Microbiol.* **7**:235–242.
 17. Kopf, M., H. Baumann, G. Freer, M. Freudenberg, M. Lamers, T. Kishimoto, R. Zinkernagel, H. Bluethmann, and G. Koehler. 1994. Impaired immune and acute phase responses in IL-6-deficient mice. *Nature (London)* **368**:339–342.
 18. Kung, V. T., and G. M. K. Humphries. 1987. Kinetic ELISA in microtitre plates. *Clin. Chem.* **33**:1573–1574.
 19. Maskell, D. J., K. J. Sweeney, D. O'Callaghan, C. E. Hormaeche, F. Y. Liew, and G. Dougan. 1987. *Salmonella typhimurium aroA* mutants as carriers of the *Escherichia coli* heat-labile enterotoxin B subunit to the murine secretory and systemic immune systems. *Microb. Pathog.* **2**:211–221.
 20. McGhee, J. R., J. Mestecky, M. T. Dertzbaugh, J. H. Eldridge, M. Hirasawa, and H. Kiyono. 1992. The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* **10**:75–88.
 21. Ndububa, D. A., G. E. Erhabor, and D. O. Akinola. 1992. Typhoid and paratyphoid fever: a retrospective study. *Trop. Gastroenterol.* **13**:56–63.
 22. O'Callaghan, D., D. Maskell, J. Tite, and G. Dougan. 1990. Immune responses in BALB/c mice following immunization with aromatic compound or purine-dependent *Salmonella typhimurium* strains. *Immunology* **69**:184–189.
 23. Okada, M., N. Sakaguchi, N. Yoshimura, H. Hara, K. Shimizu, N. Yoshida, K. Yoshizaki, S. Kishimoto, Y. Yamamura, and T. Kishimoto. 1983. B cell growth factors and B cell differentiation factor from human T hybridomas. Two distinct kinds of B cell growth factor and their synergism in B cell proliferation. *J. Exp. Med.* **157**:583–590.
 24. Ramsay, A. J., A. J. Husband, I. A. Ramshaw, S. Bao, K. I. Matthaei, G. Koehler, and M. Kopf. 1994. The role of interleukin-6 in mucosal IgA antibody responses in vivo. *Science* **264**:561–563.
 25. Ramsay, A. J., and M. Kohonen-Corish. 1993. Interleukin-5 expressed by a recombinant virus vector enhances specific mucosal IgA responses in vivo. *Eur. J. Immunol.* **23**:3141–3145.
 26. Ramsay, A. J., I. A. Ramshaw, and M. Kopf. 1994. Enhancement of specific mucosal IgA responses by interleukins 5 and 6 encoded in recombinant vaccine vectors, p. 35–39. *In* F. Brown, R. Chanock, H. Ginsberg, and E. Norrby (ed.), *Vaccines 94*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 27. Robins-Browne, R. M., and V. Bennett-Wood. 1992. Quantitative assessment of the ability of *Escherichia coli* to invade cultured animal cells. *Microb. Pathog.* **12**:159–164.
 28. Schödel, F., S. M. Kelly, D. L. Peterson, D. R. Milich, and R. Curtiss III. 1994. Hybrid hepatitis B virus core pre-S proteins synthesized in avirulent *Salmonella typhimurium* and *Salmonella typhi* for oral vaccination. *Infect. Immun.* **62**:1669–1676.
 29. Schödel, F., and H. Will. 1990. Expression of hepatitis B virus antigens in attenuated salmonellae for oral immunization. *Res. Microbiol.* **141**:831–837.
 30. Sedgwick, J. D., and P. G. Holt. 1983. A solid phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. *J. Immunol. Methods* **57**:301–309.
 31. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
 32. Tsang, V. C., R. C. Wilson, and J. M. Peralta. 1983. Quantitative single tube kinetic dependent K-ELISA assay. *Methods Enzymol.* **92**:391–403.
 33. Turner, S. J., F. R. Carbone, and R. A. Strugnell. 1993. *Salmonella typhimurium* $\Delta aroA \Delta aroD$ mutants expressing a foreign recombinant protein induce specific major histocompatibility complex class I-restricted cytotoxic T cells in mice. *Infect. Immun.* **61**:5374–5380.
 34. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides extraction with phenol-water and further applications of the procedure. *Methods Carbohydr. Chem.* **5**:83–91.

Editor: J. R. McGhee