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Characterization of a *Salmonella typhimurium aro* Vaccine Strain Expressing the P.69 Antigen of *Bordetella pertussis*

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The P.69 *Bordetella pertussis* protective antigen was expressed by use of the *trc* promoter from the chromosome of a *Salmonella typhimurium aro* vaccine strain, BRD509, by integrating the *prn* gene, encoding the 93-kDa precursor of this protein, into the *aroC* locus. P.69 was detected on the cell surface of the *S. typhimurium* strain (BRD640) by agglutination and immunoelectron microscopy. BALB/c mice immunized orally or intravenously with BRD640 showed a significant level of protection against an aerosol challenge with virulent *B. pertussis*, compared with control animals. No anti-P.69 antibodies in the serum or anti-P.69 antibody-secreting cells in the lungs were detected in BRD640-vaccinated animals, although cells isolated from spleens showed a P.69-dependent cell proliferative response. In contrast, low levels of anti-P.69 antibodies in the serum and anti-P.69 antibody-secreting cells in the lungs were detected in immunized mice following a *B. pertussis* challenge.

Whooping cough is a serious respiratory disease caused by *Bordetella pertussis* and, more rarely, *B. parapertussis*. Although the current whooping cough vaccine, composed of killed whole cells of *B. pertussis*, has greatly reduced the incidence of whooping cough in the United Kingdom and United States (7, 29, 42), fears concerning the side effects associated with vaccination have led to routine immunization being discontinued in many countries. As a consequence, less toxic acellular pertussis vaccines are being developed as alternatives to the current vaccine (29, 42). Both the currently available whole-cell vaccine and acellular vaccines are administered parenterally, and although they can elicit protection against the clinical manifestations of *B. pertussis* infection, they do not appear to be effective at preventing infection per se (21). Thus, the vaccines do not prevent the spread of infection and therefore do not elicit herd immunity. Vaccines that evoke local immune responses in the respiratory tract may be more effective in this regard.

The concept of the common mucosal immune system indicates that it should be feasible to vaccinate orally and stimulate local immunity at distant sites, such as the respiratory tract (5). The use of oral vaccines would also obviate the use of needles and the concomitant risk of blood-borne diseases, such as hepatitis B and human immunodeficiency virus. Live attenuated strains of *Salmonella* spp. have recently attracted much attention as potential oral vaccines against salmonellosis and as carriers of heterologous antigens to the immune system (11, 20, 30). Virulent *Salmonella* strains can be attenuated by the introduction of defined mutations into genes that are essential for the organisms to establish a clinically significant infection. Examples of attenuating lesions include genes involved in metabolism, such as *aro* genes, which are involved in aromatic compound biosynthesis (10, 13, 22, 24, 31, 33), genes involved in the global regulation of gene expression, such as *ompR* (18), and *cya* and *crp* (15). One attenuated strain of *Salmonella typhi*, known as Ty21a, is already licenced for use as an oral typhoid vaccine in many countries (28). Of the genetically defined attenuated strains available for experimental use, perhaps *aro* mutants have been studied most extensively. *S. typhimurium aro* mutants have been shown to be effective oral vaccines in mice (22, 24, 30, 31, 35), sheep (33), chickens (13), and cattle (23, 48) and are currently being evaluated in human volunteers (10). Oral vaccination with *S. typhimurium aro* mutants can induce secretory, humoral, and cellular immune responses (24, 30) as well as a period of short-term, nonspecific immunity that lasts several weeks after immunization and protects mice against a challenge with antigenically distinct organisms, such as *Listeria monocytogenes* (36). *S. typhimurium aro* mutants have been used as carriers to deliver to the immune system a variety of heterologous antigens, such as the B subunit of the *Escherichia coli* heat-labile toxin (30), the circumsporozoite antigen of plasmodia (2, 43), and the tetanus toxin C fragment (20).

P.69 is a surface-located protein of *B. pertussis* that may play a role in the adhesion to or invasion of eucaryotic cells by *B. pertussis* (27, 39). Purified preparations of P.69, natural or recombinant, can be used as vaccines to induce protective immune responses in experimental animals (41, 47). Thus, P.69 is being considered a candidate antigen for inclusion in new acellular whooping cough vaccine preparations. P.69 is encoded by the *prn* gene and is synthesized as a 93-kDa precursor, which is processed to yield P.69 (8). As a step towards the development of an oral whooping cough vaccine, we describe in this paper the expression of the *prn* gene from the chromosome of an *S. typhimurium aro* vaccine strain and the characterization of this strain as an experimental oral vaccine against *B. pertussis* infection in mice.

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MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. *S. typhimurium* LB5010 *galE* ($r^- m^+$) has been described (6). BRD509 is an *aroA aroD S. typhimurium* strain derived from *S. typhimurium* SL1344 (22) and was constructed as follows. An *aroA* deletion was introduced in *S. typhimurium* SL1344 by transduction with a P22 lysate prepared on C5 *aroA554*::Tn*10* as previously described (22). One isolate that was aromatic compound dependent was used to prepare tetracycline-sensitive derivatives by selection on modified Bochner medium (35). A tetracycline-sensitive, aromatic compound-dependent isolate was used to prepare the double *aro* mutant as follows. An *aroD* deletion was introduced into this isolate by transduction with a P22 lysate prepared on C5 *aroD553*::Tn*10* as previously described (31). Tetracycline-sensitive derivatives were isolated after selection on Bochner medium. Several of these were transformed with plasmid pAB51, which harbors the intact *aroA* gene from *S. typhimurium*. This plasmid has been shown to be able to complement deletions in *aroA* (10). One isolate that was still aromatic compound dependent when it harbored pAB51 was designated BRD509. It was consequently demonstrated in Southern hybridization studies that BRD509 harbored deletions in both *aroA* and *aroD* (data not shown). BRD207 is an *S. typhimurium* LT2 derivative harboring a *polA* mutation and is thus unable to support the replication of ColE1-derived plasmids (22, 48).

B. pertussis BBC26, a streptomycin-resistant derivative of strain CN2992 (39), was used in aerosol challenge experiments.

Chromosomal integration vector pDEL2 has been described extensively elsewhere (49). Plasmid pAYL1 is a pKK233-2 (Pharmacia, Scunthorpe, United Kingdom)-based plasmid encoding the *prn* gene. *prn* is encoded on a ca. 3.0-kb *Afl*III-*Mlu*I fragment of *B. pertussis* DNA. A *Hin*dIII linker was added to the *Mlu* end, and the fragment was cloned into the *Nco*I-*Hin*dIII site on pKK233-2 to create plasmid pAYL1. The *prn* gene in pAYL1 is under the control of the *trc* promoter. High-level expression of P.93 is lethal in *E. coli*, and the plasmid is unstable in the absence of antibiotics.

Growth conditions, immunization, and in vivo growth. *S. typhimurium* strains were grown statically overnight in L broth (17), recovered by centrifugation, and resuspended in sterile phosphate-buffered saline (PBS) (pH 7.2) to approximately 1×10^{10} to 5×10^{10} CFU/ml for oral immunization and to approximately 1×10^5 CFU/ml for intravenous (i.v.) immunization. The cell suspension (0.2 ml) was administered to 6- to 8-week-old female BALB/c mice (Charles River, Margate, United Kingdom) by gavage tube for oral immunization or by tail injection for i.v. immunization as described previously (30). Viable counts were performed on all inocula.

The course of the colonization of murine tissue was monitored by performing viable counts on homogenates of spleens, livers, mesenteric lymph nodes, and Peyer's patches removed at intervals after immunization as previously described (30, 37). Sera were obtained by tail bleed or heart puncture on various days.

Western blotting (immunoblotting). *S. typhimurium* strains with and without the *prn* gene were grown to the mid-log phase in L broth. The optical densities at 650 nm of the cultures were determined and adjusted so that all cultures contained equal numbers of cells. Cells were pelleted and lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and the proteins were separated by SDS-PAGE (26). The proteins were electroblotted to nitrocellulose membranes and probed with P.69-specific monoclonal antibody BB05 (51). After the addition of a rabbit anti-mouse immunoglobulin–horseradish peroxidase conjugate (1/1,000; DAKO, High Wycombe, United Kingdom), the bands were visualized with 4-chloro-1-naphthol (Sigma) as the substrate.

Immunoelectron microscopy. Bacteria were prepared for and analyzed by electron microscopy as previously described (4). Bacteria were probed before or after being embedded with P.69-specific monoclonal antibody F6E5 and then with 10-nm protein A-gold probes (Bioclinical Services, Cardiff, United Kingdom).

Aerosol challenge. Immunized and control mice were challenged by exposure to aerosols of *B. pertussis* BBC26, and the course of the infection in the lungs of infected mice was determined by performing viable counts on lung homogenates as previously described (40).

ELISA. The serum immune response to P.69 and lipopolysaccharide (LPS) was determined by an enzyme-linked immunosorbent assay (ELISA). The P.69 ELISA has been described (41). In brief, 96-well microtiter plates (EIA; Costar, NBL, Northumbria, United Kingdom) were coated with recombinant P.69 (50 μl of a 1-μg/ml solution in PBS). Appropriate dilutions of serum were added and, after a wash, an anti-mouse immunoglobulin-horseradish peroxidase conjugate (1/1,000; DAKO) was added. *O*-Phenylenediamine was used as the substrate, and optical densities at 492 nm were determined in a Titertek Multiscan MCC ELISA reader. The assay for anti-LPS responses was performed identically, except that the plates were coated with *S. typhimurium* LPS (50 μl of a 1-μg/ml solution in PBS; Sigma). Titers were taken to be the highest dilution of a serum sample giving a reading twice that of a similarly diluted serum sample obtained prior to immunization; values below 0.1 were not included.

Assay for P.69-specific antibody-secreting cells (ASC) in murine lungs. Local antibody production in murine lungs was determined by the ELISPOT technique (16, 45). Lymphocytes were isolated from murine lungs as follows. Lungs were washed briefly in PBS to remove traces of blood and then were finely chopped with a scalpel blade. One milliliter of PBS containing 10 mM $MgCl_2$, 0.5 U of collagenase A (Boehringer Mannheim, Lewes, United Kingdom) per ml, and 0.25 mg of DNase I (Boehringer) per ml was added for each pair of lungs, and the mixture was incubated at 37°C with gentle agitation for 45 min. The mixture was then passed through 40-gauge mesh. Lumps were pressed through the mesh with the plunger from a 5-ml syringe. The cell suspension was placed in a centrifuge tube and allowed to stand for several minutes to allow large debris to settle. The supernatant was removed, and the cells were pelleted and washed several times. Erythrocytes and nonviable cells were removed by centrifugation on a Ficoll-Isopaque gradient (LSM; Flow Laboratories Ltd., Hertfordshire, United Kingdom). After a wash, cell viability was determined by trypan blue exclusion. Cells were finally suspended in RPMI 1640 complete medium (10% fetal calf serum, penicillin [100 IU/ml], streptomycin [100 μg/ml], 2 mM L-glutamine; Flow).

The ELISPOT assay was performed essentially as described previously (45). In brief, 24-well tissue culture plates (Costar) were coated overnight with recombinant P.69 (0.5 ml of a 1-μg/ml solution in PBS); after washing and blocking were done, 0.5-ml volumes of dilutions of the lymphocyte suspensions in RPMI 1640 complete medium were added to

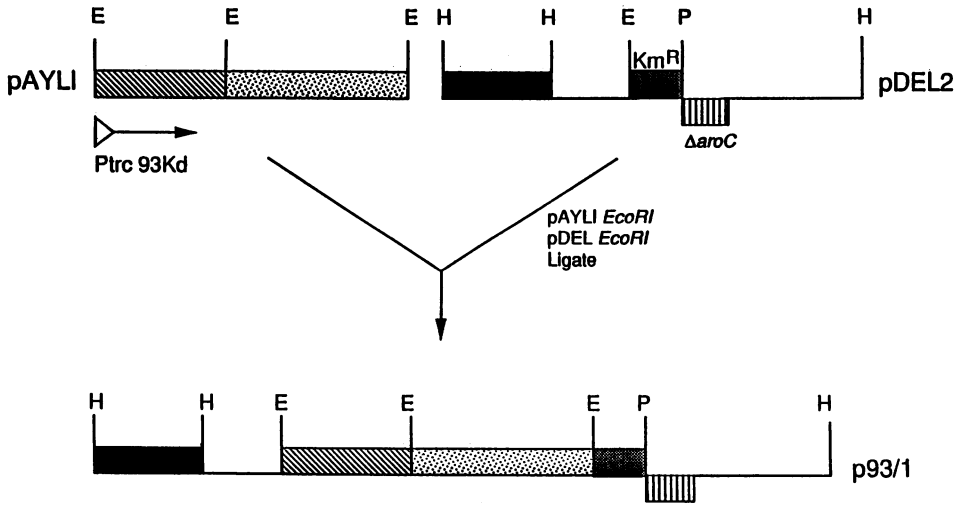

FIG. 1. Construction of the *prn* integration vector plasmid. pAYL1 comprises pKK233-2 (▨) and a ca. 3.0-kb fragment of *B. pertussis* genomic DNA encoding the *prn* gene (▧). pDEL2 is the chromosomal integration vector; symbols: ■, pUC18 vector sequences; ——, *S. typhimurium* chromosomal DNA. The positions of the truncated *aroC* gene and the Km^r cassette are also shown. Both plasmids were digested with *Eco*RI and ligated to form pP.93/1. Abbreviations: E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II.

the wells and incubated at 37°C in 10% CO_2 for 3 h. After a wash, goat anti-mouse immunoglobulin G (IgG), IgA, or IgM (1/1,000; Sigma) and rabbit anti-goat IgG–alkaline phosphatase (1/1,000; Sigma) were added sequentially. Finally, the substrate solution (0.5 μl of a 1-mg/ml 5-bromo-4-chloro-3-indolyl phosphate solution in 2-amino-2-methyl-1-propanol buffer [Sigma]) was added, and the plates were incubated until blue spots were visible under low-power microscopy.

T-cell proliferation assay. Spleens were removed from immunized mice, and cells were freed by washing of the organs between the frosted ends of glass slides. Large debris was removed, and erythrocytes were hypotonically lysed. Proliferation assays were performed on washed lymphocytes as previously described (50).

RESULTS

Expression of P.69 from the chromosome of *S. typhimurium aroA* vaccine strain BRD509. P.69 is a processed protein derived from a 93-kDa precursor by the proteolytic removal of an N-terminal signal sequence and cleavage at the carboxy terminus (8). The carboxy-terminal region of P.93 is believed to assist in the translocation of the P.69 polypeptide to the cell surface (unpublished results). pAYL1 is a pKK233-2-based recombinant plasmid that encodes the complete *prn* gene with the ATG initiation codon inserted at the *Nco*I site of pKK233-2 (Fig. 1). P.93 is expressed from the *trc* promoter in this construct. *E. coli* cells harboring pAYL1 exhibit plasmid instability. Thus, to stabilize the expression of *prn*, we integrated the gene into the *S. typhimurium* chromosome by using an *aroC*-based chromosomal integration system that has been described (49). pAYL1 DNA was digested with *Eco*RI, and fragments were cloned into the single *Eco*RI site on *aroC*-based integration vector pDEL2 and transformed into *E. coli* TG1. Only a few kanamycin-resistant (Km^r) colonies were obtained. Plasmids isolated from transformants all had the same physical structure (Fig. 1), with the entire pAYL1 plasmid being inserted into pDEL2. Plasmids containing only the *Eco*RI fragment encoding P.93 were not isolated. Several attempts to obtain such constructs were unsuccessful; the reason is unknown. The pKK233-2 vector carries the strong *rrnB* transcriptional terminator, and it is possible that the isolation of the *prn* gene from *rrnB* results in lethal readthrough into adjacent genes. One of the isolated plasmids, pP.93/1, was selected for further study and was introduced into DNA polymerase I-deficient *S. typhimurium* BRD207 by electroporation. Since pP.93/1 was constructed with a ColE1 replicon and requires DNA polymerase I for replication, it could not replicate in BRD207, and colonies selected for kanamycin resistance were chromosomal integrants. Chromosomal integration into *aroC* was verified by Southern blotting (data not shown). A bacteriophage P22 lysate was prepared from this strain and used to transduce the *prn* gene into *S. typhimurium galE* mutant LB5010 and *aroA-aroD* strain BRD509. Single Km^r BRD509 and LB5010 colonies were isolated and shown to produce P.69 by Western blotting (see below). Analysis of chromosomal DNA from BRD509 (*prn*) and LB5010 (*prn*) by Southern blotting revealed the disruption of *aroC* and the presence of the integrated *prn* gene in the *aroC* locus of BRD509 (Fig. 2).

Characterization of *S. typhimurium prn* strains. *E. coli* strains harboring pAYL1 export process P.69 protein outside the cell, where it remains attached in significant quantities to the cell surface. Such *E. coli* cells can readily be agglutinated with anti-P.69 agglutinating sera (data not shown). BRD509 and LB5010 cells harboring the *prn* gene (BRD640 and BRD641, respectively) were tested for their ability to be agglutinated by polyclonal anti-P.69 sera. BRD641 cells were agglutinated strongly, whereas LB5010 cells failed to be agglutinated. In comparison, BRD640 cells were agglutinated only weakly and variably, whereas BRD509 cells failed to be agglutinated. Poor agglutination of BRD640 may have been due to the presence of smooth LPS at the surface of these cells, compared with BRD641, which is a rough strain.

Western blotting was used to detect P.69 polypeptide in the *S. typhimurium* strains. Both BRD640 and BRD641 expressed significant levels of processed P.69 protein, whereas BRD509 and LB5010 expressed no detectable P.93 or P.69 polypeptide (Fig. 3). This result further suggests that the LPS of BRD640 (*prn*) interferes with agglutination, since

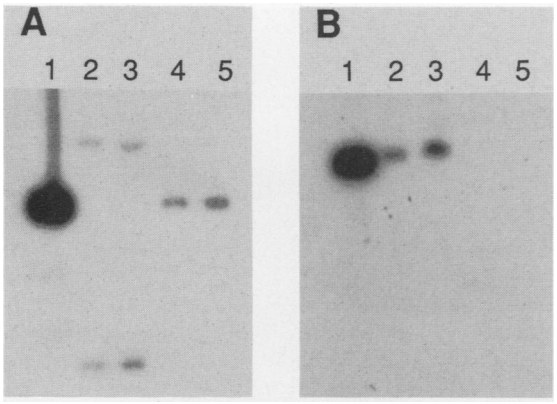

FIG. 2. Southern blot showing the disruption of *aroC* (A) and the presence of *prn* in P.69-containing *S. typhimurium*. (A) Chromosomal DNA (4 μg) from *S. typhimurium* strains digested with *Hin*dIII and probed with a ^{32}P-labelled 5.8-kb *Hin*dIII fragment of *S. typhimurium* genomic DNA containing the *aroC* gene. Lanes: 1, positive control, probe fragment (0.5 μg); 2, BRD640 (BRD509 *prn*); 3, BRD641 (LB5010 *prn*); 4, BRD509; 5, LB5010. (B) Chromosomal DNA digested with *Pst*I (4 μg) and probed with a 1.8-kb *Pst*I fragment of *B. pertussis* DNA from within the *prn* gene. Lanes: 1, positive control, 0.5 μg of probe DNA; 2 to 5, as in panel A.

this strain expresses levels of P.69 comparable to those expressed by BRD641 (*prn*). BRD640 was agglutinated well by anti-O4 and -O5 *S. typhimurium* typing serum, which is directed against LPS, and smooth LPS ladders were detected in silver-stained polyacrylamide gels (data not shown).

Further confirmation that processed P.69 protein was exported to the BRD640 cell surface was obtained by immunoelectron microscopy (Fig. 4). BRD640 and BRD509 cells were prepared and labelled with anti-P.69 mouse antibody and protein A-gold probes as described in Materials and Methods. In the postembedding labelled cells (Fig. 4A and B), the probes were associated with the outer membrane of BRD640 but not BRD509. Very few probes were found inside the cells; most were at the outer surface. Negative staining (Fig. 4C and D) revealed an even distribution of the probes over the surface of BRD640. These results show that P.69 is correctly localized in *S. typhimurium* and that the protein is at least partially expressed on the surface of BRD640.

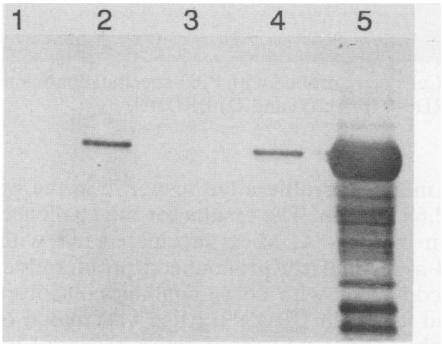

FIG. 3. Expression of P.69 in *S. typhimurium*. Whole-cell extracts of *S. typhimurium* with and without the *prn* gene were separated by SDS-PAGE, electroblotted to nitrocellulose membranes, and probed with P.69-specific monoclonal antibody BB05. Lanes: 1, BRD509; 2, BRD640 (BRD509 *prn*); 3, LB5010; 4, BRD641 (LB5010 *prn*); 5, purified P.69 (1 μg).

Characterization of BRD640 in vivo. Since P.69 was clearly surface located when expressed from the full P.93 gene in *S. typhimurium*, the ability of BRD640 to colonize the reticuloendothelial system of mice following oral or i.v. inoculation was examined. BRD640 and BRD509 were inoculated orally into groups of BALB/c mice, and the Peyer's patches, mesenteric lymph nodes, livers, and spleens of infected mice were examined on various days after inoculation. The results are shown in Table 1. It is clear that both BRD640 and BRD509 were able to colonize all organs examined with similar efficiencies. Thus, the expression of P.69 at the *S. typhimurium* cell surface does not impair the ability of the bacteria to translocate from the gut into deeper tissues. Selected animals that had been vaccinated with BRD640 were challenged with mouse-virulent *S. typhimurium* SL1344 4 and 8 weeks after oral or intravenous vaccination. These mice were found to be well protected against these challenges (data not shown), suggesting that the expression of P.69 at the *S. typhimurium* cell surface does not significantly interfere with the murine immune response to the *S. typhimurium* vaccine strain.

Aerosol *B. pertussis* challenge of mice vaccinated with BRD640 or BRD509. Groups of mice vaccinated orally or i.v. with BRD640 or BRD509 were aerosol challenged with *B. pertussis* to assess whether they were protected. Protection in this model is assessed by the ability of immunized animals to clear *B. pertussis* from their lungs on the days following the aerosol challenge (40). The results of a typical experiment are shown in Fig. 5A. In groups of mice vaccinated i.v. with BRD509 or saline, *B. pertussis* multiplied, reaching a peak at day 7. Animals orally vaccinated with BRD509 had a reduced level of colonization at 7 days after challenge compared with the other two control group animals. This result may have been due to a nonspecific protective mechanism displayed in orally vaccinated animals. This nonspecific protection disappeared when the aerosol challenge was delayed for several weeks after the final vaccination (Fig. 5B). Groups of mice vaccinated orally or i.v. with BRD640 consistently displayed evidence of protection against an aerosol challenge with *B. pertussis*. *B. pertussis* failed to grow in the lungs of these mice and was consistently present at a lower level than in the lungs of the various control group mice.

Immune response to P.69 delivered by BRD640 to the murine immune system. Initially, mice were examined for the presence of serum antibodies to P.69 by an ELISA. Surprisingly, no serum response to P.69 was detected in mice immunized orally or i.v. with BRD640 prior to an aerosol challenge (Table 2). A strong serum anti-*S. typhimurium* LPS response was present in mice immunized i.v. or orally with BRD509 or BRD640 (data not shown). This result correlates well with the finding that these animals were protected against salmonellosis.

Local B-cell responses in the lungs of vaccinated mice were examined by the ELISPOT assay. No anti-P.69 ASC were detected in vaccinated animals prior to a challenge with *B. pertussis* (data not shown).

The serum of vaccinated and control animals that had been challenged with an aerosol of *B. pertussis* cells was examined for anti-P.69 antibodies. An anti-P.69 antibody response was detected in the serum of all groups of mice (Table 2). The antibody titer was twofold higher in groups of mice that had been vaccinated with BRD640 than in those that had been vaccinated with BRD509. This result suggests

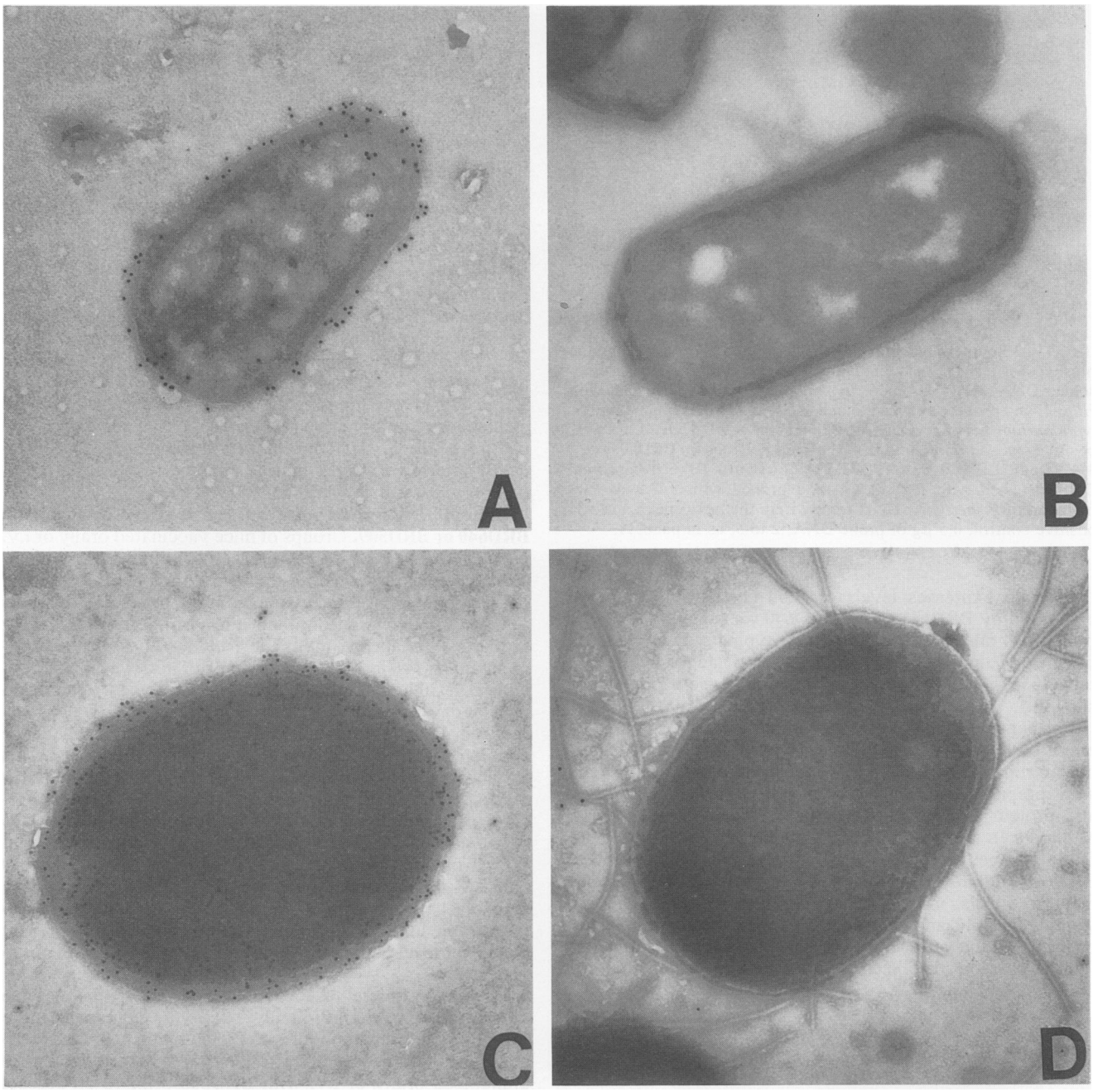

FIG. 4. Localization of P.69 in *S. typhimurium* by immunoelectron microscopy. Cells were probed with P.69-specific monoclonal antibody F6E5 postembedding (A and B) or with negative staining (C and D). (A and C) BRD640 (*prn*); (B and D) BRD509.

that some priming may have occurred during vaccination. However, this response was much lower than might have been expected for animals vaccinated twice. The serum antibody response is much higher in mice vaccinated with purified P.69 (41).

Small numbers of anti-P.69 ASC appeared in the lungs of all groups of mice after an aerosol challenge (Table 3). The levels of responses were similar in all groups of animals, with a preponderance of IgM-secreting cells. This result probably represents a primary response to P.69 present in the *B. pertussis* challenge. Cells were prepared from the spleens of vaccinated and challenged animals and assayed for their ability to mount a proliferative response in the presence of purified P.69 antigen. The results for the challenged animals are shown in Table 4. Mice vaccinated i.v. with BRD640 displayed a particularly pronounced proliferative response to P.69, compared with corresponding control group mice vaccinated i.v. with BRD509. Mice vaccinated orally with BRD640 showed a lower proliferative response to P.69, but it was still higher than that of corresponding control group mice vaccinated orally with BRD509. Thus, the mounting of a T-cell proliferative response to P.69 correlates well with protection of mice against a *B. pertussis* challenge. Parallel results were obtained with spleen cells isolated from mice

TABLE 1. Growth of *S. typhimurium* in the organs of mice following oral inoculation[a]

Strain	Day	Mean log CFU/organ (±2 SEM) in[b]:			
		PP	MLN	Livers	Spleens
BRD640	7	4.0 (0.1)	2.6 (0.5)	2.7 (0.8)	2.6 (1.3)
BRD509	7	3.5 (0.2)	3.1 (0.8)	1.35 (0.5)	1.0 (0.2)
BRD640	28	0.8 (0.5)	1.72 (0.2)	1.0 (0.8)	0.5 (0.2)
BRD509	28	1.1 (0.5)	2.0 (0.1)	0.7 (0.6)	0.4 (0.1)

[a] Mice received 2.45×10^{10} CFU of BRD640 or 1.3×10^{10} CFU of BRD509 orally on day 0.
[b] Mean counts for groups of four mice. PP, Peyer's patches; MLN, mesenteric lymph nodes.

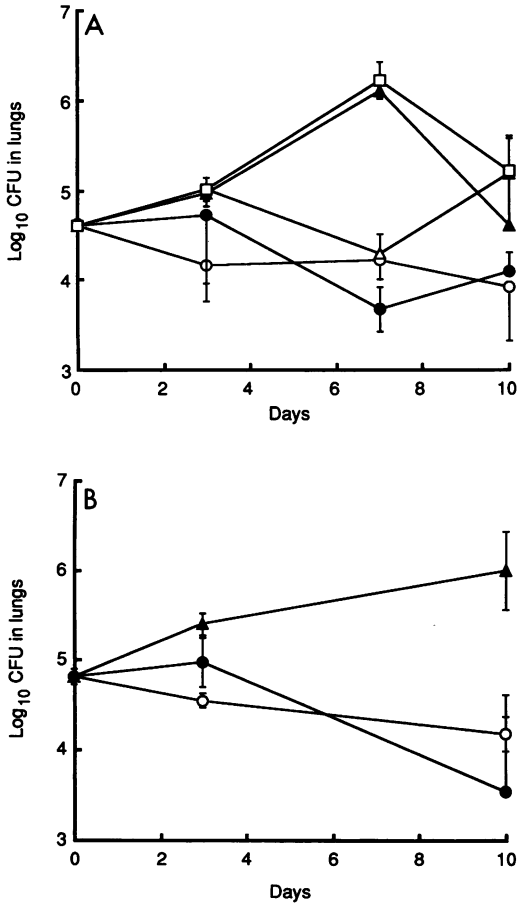

FIG. 5. Growth of *B. pertussis* in the lungs of mice challenged 14 days (A) or 28 days (B) after the final immunization with *S. typhimurium*. (A) Mice were immunized orally or i.v. on days 0 and 28 with either BRD640 (*prn*) or BRD509. Orally immunized mice received 3×10^9 to 5×10^9 CFU per dose, and i.v. immunized mice received ca. 1×10^4 CFU per dose. Mice were aerosol challenged with *B. pertussis* 14 days (day 42) after the second immunization. Symbols: ○, oral BRD640; △, oral BRD509; ●, i.v. BRD640; ▲, i.v. BRD509; □, control. (B) Mice were immunized orally with ca. 5×10^9 CFU of BRD509 or BRD640. The BRD640-immunized mice were split into two groups, and one group and the BRD509-immunized mice were boosted orally on day 28. Mice were aerosol challenged 28 days later (day 56). Symbols: ○, one dose of BRD640; ●, two doses of BRD640; ▲, BRD509. Points represent the mean counts for homogenates from four pairs of lungs (±2 standard errors of the mean).

TABLE 2. Serum antibody response to P.69 in mice immunized with BRD509 or BRD640

Group	Titer on the following days after final immunization[a]:			
	8	17	24	31
Oral BRD640	<100	200	800	800
Oral BRD509	<100	<100	400	400
i.v. BRD640	<100	200	800	800
i.v. BRD509	<100	100	400	400
Control	<100	100	400	400

[a] Mice were aerosol challenged with *B. pertussis* on day 14.

that were identically immunized but not challenged (data not shown).

DISCUSSION

In this paper, we describe the construction and characterization in vivo of an *S. typhimurium aro* mutant expressing the protective P.69 antigen of *B. pertussis*. The P.69 polypeptide is a processed product derived by cleavage of a 93-kDa precursor protein (8). The generation of P.69 in vivo involves the removal of the carboxy-terminal portion of P.93, which is required for secretion across the bacterial cell envelope. Hence, we inserted the entire *prn* gene, expressed from the *trc* promoter, into the *S. typhimurium* chromosome at the *aroC* locus. This integration step resulted in the disruption of the *aroC* gene. Thus, BRD640 harbors three independent attenuating *aro* lesions. The *prn* gene was efficiently expressed in *S. typhimurium*, and fully processed P.69 protein was exported to the cell surface, since *S. typhimurium* strains expressing P.69 could be agglutinated with polyclonal anti-P.69 sera. Interestingly, BRD640, which expresses smooth LPS, was agglutinated much less efficiently than rough strain BRD641. Thus, P.69 antigen was apparently significantly masked by LPS side chains at the *S. typhimurium* cell surface. This fact could partially explain the inefficient induction of anti-P.69 antibodies by BRD640.

Purified P.69 is immunogenic in mice and can induce significant protection against an aerosol challenge with virulent *B. pertussis* organisms. Native P.69 purified from *B. pertussis* or recombinant P.69 purified from *E. coli* (41), yeast (unpublished observation), or baculovirus (unpublished observation) expression systems is protective in this model. Mice vaccinated with BRD640 displayed an enhanced ability to clear *B. pertussis* cells following an aerosol challenge, compared with controls. This protection could not be correlated with the presence of anti-P.69 antibodies in the serum or anti-P.69 ASC in the lungs of the mice, since

TABLE 3. Anti-P.69 ASC in the lungs of mice 12 days after an aerosol challenge

Group	No. of ASC of the following classes/10^8 lymphocytes:		
	IgG	IgA	IgM
Oral BRD640	100	80	150
Oral BRD509	105	100	421
i.v. BRD640	307	100	154
i.v. BRD509	ND[a]	ND	ND
Control	91	182	273

[a] ND, not detected.

TABLE 4. Proliferation of spleen cells isolated from *S. typhimurium*-immunized mice in response to P.69[a]

P.69 (μg/ml)	[^{3}H]thymidine incorporation, in cpm, in the following groups:				
	Oral BRD640	Oral BRD509	i.v. BRD640	i.v. BRD509	Control
0.83	4,067 (19)	1,599 (3.1)	76,733 (76)	3,065 (1.1)	3,215 (3.5)
0.29	1,537 (7)	1,584 (2.3)	77,331 (77)	3,508 (1.3)	2,372 (2.6)
0.09	462 (2)	937 (1.6)	73,021 (73)	3,681 (1.4)	953 (1.0)
0	214	523	1,000	2,685	904

[a] Spleen cells were isolated from four mice per group 20 days after an aerosol challenge (day 52) and pooled. Cells were restimulated in vitro with various concentrations of recombinant P.69. Proliferation was measured as the incorporation of [^{3}H]thymidine during the last 18 h of a 4-day culture period. Values in parentheses represent the fold increase in proliferation over background proliferation.

animals vaccinated with BRD640 failed to produce anti-P.69 antibodies, despite producing significant levels of anti-*S. typhimurium* LPS antibodies.

Adult mice immunized parenterally with a single dose of filamentous hemagglutinin (FHA) (25) or P.69 (41) adsorbed to aluminium hydroxide produce high levels of circulating antibodies and show a reduction in *B. pertussis* counts in the lungs following an aerosol challenge similar to that resulting from a single oral immunization with BRD640. Circulating antibodies against pertussis components have been demonstrated to facilitate the removal of *B. pertussis* from the lungs. Serum P.69-specific immunoglobulins can transude into the lower respiratory tract (47), and passively administered anti-P.69 (47) and anti-FHA (25) antibodies provide protection in the aerosol challenge model.

Stimulation of the local respiratory immune system is also an effective means of evoking pulmonary antipertussis defenses. Intranasal immunization of mice with FHA (46) or P.69 (38a) induces specific secretory IgA responses and promotes enhanced clearance of *B. pertussis* from the lungs. Our results indicate that cellular immunity also provides some protection against *B. pertussis* infection in murine lungs.

However, none of these immunization regimens provokes the strong immunity against lung colonization seen in mice that have recovered from a *B. pertussis* pulmonary infection or that have been immunized with a live attenuated *B. pertussis* strain (3, 34, 40). Such immune mice rapidly eliminate *B. pertussis* from their lungs. Therefore, full protection against *B. pertussis* infection may require the induction and cooperation of humoral, secretory, and cell-mediated antipertussis immune responses. On the other hand, the strong immunity resulting from an infection may be due to the exposure of the immune system to a larger number of immunogens. Vaccines containing multiple pertussis antigens are more efficacious than single-component vaccines in both humans and animals (1, 42).

The reason why BRD640 failed to induce anti-P.69 antibodies remains unclear. P.69 is produced in this strain in relatively large amounts and is present at the surface of the *S. typhimurium* vaccine strain. Low doses of purified recombinant P.69 induce antibodies in mice when injected in an adjuvant (41). Increasing the levels of expression of P.69 in *S. typhimurium* might enable the vaccine strain to seroconvert mice, and we intend to test this possibility. Despite the lack of a humoral antibody response to P.69, cells isolated from the spleens of BRD640-vaccinated animals proliferated significantly when restimulated in vitro with purified P.69 antigen, indicating that anti-P.69 T cells are effectively primed in mice immunized with BRD640.

There are other examples of protective immunity induced by *S. typhimurium* expressing heterologous antigens in the absence of significant levels of antibodies. Mice immunized with an attenuated *S. typhimurium* strain expressing the circumsporozoite (CS) protein of *Plasmodium berghei* were more resistant to sporozoite challenge. Protection appeared to be mediated by $CD8^+$ cytotoxic T cells (2, 43). Our model indicates that T cells are the prime mediator of immunity to *B. pertussis* in mice vaccinated with *S. typhimurium* expressing P.69.

The importance of cell-mediated immunity in protection from intracellular pathogens is well established, and the recent rediscovery that *B. pertussis* can invade and survive in eucaryotic cells (12, 14, 19, 39) suggests that cell-mediated immunity may be important in fighting *B. pertussis* infection. It has not been established whether *B. pertussis* survives intracellularly in the murine respiratory tract. However, *B. pertussis* has been demonstrated to interact with macrophages in vitro (38) and in vivo (12, 44), and it is tempting to speculate that activated macrophages play a role in immunity to pertussis.

The gene coding for *B. pertussis* FHA was introduced into an attenuated *S. dublin* strain (32). FHA was expressed in *S. dublin*, but its cellular location was not reported. Low levels of secretory (gut washings) and circulating anti-FHA antibodies were induced in mice orally vaccinated with this *S. dublin* strain. Whether these antibodies were significant was not determined, because a challenge with *B. pertussis* was not performed. The proliferation of lymphocytes exposed to FHA also was not investigated, so it is difficult to make comparisons between our data and those of Molina and Parker (32). One important difference is that in our experiments, P.69 was stably expressed from the bacterial chromosome, whereas the FHA gene was carried on a plasmid that was rapidly lost in vivo in the absence of antibiotics.

Why different antigens give rise to different immune responses when expressed in vivo by *Salmonella* strains is unknown but may be due to differences in cytokine induction or antigen processing. For example, mice immunized with *S. typhimurium* expressing the leishmanial gp63 protein appear to show a predominantly TH_1 helper T-cell response (52).

It is interesting that nonspecific protection from *B. pertussis* infection was induced by oral but not i.v. immunization with *S. typhimurium* alone. This result indicates that nonspecific as well as specific immunity can be activated at different mucosal sites without impinging on the systemic immune system. Nonspecific immunity to heterologous pathogens, such as *Listeria* spp., was demonstrated in mice challenged 2 to 3 weeks after immunization with attenuated *S. typhimurium* (24, 36). When the delay between immunization and *B. pertussis* challenge was extended, the nonspecific protection disappeared.

Our experiments demonstrate the feasibility of developing *Salmonella* strains expressing pertussis antigens as an oral whooping cough vaccine. It is thought that it will be necessary to use a combination of several pertussis antigens to immunize humans successfully against whooping cough. Inactivated pertussis toxin, P.69, and FHA are the three main candidates at present. We have constructed attenuated *S. typhi* strains that express foreign antigens from the chromosome (10). Therefore, it should be feasible, by use of our system, to construct strains of *S. typhi* that express multiple pertussis antigens and that could be evaluated as oral whooping cough vaccines in humans.

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