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Specific Lung Mucosal and Systemic Immune Responses after Oral Immunization of Mice with *Salmonella typhimurium aroA*, *Salmonella typhi* Ty21a, and Invasive *Escherichia coli* Expressing Recombinant Pertussis Toxin S1 Subunit

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Pertussis toxin (PT) is considered an essential protective component for incorporation into new generation vaccines against *Bordetella pertussis*, the causative agent of whooping cough. Traditionally, antipertussis vaccination has employed an intramuscular route. An alternative to this approach is to stimulate mucosal and systemic immune responses by oral immunization with live vaccine carrier strains of *Salmonella* spp. or *Escherichia coli*. Recombinant S1 subunit of pertussis toxin was expressed in the attenuated *aroA* mutant of *Salmonella typhimurium*, SL3261, in the human typhoid vaccine strain *Salmonella typhi* Ty21a, and in *E. coli* CAG629 containing the *Shigella flexneri* plasmid pWR110, which encodes bacterial invasiveness of epithelial cells. Expression of recombinant PT S1 subunit (rPT-S1) did not affect in vitro invasiveness of the tested strains, which retained the ability to adhere to and invade the embryonic human intestinal cell line HI-407. Following oral immunization of mice with the live vaccine strains expressing rPT-S1, immunoglobulin G (IgG), IgA, and IgM responses were monitored. IgG specific to PT was detected in serum samples of mice, while IgG and IgA specific to PT were detected in lung washes after oral immunization with living *Salmonella* spp. or *E. coli* (pWR110) expressing rPT-S1. Utilization of live oral vaccines expressing *B. pertussis* antigens, which stimulate both a systemic and lung mucosal response, may provide an attractive alternative to purified component vaccines against whooping cough.

Bordetella pertussis is the causative agent of whooping cough, a particularly severe disease of infants characterized by repeated bouts of paroxysmal coughing. Until recently, this disease has been controlled through vaccination. However, mounting concern about side effects associated with immunization with heat-killed, whole-cell vaccine preparations has lead to decreased vaccine acceptability and a disturbing rise in the incidence of the disease in recent years. There is, therefore, an urgent need to develop new, nonreactogenic, and effective vaccines against pertussis.

Virulence factors produced by *B. pertussis* that are candidates for inclusion in new generation vaccines include pertussis toxin (PT) and filamentous hemagglutinin (FHA) (22). The importance of these antigens as vaccine components has been emphasized in several animal models (11, 19). Unfortunately, human trials with acellular vaccine preparations composed of both of these components failed to elicit a fully efficacious response despite their ability to induce humoral immunity (1, 16, 34).

Antipertussis vaccination has traditionally employed an intramuscular route, mainly stimulating a systemic response. An alternative approach would be to stimulate an immune response directly at the lung epithelial surface. This would have the additional benefit of helping to reduce *B. pertussis*. colonization. We have recently demonstrated that the stimulation of gut immunity by using recombinant vaccine carrier strains of *Salmonella typhimurium* and invasive *Escherichia coli* expressing plasmid-encoded FHA results in the production of specific mucosal immunity at the lung epithelial surface (6, 7). As an extension of these observations to other potential *B. pertussis* vaccine components, we report here expression of a recombinant S1 subunit of PT (rPT-S1) in the attenuated *aroA* mutant of *S. typhimurium*, SL3261, in the human typhoid vaccine strain *Salmonella typhi* Ty21a, and in an *E. coli* strain containing the *Shigella flexneri* plasmid pWR110, which codes for bacterial invasiveness of epithelial cells. Both PT-specific systemic and lung mucosal antibody responses were obtained following oral immunization with these live vaccine carrier strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used in this work were *B. pertussis* Tohama I (29), *E. coli* JM109 (37), *E. coli* CAG629 *lon htpR165*-Tn*10* (C. Gross), *E. coli* K-12 395-1 (27), *S. typhimurium aroA* mutant SL3261 (10), *S. typhimurium* SL5283 (B. Stocker), a *galE503* derivative of LB5000 *hsdSB121 leu-3121* (26), and *S. typhi* Ty21a (5). The plasmids used in this work were pTX42 (14), which was a kind gift from J. Keith and was used as a source of the PT operon; pUC18 (37), pWR110, and R64*drd*11 (8, 27, 28); and pJLA506 (M. Walker), a derivative of the expression plasmid pJLA503 (31) with a modified multiple-cloning site.

E. coli was grown on Luria agar or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) agar or in Luria broth (25), and *S. typhimurium* strains were grown on Luria agar or in Luria broth (25). *S. typhimurium* Ty21a was grown on Luria agar or in brain heart infusion broth containing 0.001% galactose. Broth cultures were grown with shaking at 200 rpm. *B. pertussis* Tohama I was grown on Bordet Gengou

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agar base (Difco) supplemented with 1% glycerol and 15% (vol/vol) defibrinated horse blood or in SS-X broth (33). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 50 μg/ml; and kanamycin, 50 μg/ml. Broth cultures were aerated by shaking at 300 rpm in a New Brunswick Environmental Incubator Shaker.

DNA manipulations, plasmid transfer, and induction experiments. Restriction endonucleases and T4 DNA ligase were used as previously described (25). Plasmid isolation and transformation were performed as described by Sambrook et al. (25). *Taq* polymerase was used as described by Scharf (30). The polymerase chain reaction primers S1-5′*Nde*I (5′-GGCATATGCGTTGCACTCGGGCAATT-3′) and S1-3′*Xba*I (5′-CCAGGTCTAGAACGAATA-3′) used for cloning the S1 subunit cistron as a *Nde*I-*Xba*I fragment (*Nde*I and *Xba*I sites, respectively, are underlined) were synthesized with an Applied Biosystems Model 380B DNA synthesizer in accordance with the manufacturer's instructions. Conjugal transfer of pWR110 (Km^r) to *E. coli* CAG629 was accomplished by using the mobilizing helper plasmid R64-*drd*11 (Tc^r) as previously described by Sansonetti et al. (27, 28). Induction experiments were performed as previously described by Guzmán et al. (7). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell extracts was performed as described by Laemmli (12).

Electron microscopy. For postembedding labelling, cells of the respective strains were fixed directly in Luria broth with a fixation solution containing 0.5% (vol/vol) formaldehyde and 0.3% (vol/vol) glutaraldehyde in phosphate-buffered saline (PBS; 0.1 M phosphate buffer, 0.9% NaCl [pH 7.0]) for 1 h on ice. After being washed with PBS containing 10 mM glycine, the samples were embedded into 1.5% (wt/vol) agar. After solidification of the agar, small cubes were cut and embedded with progressive lowering of the temperature by using Lowicryl K4M resin (23). Immunolabelling was done with a 1:25 dilution of the purified rabbit PT-specific immunoglobulin G (IgG) antibody (200 μg of IgG protein per ml) (36) by incubating ultrathin sections on drops of the diluted antibody for 5 h at room temperature. After being washed with PBS, the bound antibodies were made visible with protein A-gold complexes (10-nm-diameter gold particle size, 30-min incubation time), prepared by established procedures (32). Subsequently, the sections were washed with PBS containing 0.01% Tween 20, and the sections were air dried prior to being stained with 4% (wt/vol) uranyl acetate for 3 min. Electron micrographs were examined, and micrographs were taken with a Zeiss EM10B electron microscope at an acceleration voltage of 80 kV and at calibrated magnifications.

Tissue culture methods and in vitro adhesion and invasion assays. The human embryonic intestinal cell line Intestine 407 (ATCC CCL 6, designated HI-407 in this work; GIBCO Laboratories, Eggenstein, Germany) was maintained in Dulbecco's modified Eagle medium (DME; GIBCO) with 10% fetal calf serum (GIBCO), 5 mM glutamine (Flow), and 1 mM pyruvate (Flow), in an atmosphere containing 5% CO_2 at 37°C. Trypsinized cells were seeded at a concentration of approximately 2×10^5 cells per coverslip (15 by 15 mm) in tissue culture plates (12 by 4.5 cm^2; Flow). These were incubated for 18 to 24 h and then washed three times in PBS (per liter, 8.0 g of NaCl, 2.0 g each of KCl, $Na_2HPO_4 \cdot 2H_2O$, and KH_2PO_4 [pH 7.4]). For infection of monolayers, bacteria were harvested by centrifugation in the exponential growth phase, resuspended to a density of 10^8 CFU/ml in DME, overlaid onto the monolayer of cells on coverslips, and incubated for 45 min at 37°C with 5% CO_2. Unattached and loosely attached bacteria were removed by washing the monolayers three times with PBS. For adhesion assays, the monolayers were then stained with Giemsa solution and examined by light microscopy, and the mean number of bacteria per HI-407 cell was calculated by averaging the number of bacteria adhering to 40 cells by using light microscopy. For invasion assays, DME containing gentamicin (100 μg/ml) was then added; this concentration of gentamicin killed extracellular bacteria and thereby prevented any reinfection of cells. Monolayers were incubated for an additional 3-h period and then washed three times with PBS. The number of invading bacteria was determined by lysing the eukaryotic cells with the addition of 1 ml of 1% Triton X-100 (vol/vol) to each well and calculating viable counts on Luria agar plates.

Mouse immunization. Six- to seven-week-old female BALB/c mice were immunized and caged separately in groups of five. Mice were immunized according to the protocols given in Table 2. The groups were immunized with one dose on day 0 and boosted with identical doses 30 and 40 days later. The animals were sacrificed 10 days after the last booster, and the samples were collected. For immunization with live bacteria, *E. coli* CAG629 and *S. typhimurium* SL3261 were grown in Luria broth, while *S. typhi* Ty21a was grown in brain heart infusion broth containing 0.001% galactose and *B. pertussis* Tohama I was grown in SS-X medium. An overnight liquid culture of bacteria was diluted 100-fold with broth containing an appropriate antibiotic for selection, and the fresh culture was incubated at 37°C until it reached early logarithmic phase. The cultures were centrifuged, and the bacteria were resuspended in PBS to an optical density which gave the appropriate viable count (as determined previously for each strain). For oral immunization, immediately before immunization an equal volume of 3% sodium bicarbonate in PBS (pH 8.0) was added to the suspension. Mice that had been deprived of water for 6 to 8 h were then gently fed with 50 μl of the bacterial suspension. For intraperitoneal (i.p.) immunizations, bacteria were suspended in PBS and, if required, were heat killed by treatment at 60°C for 30 min.

Sacrificed mice were exsanguinated by cutting the brachial artery, and the serum was separated and stored at −20°C. Lung washes were collected after pertracheal cannulation and gentle washing with 0.7 ml of ice-cold PBS containing 2 mM phenylmethylsulfonyl fluoride as a protease inhibitor. About 0.5 ml of lung wash was recovered from each mouse. Lung washes were centrifuged at $3,000 \times g$ for 5 min at 4°C to remove debris and stored at −20°C.

Immunological techniques. Monoclonal antibody E19 (36), which reacts with the S1 subunit of PT, was used in Western blot (immunoblot) experiments carried out essentially as described by Burnette (3). Proteins were transferred to a nitrocellulose membrane by using 25 mM Tris–192 mM glycine–20% methanol (pH 8.3) as transfer buffer and a 10% solution of 0.3% low-fat milk in PBS (pH 7.4) as a blocking reagent. E19 hybridoma supernatant fluid was used as the first antibody. The detection system used was Bio-Rad horseradish peroxidase-conjugated goat anti-mouse IgG and 4-chloro-1-naphthol as substrate.

For the determination of subclass-specific antibodies against the S1 subunit in serum and lungs washes, enzyme-linked immunosorbent assays (ELISAs) were performed as follows. Nunc Maxisorp Immunomodule 96-well plates were coated with PT (kindly supplied by S. Cryz) diluted in 0.1 M $NaHCO_3$ (pH 9.6) (60 ng in 50 μl per well) and incubated at 4°C overnight. The wells were blocked with 100 μl of 10%

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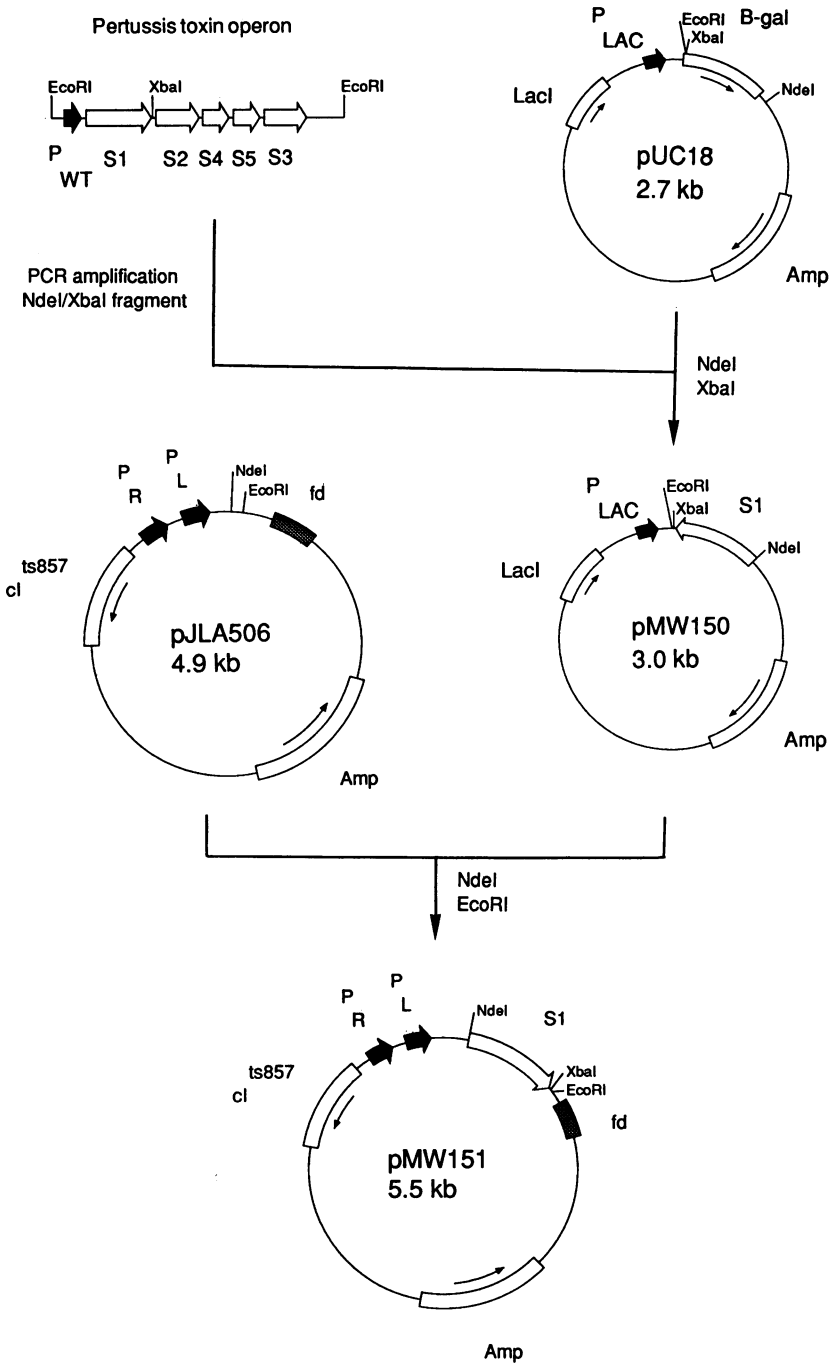

FIG. 1. Construction of plasmids specifying rPT-S1 expression. The PT operon was cloned from plasmid pTX42 (14). PT subunit genes S1 to S5 (open arrows) and the PT promoter (closed arrow) are indicated. Plasmid pUC18 contains the lactose repressor gene (LacI [open box]), ampicillin resistance gene (Amp [open box]), *lac* promoter (P_{LAC} [closed arrow]) and β-galactosidase alpha fragment sequence (B-gal [open box]). The expression vector pJLA506 contains the lambda p_L and p_R promoters in tandem (P_L and P_R [closed arrows]), temperature-sensitive lambda repressor gene (cI^{ts857} [open box]), fd transcriptional terminator (fd [cross-hatched box]), and ampicillin resistance gene as described for pUC18. The direction of transcription is indicated by either thin arrows or the orientation of open arrows. Plasmids are not drawn to scale, and only relevant restriction sites are shown.

fetal calf serum in PBS for 2 h at 37°C. Plates were subsequently washed three times with PBS, and 100 μl of serum samples (diluted 1:25) or lung washes (diluted 1:10) in 10% fetal calf serum in PBS was added to each well. After 2 h at 37°C, the plates were again washed, and 100 μl of alkaline phosphatase-conjugated goat anti-mouse antibodies for IgG, IgM, or IgA heavy chains (Southern Biotechnology Associates, Inc.) diluted 1:300 in 10% fetal calf serum in PBS was added to each well and incubated for 2 h at 37°C. The plates were again washed and then developed by the addition

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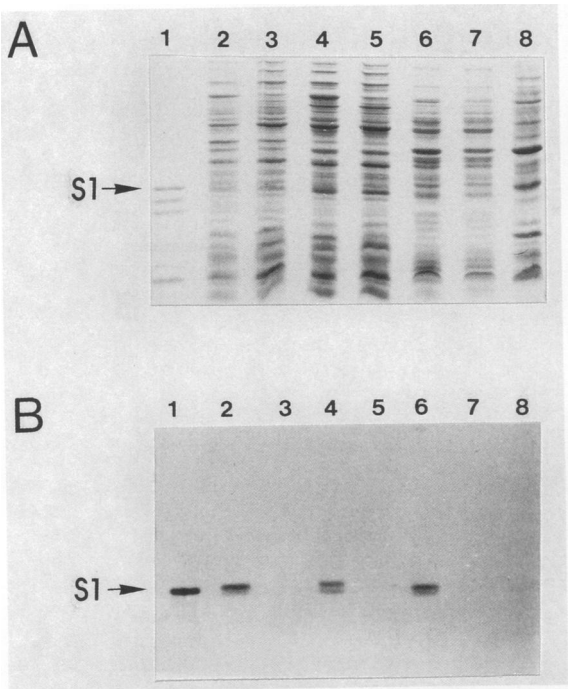

FIG. 2. Analysis of rPT-S1 expression by recombinant vaccine strains. Whole-cell extracts of *S. typhimurium* SL3261(pMW151) (lanes 2 and 3), *S. typhi* Ty21a(pMW151) (lanes 4 and 5), and *E. coli* CAG629(pMW151) (lanes 6 and 7) are shown, as are an extract of *B. pertussis* Tohama I (lane 8) and purified PT (lane 1). Bacteria in samples loaded onto lanes 2, 4, and 6 were induced at 42°C for 1 h, while for samples in lanes 3, 5, and 7, bacteria were grown at 30°C. (A) Coomassie blue-stained gel of whole-cell extracts; (B) Western blot analysis of whole-cell extracts using the anti-PT monoclonal antibody E19. The positions are of the PT S1 subunit are indicated by arrows. All lanes in panels A and B were loaded with the same amount of material except lane 1, in which 50-fold-less PT was loaded for panel B than for panel A.

of 100 μl of substrate solution (1.0 mg of *p*-nitrophenylphosphate, disodium salt, in diethanolamine buffer [pH 9.8]) per well. After 30 min at room temperature, the reaction was stopped by the addition of 50 μl of 3.0 M NaOH, and the A_{405} was determined with a Titertek Multiskan MCC microplate reader (Flow). All the samples were processed simultaneously on the same day, each serum or lung wash sample was individually assayed, and normal nonimmunized mouse sera or lung washes were used as the blank for the ELISA readings. Results are expressed as the mean values for each group.

RESULTS

Construction of rPT-S1 expression plasmids. The PT operon, originating from plasmid pTX42 (14), was used as a polymerase chain reaction template to amplify the PT S1 subunit. The polymerase chain reaction primers S1-5′*Nde*I and S1-3′*Xba*I were used to amplify a DNA fragment containing the ATG start codon of the S1 gene incorporated into the *Nde*I restriction endonuclease cleavage site of the 5′ primer. The 3′ primer contains the *Xba*I site found at the 3′ end of the wild-type S1 subunit DNA sequence, which incorporates the TAG stop codon of the gene. To facilitate screening clones containing the PCR product, the *Nde*I-*Xba*I fragment containing the S1 structural gene was digested with *Nde*I and *Xba*I restriction enzymes and cloned firstly into analogous sites found in pUC18 to produce pMW150. Subsequently, the S1 structural gene was purified and cloned as an *Nde*I-*Eco*RI fragment into the corresponding sites of expression vector pJLA506 to construct pMW151 (Fig. 1). This plasmid contains the S1 structural gene downstream of the efficient *atpE* ribosome binding site and tandem lambda p_L and p_R promoters under the control of the temperature-sensitive *c*I repressor protein, i.e., under a heat-inducible expression system.

Production of rPT-S1 in *Salmonella* spp. and *E. coli*. The expression plasmid pMW151 was transformed into the *lon* and *htpR* protease-deficient *E. coli* CAG629 and *S. typhimurium* SL5283. Plasmid DNA isolated from the latter was used to transform *S. typhimurium* SL3261 and *S. typhi* Ty21a. After induction at 42°C for 1 h, whole-cell extracts were subjected to SDS-PAGE and Western blotting with the S1-specific monoclonal antibody E19 (36). rPT-S1 and a degradation product of rPT-S1 migrating as a slightly lower band were detected at levels significantly higher than those found for *B. pertussis* Tohama I (Fig. 2). Lower levels of rPT-S1 were detected in recombinant strains after induction at 37°C for 24 h (results not shown).

Immunoelectron microscopy of *E. coli* CAG629 and *S. typhi* Ty21a containing either pJLA506 or pMW151 demonstrated that, after induction, inclusion bodies are not formed and that rPT-S1 is accumulated at the periphery of pMW151-bearing strains of *E. coli* CAG629 and *S. typhi* Ty21a (Fig. 3A, B, D, and E), indicating that the leader peptide of the S1 subunit is correctly processed, as previously described (4). Outer membrane ghosts of *E. coli* CAG629(pMW151) are labelled with gold particles, indicating that the rPT-S1 subunit is translocated through the cytoplasmic membrane into the periplasmic space and may be associated with the outer membrane (Fig. 3C). For *S. typhimurium* SL3261(pMW151), the label is detectable only in the cytoplasm (Fig. 3F). Other recombinant proteins including the Shiga toxin B subunit and a mycobacterial outer membrane protein were also expressed exclusively in the cytoplasm of this strain, whereas in wild-type *S. typhimurium*, these proteins were localized at the cell periphery (22a). It is not yet clear whether the *aroA* mutation or other strain-specific differences influence secretion of rPT-S1 in SL3261.

Ability of recombinant strains to adhere to and to invade the embryonic human intestinal cell line HI-407. The presence of plasmid pMW151 affected neither the ability of *S. typhimurium* SL3261 and *S. typhi* Ty21a to adhere to the embryonic human intestinal cell line HI-407 nor the ability to invade such cells (Table 1). The *S. flexneri* plasmid pWR110, which carries genes required for bacterial invasion of epithelial cells, was conjugally transferred to *E. coli* CAG629 (pMW151) to produce *E. coli* CAG629(pMW151 and pWR110). The presence of pWR110 resulted in the conversion of this strain to an invasive phenotype. Adhesion and invasion assays gave comparable results, although *E. coli* CAG629(pMW151 and pWR110) gave a higher adhesion assay response than either *Salmonella* spp. but a comparatively lower invasion assay response (Table 1). This anomaly was not observed in similar experiments conducted with another *E. coli* strain (6) and may represent strain-dependent differences which result in a decreased ability to survive intracellularly.

Assessment of specific anti-PT antibody responses in vaccinated mice. After the introduction of the rPT-S1 expression plasmid pMW151, both types of vaccine delivery system tested in this study, attenuated *Salmonella* spp. and *E. coli*

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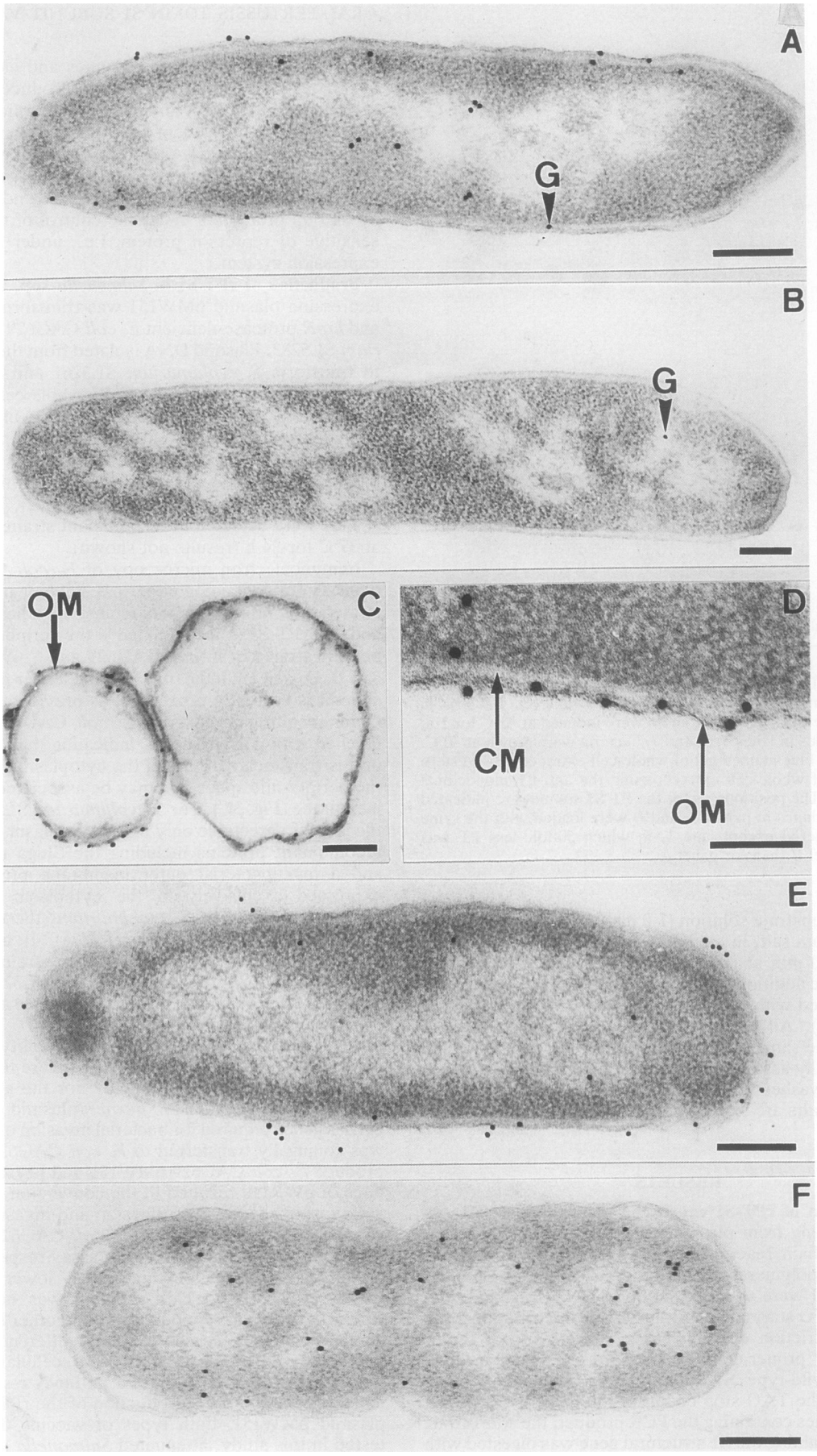

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TABLE 1. In vitro adhesiveness and invasiveness of strains used in vaccination protocols

Strain	Adhesiveness[a]	Invasiveness[b]
SL3261	18.2 ± 4.3	4.8×10^3 ± 1,166
SL3261(pMW151)	17.8 ± 3.8	6.8×10^3 ± 909
Ty21a	11.0 ± 3.4	1.6×10^4 ± 2,582
Ty21a(pMW151)	10.6 ± 3.1	1.3×10^4 ± 2,160
CAG629	3.7 ± 2.5	<25[c]
CAG629(pMW151)	3.2 ± 2.7	<25[c]
CAG629(pMW151 and pWR110)	31.7 ± 7.0	4.0×10^2 ± 18

[a] Mean number of attached bacteria per HI-407 cell ± standard deviation.
[b] CFU of viable intracellular bacteria per coverslip ± standard deviation.
[c] Limit of detection.

TABLE 2. Mouse immunization protocols

Group[a]	Organism or strain	Dose[b]	Route[c]	Viability of bacteria
a	*B. pertussis* Tohama I	10^6	i.p.	Heat killed
b	SL3261	10^6	i.p.	Live
c	SL3261(pMW151)	10^6	i.p.	Live
d	SL3261	10^9	Oral	Live
e	SL3261(pMW151)	10^9	Oral	Live
f	Ty21a(pMW151)	10^9	Oral	Live
g	CAG629(pMW151)	10^9	Oral	Live
h	CAG629(pMW151 and pWR110)	10^9	Oral	Live

[a] Group designation as in Fig. 4.
[b] Total number of bacteria per mouse.
[c] i.p., intraperitoneal injection; Oral, orally administered dose.

bearing the *S. flexneri* invasion plasmid pWR110, elicited anti-PT antibody responses (Table 2; Fig. 4). The i.p. immunization of mice with either 10^6 heat-killed *B. pertussis* cells or 10^6 live *S. typhimurium* SL3261(pMW151) cells induced similar, high serum and lung mucosal IgG antibody levels but insignificant amounts of IgA. However, higher IgM responses were engendered by live *S. typhimurium* SL3261 (pMW151) compared with heat-killed *B. pertussis* after i.p. immunization (compare groups a and c in Fig. 4).

Oral immunization with 10^9 live *S. typhimurium* SL3261 (pMW151), *S. typhi* Ty21a(pMW151), or *E. coli* CAG629 (pWR110 and pMW151) elicited high systemic IgG and low systemic IgA levels, similar to those levels found after i.p. immunizations (compare groups a and c with groups e, f, and h in Fig. 4A), and elevated anti-pertussis toxin IgA levels in lung washes. The amounts of IgG and IgA in lung washes were found to be highest after oral immunization with *S. typhimurium* SL3261(pMW151), followed by *S. typhi* Ty21a (pMW151), and were lowest after immunization with *E. coli* CAG629(pWR110 and pMW151) (compare groups e, f, and h in Fig. 4B). This variation may reflect strain differences in the ability to persist in mice. The absence of plasmid pWR110 from *E. coli* CAG629(pMW151) was associated with a marked decrease in the anti-PT response (compare groups g and h in Fig. 4). Presumably a lack of the invasion plasmid prevents an adequate antigen presentation to the gut immune system by *E. coli* CAG629(pMW151). Significant IgM responses were not apparent for any strain after oral immunization (groups d to h in Fig. 4).

DISCUSSION

Whooping cough is a severe respiratory tract disease. Traditional antipertussis vaccination has employed the intramuscular route. However, it may be desirable to develop vaccines which stimulate not only specific serum responses but also specific IgA in the respiratory tract. The production of lung mucosal secretory antibodies may interfere with the attachment of *B. pertussis* and subsequent colonization and thereby help prevent infection as well as disease. Such a vaccine would additionally favor eradication of a disease for which there is only a human reservoir. Serological studies of patients with whooping cough have revealed increases in both IgG and IgA levels against specific *B. pertussis* virulence determinants. On the other hand, whole-cell vaccines engender weak, if any, specific IgA (34). The mucosal immunological network contains subpopulations of lymphoid cells with the ability to migrate from the intestine to other distant mucosa (i.e., the respiratory tract) (2, 15, 24). Therefore, the development of enteric delivery systems for pertussis antigens which stimulate both a systemic and lung mucosal response may not only result in the prevention of infection and disease, and thus provide an attractive alternative to purified component vaccines against whooping cough, but also eliminate the need for purification of individual vaccine components for this purpose. The purification of individual vaccine components may be cost prohibitive for use in Third World countries.

We have recently demonstrated that stimulation of gut immunity by using recombinant *S. typhimurium aroA* SL3261 expressing the 220-kDa FHA protein resulted in a specific secretory IgA response at the lung mucosal surface (6, 7). Another group of investigators was unsuccessful in engendering a lung mucosal immune response after oral immunization of mice with *Salmonella* spp. expressing truncated FHA (17, 20). The inability to detect lung mucosal responses in these previous studies may reflect problems of plasmid instability or the expression level of recombinant FHA obtained. Our ability to detect a mucosal immune response may also have been due to an increased immunogenicity of the native protein compared with the truncated forms of FHA expressed in the earlier studies (17, 20).

PT is considered an essential protective component for new generation vaccines against whooping cough. In our present study, rPT-S1 was expressed in the attenuated *aroA* mutant of *S. typhimurium*, SL3261, in the human typhoid vaccine strain *S. typhi* Ty21a, and in *E. coli* CAG629 containing the *S. flexneri* plasmid pWR110, which encodes for bacterial invasiveness of epithelial cells. Expression of rPT-S1 did not affect in vitro invasiveness of HI-407 cells by the tested strains. Following oral immunization of mice with the live vaccine strains expressing rPT-S1, IgG specific to PT was detected in serum samples of mice while IgG and IgA

FIG. 3. Immunoelectron microscopic localization of the rPT-S1 subunit. Ultrathin sections were treated with polyclonal rabbit anti-PT IgG antibodies; bound antibodies were visualized with protein A-gold complexes. Most of the gold particles (labelled G) are associated with the cell periphery in the strains *E. coli* CAG629(pMW151) (A, C, and D) and *S. typhi* Ty21a(pMW151) (E), whereas for *S. typhimurium* SL3261 (pMW151), the label is located in the cytoplasm (F). Incubation of *E. coli* CAG629(pJLA506) with the antibodies and the protein A-gold particles as a control experiment showed no label (B). Bars represent 0.2 μm in panels A, B, C, E, and F and 0.1 μm in panel D. Abbreviations: CM, cytoplasmic membrane; OM, outer membrane.

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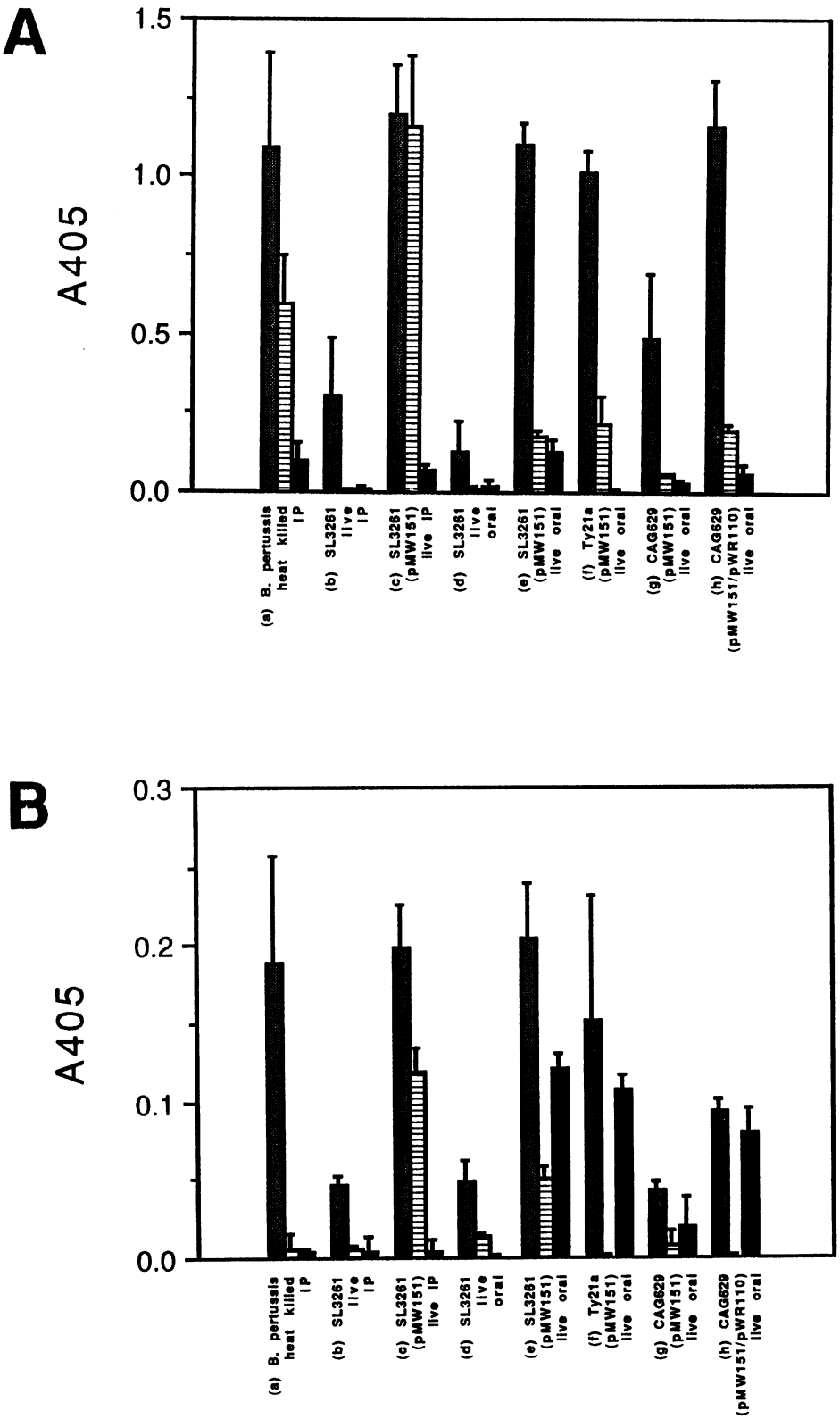

FIG. 4. Levels of PT-specific antibodies in serum (A) and lungs (B) after immunization of mice. Standard deviations are indicated by vertical lines. ▨, IgG; ▤, IgM; ■, IgA.

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specific to PT were detected in lung washes. These immune responses were detected irrespective of whether rPT-S1 was localized in the periplasmic space (*E. coli* CAG629 and *S. typhi* Ty21a) or in the cytoplasm (*S. typhimurium* SL3261).

It has been demonstrated that i.p. immunization of mice with crude preparations of recombinant S1 subunit resulted in an anti-PT response, but mice were not significantly protected from intracerebral challenge (4). It will be important to determine whether oral immunization of mice with recombinant *Salmonella* spp. or invasive *E. coli* expressing rPT-S1 either alone or in conjunction with such strains expressing FHA, stimulating both systemic and lung mucosal responses, results in a protective immune response in the intranasal model of infection (21). An immune response against the S2 to S5 subunits of the PT B oligomer may be necessary to achieve protection.

Live antigen delivery systems resistant to antibiotics are considered by the U.S. Food and Drug Administration to be unacceptable for human use. Non-antibiotic-resistance transposon systems which allow the stable integration and expression of potential vaccine components, for instance pertussis holotoxin in *Bordetella bronchiseptica* (35), in antibiotic-sensitive delivery systems are, however, available (9). Similar antibiotic-resistance "mini-transposons" have been used to successfully integrate a gene encoding the circumsporozoite protein of *Plasmodium yoelii* into the chromosome of *S. typhimurium* and elicit an immune response against the circumsporozoite protein in mice (4a). Experiments designed to express the other PT subunits, S2 to S5, and to incorporate such expression systems into the chromosome of the vaccine delivery strains described above are in progress.

In this report, and in the previous studies made by Guzmán et al. (6, 7), we have demonstrated the potential of recombinant enteric vaccine carrier systems to stimulate lung mucosal immune responses to *B. pertussis* antigens. These results may lead to the future development of oral vaccines against whooping cough and other infections of the respiratory tract. Particularly encouraging is the anti-PT response engendered by *S. typhi* Ty21a(pMW151), since Ty21a has been approved for human use by the U.S. Food and Drug Administration and is known to be a safe vaccine (13). However, the apparent failure of Ty21a to engender an anti-O antigen immune response in infants by using standard doses efficacious in school children (18) indicates that alternative strains may be required as vaccine delivery vehicles for infants.

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