Expression and Immunogenicity of Pertussis Toxin S1 Subunit-Tetanus Toxin Fragment C Fusions in *Salmonella typhi* Vaccine Strain CVD 908

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Salmonella typhi vaccine strain CVD 908 can deliver heterologous antigens to the host immune system following mucosal immunization. Stable expression of foreign proteins in *Salmonella* cells often requires antigen-specific engineering strategies. Fusion of antigens to stabilizing proteins has proven to be a successful strategy for rescuing otherwise unstable proteins. We designed plasmids to allow the fusion of antigens to the amino terminus or carboxyl terminus of fragment C of tetanus toxin, separated by a 4-amino-acid hinge region. Towards the ultimate goal of developing a live oral diphtheria-pertussis-tetanus vaccine, we used these plasmids to stably express the S1 subunit of pertussis toxin in CVD 908. Driven by the anaerobically inducible *nirB* promoter, the S1 subunit alone was expressed poorly in *Salmonella* cytoplasm. In contrast, hybrid proteins with S1 fused to either the amino or carboxyl terminus of fragment C were expressed at a high level in CVD 908 and were recognized in Western blot (immunoblot) analysis by monoclonal antibodies directed to S1 and to fragment C. Mice were immunized by the oral or intranasal routes with CVD 908 derivatives harboring these recombinant plasmids. All fusion proteins elicited serum antibody responses to fragment C following intranasal immunization, whereas oral inoculation did not. The configuration of antigens constituting the fusion was critical; S1 fused to the amino terminus of fragment C was less effective than S1 fused to the carboxyl terminus in generating anti-fragment C antibodies. CVD 908 expressing truncated S1 fused to the carboxyl terminus of fragment C elicited neutralizing serum pertussis antitoxin following intranasal immunization of mice.

Dissatisfaction with the current inactivated whole-cell pertussis component of the diphtheria-tetanus-pertussis (DTP) vaccine has led to the development of acellular vaccine formulations composed of purified *Bordetella pertussis* components administered parenterally (27, 34). Alternatively, delivery of these components could be achieved via expression in an orally administered attenuated *Salmonella* live vector vaccine strain. This strategy provides several advantages: (i) it avoids the expensive and potentially denaturing process of antigen purification, (ii) genetic manipulation of *Salmonella* strains should allow concurrent delivery of multiple antigens, and (iii) mucosal immunization with *Salmonella* strains would elicit mucosal and cell-mediated immune responses, in addition to stimulating serum antibodies (5, 7, 28).

Salmonella typhi CVD 908, developed as a live oral typhoid vaccine, is a promising candidate for use in the delivery of heterologous antigens (20, 21, 40). The progression of infection following oral administration of this vaccine is severely limited because of attenuating deletions in *aroC* and *aroD*, which render this strain nutritionally dependent on the aromatic precursors *para*-aminobenzoic acid and 2,3-dihydroxy-

benzoate, which are not available in sufficient concentration in human tissues (19). CVD 908 has been utilized successfully as a live vector of viral (10), bacterial (12), and protozoal (15) antigens.

Towards the goal of achieving a safe and effective multivalent oral DTP vaccine, we exploited CVD 908 as a live vector of tetanus and pertussis antigens. Previous studies have demonstrated the successful expression and protective ability of the nontoxic, immunogenic fragment C (FragC) of tetanus toxin in *Salmonella typhimurium* (4, 8). Recent studies in this laboratory have established that intranasal administration of *S. typhi* CVD 908 expressing FragC protected mice from a lethal challenge with tetanus toxin (11).

Here, we describe our efforts to express in a stable fashion a pertussis antigen, the S1 subunit of pertussis toxin. Pertussis toxin is one essential virulence factor of *B. pertussis* (33, 45), and inactivated pertussis toxin is considered an essential component of the new parenteral acellular vaccines. Indeed, one acellular vaccine consisting of pertussis toxoid alone conferred a moderate level of protective efficacy in a controlled field trial with infants in Sweden (43). Pertussis toxin is composed of five subunits, S1 through S5 (41). S1 is responsible for the enzymatic ADP ribosylation activity of holotoxin, while S2 through S5 form the B oligomer which binds to host cells and delivers the enzymatic subunit (24, 42). Toxin-neutralizing epitopes reside within the S1 subunit. Monoclonal antibodies against S1 neutralize toxin enzymatic activity in vitro and passively protect mice against challenge with *B. pertussis* (35–37). Furthermore, purified fusion proteins consisting of S1 fused to either diphtheria toxin or FragC of tetanus toxin elicited neutralizing

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
<i>S. typhi</i> CVD 908	Ty2 Δ aroC1019 Δ aroD1013	20
<i>S. typhimurium</i> SL3261	Lt2 aroA	19
pTET <i>nir</i> 15	pBR322 derivative carrying the FragC gene under control of the <i>nirB</i> promoter	32
pUC-9K/129G	Pertussis toxin operon with mutant S1 subunit (9K/129G)	34
pNFV-2	pTET <i>nir</i> 15 derivative with hinge region inserted at the 5' end of FragC	This work
pNFV-3	pNFV-2 derivative in which the S1 gene has been fused to the 5' end of FragC	This work
pNFV-4	pTET <i>nir</i> 15 derivative in which the FragC gene has been replaced with the S1 gene	This work
pOG214	pTET <i>nir</i> 15 derivative with a hinge region inserted at the 3' end of the FragC gene	14
pCFV-1	pOG214 derivative in which the S1 gene has been fused to the 3' end of the FragC gene	This work
pCFV-2	pCFV-1 derivative in which a truncated S1 gene has been fused to the 3' end of FragC	This work

antibodies in animal models following parenteral immunization (2, 3).

By varying the design of protein fusion constructions, we sought to optimize the stable expression and immunogenicity of S1 in CVD 908. We designed plasmids which allow antigen insertion to either the amino terminus or carboxyl terminus of FragC, thereby resulting in S1 fusions in both configurations. One protein fusion of S1 to FragC was particularly stable in CVD 908 and allowed induction of a serum antibody response in mice to both heterologous antigens following mucosal (intranasal) immunization with the CVD 908 live vector expressing this fusion.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains and plasmids used in these studies are listed in Table 1. Bacteria were grown in Luria broth base (Gibco BRL, Gaithersburg, Md.) unless otherwise noted. *S. typhi* CVD 908 and *S. typhimurium* SL3261 strains were grown with the addition of 0.0001% 2,3-dihydroxybenzoate (Sigma, St. Louis, Mo.). Carbenicillin was used at a concentration of 50 μ g/ml where indicated.

DNA manipulations. Restriction enzymes, ligase, and polymerase were purchased from Gibco BRL or New England Biolabs (Beverly, Mass.) and used according to the manufacturers' instructions. The electroporation of *Salmonella* strains was performed in a Gene Pulser apparatus (Bio-Rad, Melville, N.Y.) set at 2.5 kV, 400 Ω , and 25 μ F. Electroporation-competent cells were made by washing stationary-phase cells three times in sterile distilled H₂O and resuspending them in distilled H₂O to a final volume of 1/100 of the original culture volume. PCRs were performed in a MiniCycler PTC-150 (MJ Research, Watertown, Mass.) with Vent DNA polymerase (New England Biolabs).

Plasmid constructions. Oligonucleotide primers were synthesized at the Biopolymer Laboratory of the University of Maryland School of Medicine on a 380B DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.) and are listed in Table 2. With pTET*nir*15 used as a template, primers Ptx3 and P4 were used to generate pNFV-2, which contains a unique *Nsi*I site within the ATG start codon and encodes the hinge region in frame to the 5' terminus of the FragC gene. The S1 gene was amplified from pUC-9K/129G with primers Ptx4 and Ptx5. Primer Ptx4 adds an *Nsi*I site and ATG codon to the 5' end of the S1 gene at bp 609 (based on the published sequence with GenBank accession number M13223 [29]). Primer Ptx5 adds an *Nsi*I site to the 3' end of the S1 gene, allowing its in-frame insertion into pNFV-2. A second construct, pNFV-4, was generated by insertion of the S1 gene as an *Nsi*I-*Xba*I fragment into pNFV-3 digested with *Nsi*I and *Nhe*I, thus replacing the FragC gene.

In order to allow cloning of the S1 gene at the 3' end of FragC in pOG214, S1

was amplified from pUC-9K/129G with primers Ptx1 and Ptx7. Primer Ptx1 includes a *Hind*III site at the 5' end of S1, and primer Ptx7 includes the stop codon of the S1 gene and an *Xba*I site at the 3' end of the S1 fragment to allow cloning into pOG214 to create pCFV-1. Another construct, pCFV-2, was generated by replacing the S1 gene in pCFV-1 with a truncated form of the S1 gene. The truncated S1 gene was generated by deletion of the 147-bp 3'-terminal *Sau*3AI-*Xba*I fragment.

Western blot (immunoblot) analysis. Bacteria were harvested from broth cultures, and samples were normalized for cell number. Samples containing approximately 10⁷ bacteria, were mixed with sodium dodecyl sulfate (SDS) sample buffer, boiled for 10 min, and electrophoresed on SDS-12% polyacrylamide gels. Gels were stained with Ultra-Pure BluePrint Fast-PAGE stain (Gibco BRL) for visualization of protein bands or transferred to membranes for Western immunoblot analysis. Western blotting was performed with the Immuno-Lite chemiluminescent assay kit (Bio-Rad, Richmond, Calif.) according to the manufacturer's protocol. Membranes were probed with monoclonal antibody against tetanus toxin FragC (Boehringer-Mannheim, Indianapolis, Ind.) or monoclonal antibody 18.1.7 against FragC (kindly provided by J. Halpern, Center for Biologicals Evaluation and Research [17]) or monoclonal antibody 3CX4 against the S1 subunit of pertussis toxin (25).

Protein preparation. For crude protein preparations, *S. typhi* strains harboring constructs were grown in Luria broth with antibiotics and 2,3-dihydroxybenzoate overnight. Cells were harvested by centrifugation at 10,000 $\times$ g and resuspended in sterile phosphate-buffered saline (PBS) to achieve a uniform optical density at 600 nm of 4.0. Bacteria were lysed by sonication in a Heat Systems-Ultrasonics (Plainview, N.Y.) sonicator for three bursts of 45 s each. Cell debris was discarded following centrifugation of the sonicates at 5,000 $\times$ g for 15 min. Crude protein suspensions were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) analysis to verify protein content.

Formalin-fixed bacteria. Formalin-fixed heat-killed *Salmonella* strains were prepared as described elsewhere (9). Briefly, 10⁸ organisms per ml were mixed with PBS-buffered formaldehyde (1.25% final concentration) for 3 h at room temperature and then heated to 80°C for 3 min. The bacteria were washed in sterile PBS and resuspended to a concentration of 10¹¹ cells per ml in PBS.

Mouse studies. *Salmonella* strains were inoculated into Luria broth with antibiotics plus 2,3-dihydroxybenzoate and grown at 37°C until the optical density at 600 nm reached approximately 1.0. Cells were harvested and resuspended in sterile PBS to a concentration of approximately 10¹¹ CFU/ml. Colony counts were performed on all inocula to confirm the cell numbers. Eight-week-old female BALB/c mice (Charles River, N.Y.) were randomized into groups of five mice per vaccine. For oral inoculations, mice were fasted for 12 h and fed 100 μ l of 3% sodium bicarbonate in PBS several minutes prior to dosing with 100 μ l of the bacterial sample by gastric tube. For intranasal inoculations, mice were inoculated with 50 μ l of bacterial sample by placement into the nares. For subcutaneous inoculation, 50 μ l of protein preparation was mixed with 50 μ l of complete Freund's adjuvant and injected subcutaneously into the upper back. For intravenous (i.v.) inoculation, 100 μ l of crude protein was injected into the tail vein. All mice were given doses on days 0, 28, 42, and 56.

TABLE 2. Primers used in PCRs

Primer	Sequence (5' to 3')
Ptx1	CGCAAGCTTGACGATCCTCCCGCCACC
Ptx3	GACGTGACGGGCCCCGCCCATGCGAAAGTCTCCTGTGGATGA
Ptx4	CGCATGCATGACGATCCTCCCGCCACCGTATAC
Ptx5	GGATCCATGCATGAACGAATACGCGATGCTTTCGTA
Ptx7	CGGAATTCTCCTGGTCTAGAACGAATACGCGATGCT
P4	GCAGGAAAGAACATGTGAGCCTAGGGCCAGCAAAAGGCCAGGAAC

FIG. 1. Plasmid maps of pTETnir15 derivatives. Vectors illustrate amino- and carboxyl-terminal fusions of S1 to FragC.

Blood samples were collected from individual mice by retroorbital sampling with a capillary tube on day 0, day 21 (3 weeks after the first dose), and days 38, 52, and 66 (10 days following each boosting dose). All sera were stored at -20°C .

ELISAs. Serum antibody responses to tetanus toxin and to pertussis toxin were quantitated by enzyme-linked immunosorbent assay (ELISA), as previously described (11). The tetanus toxin ELISA plates were coated with tetanus toxoid (Connaught Laboratories) at a concentration of $2\text{ }\mu\text{g/ml}$. Twofold serial dilutions of each serum sample were performed until an end point titer was reached. The end point titer was determined to be the highest dilution giving an A_{405} of greater than 0.1. This cutoff value of 0.1 was determined as the mean plus two standard deviations above the mean optical density value of 20 normal mouse serum samples run at a 1:20 dilution. Serum immunoglobulin G (IgG) antibody titers against pertussis toxin were measured as described above, with $1.0\text{ }\mu\text{g}$ of purified pertussis toxin (List Biological Laboratories, Campbell, Calif.) per ml as the coating antigen. Serum IgG antibody against *Salmonella* lipopolysaccharide (LPS) was measured as described above, with *S. typhi* LPS (Difco, Detroit, Mich.) as the coating antigen at a concentration of $5.0\text{ }\mu\text{g/ml}$. Prevacination serum samples allowed each mouse to serve as its own negative control. Geometric mean titers (GMTs) were determined for each group.

CHO cell assay. The CHO cell assay for measurement of antibody neutralization of pertussis toxin was performed essentially as described by Hewlett et al. (18). CHO cells were seeded into 96-well microtiter dishes at a concentration of 2×10^6 cells per well and allowed to adhere for 2 to 4 h. Pertussis toxin (30 μg per well; List Biological Laboratories) was incubated for 1 h at 37°C with or

without serial twofold dilutions of test antiserum in CHO cell growth medium (Ham's F-12 with 1% fetal bovine serum) in a total volume of $50\text{ }\mu\text{l}$. Pertussis toxin alone or pertussis toxin-antiserum mixtures were then added to CHO cells and incubated for 24 h at 37°C in a 5% CO_2 incubator. Cells were then stained with Giemsa stain and scored for CHO cell clustering by light microscopy.

RESULTS

Antigen constructions. Multiple constructions were designed to identify which ones optimized stable expression of the S1 subunit of pertussis toxin fused to FragC of tetanus toxin in *S. typhi* vaccine strain CVD 908. All constructions were based on the plasmid pTETnir15, which expresses the nontoxic yet fully protective FragC of tetanus toxin under direction of the *nirB* promoter (4, 17, 32). For S1 constructions, we utilized a mutant form of the S1 gene which lacks enzymatic activity. pUC-9K/129G contains the pertussis toxin operon with two point mutations in the S1 subunit gene in codons encoding critical enzymatic residues (34). The mutant S1 molecule is devoid of all enzymatic activity yet as a part of holotoxin can

FIG. 2. S1-FragC constructions expressed in *S. typhi* CVD 908. (A) Graphical illustration of genetic constructs. (B) BluPrint-stained gel of *S. typhi* CVD 908 whole-cell lysates of approximately 10^7 bacteria. Lane A, CVD 908(pTET*nir*15); lane B, CVD 908(pNFV-3); lane C, CVD 908(pCFV-1); lane D, CVD 908(pCFV-2); and lane E, CVD 908(pNFV-4).

induce protective immunity; i.e., it maintains correct T- and B-cell epitopes (30, 34).

Amino-terminal fusion vectors. A vector to facilitate the insertion of the S1 gene at the 5' end of the FragC gene was developed. Using the PCR strategy outlined in Materials and Methods, we modified pTET*nir*15 with the introduction of two changes: (i) a unique *Nsi*I site was engineered within the first ATG codon downstream of the *nirB* promoter and (ii) a linker region was inserted at the 5' end of the FragC gene encoding a hinge peptide composed of Gly-Pro-Gly-Pro. The resultant vector, pNFV-2, allows cloning of a gene of interest into the unique *Nsi*I site, placing it under control of the *nirB* promoter and fusing it in frame to the FragC gene, separated by the hinge region. The S1 gene was cloned into the unique *Nsi*I site in pNFV-2, producing plasmid pNFV-3. pNFV-3 encodes a hybrid protein with S1 fused to the amino terminus of FragC (Fig. 1).

A second vector, pNFV-4, which contained only the S1 gene, was derived from pNFV-3 by deleting the hinge and FragC genes and inserting a stop codon at the 3' terminus of the S1 gene. Thus, the S1 gene exists alone yet in an identical context as in the fusion vectors, i.e., under *nirB* direction and within the modified pTET*nir*15 replicon (Fig. 1).

Carboxyl-terminal fusion vectors. In order to test the hypothesis that the configuration of antigens can affect expression and immunogenicity, constructions in which S1 was fused to the opposite or carboxyl terminus of FragC were prepared. pOG214 is a modified version of pTET*nir*15 which includes a linker region at the 3' end of FragC encoding the in-frame hinge region and a multiple cloning site (14). The S1 gene was inserted into pOG214, creating pCFV-1, which contains the S1 gene fused in frame to the 3' terminus of the FragC gene, separated by the hinge region (Fig. 1).

A fourth construct, which encoded a truncated form of the S1 subunit fused to the carboxyl terminus of FragC, was created. A 147-bp *Sau*3AI-*Xba*I fragment was deleted from the 3'

end of the S1 gene in pCFV-1, resulting in pCFV-2. Plasmid pCFV-2 encodes a truncated form of S1, with the 49 carboxyl-terminal amino acids deleted, which is fused to the carboxyl terminus of FragC (Fig. 1).

Antigen expression in *Salmonella* strains. The four plasmid constructions were electroporated into *S. typhimurium* SL3261 and *S. typhi* vaccine strain CVD 908. Whole-cell lysates of these strains were prepared following overnight growth in liquid culture with aeration. BluPrint staining of SDS-PAGE gels containing lysates of CVD 908 derivatives clearly revealed the presence of the 50-kDa protein, corresponding to the size of FragC in strain CVD 908(pTET*nir*15) (Fig. 2B, lane A). A unique band at approximately 71 kDa, corresponding to the size of the fusion protein consisting of FragC plus truncated S1 (Fig. 2B, lane D), is visible in strain CVD 908(pCFV-2). A band of approximately 80 kDa is expected, as this is the size of a fusion protein composed of FragC plus full-length S1 encoded by pNFV-3 and pCFV-1. Sufficient protein to allow the clear visualization of this band on the BluPrint-stained gel was not produced (Fig. 2B, lanes B and C). The 27-kDa band, corresponding to the size of the S1 subunit, is not visible in strain CVD 908(pNFV-4) (Fig. 2B, lane E).

In order to determine relative expression levels and antibody binding characteristics of each antigen, Western blot analysis was performed. Membranes with immobilized cell lysates were incubated with monoclonal antibodies directed against FragC or against S1. The predicted fusion protein of approximately 80 kDa was expressed in CVD 908(pNFV-3) and CVD 908(pCFV-1) (Fig. 3, lanes D and E, respectively) and detected by monoclonal antibodies directed against both FragC and S1. A slightly smaller fusion protein, approximately 71 kDa, was detected with both antibodies in CVD 908(pCFV-2) (Fig. 3, lane F) because of the truncation of S1. A faint protein band of 27 kDa, corresponding to the size of the S1 subunit, was visualized with anti-S1 monoclonal antibody in CVD 908(pNFV-4) (Fig. 3A, lane C). Also apparent from Western blot analysis

FIG. 3. Western blot analysis of CVD 908 derivatives. Membranes were probed with monoclonal antibodies directed against pertussis toxin S1 subunit (A) or against tetanus toxin (B). Lanes A, *B. pertussis* BP338; lanes B, CVD 908; lanes C, CVD 908(pNFV-4); lanes D, CVD 908(pNFV-3); lanes E, CVD 908(pCFV-1); lanes F, CVD 908(pCFV-2); and lanes G, CVD 908(pTETnir15).

was that the quantity of protein expressed in vitro in CVD 908(pCFV-2) was greater than those expressed with the other two fusions. This strain expresses the FragC-S1 truncate fusion. Similar protein profiles were observed when the fusions were expressed in *S. typhimurium* SL3261 (Fig. 4). The high-molecular-weight material visualized in Fig. 3A, lane A, represents incompletely solubilized *B. pertussis* cells with S1 migrating as part of an aggregate.

The *nirB* promoter is induced in environments with low oxygen levels (22, 23, 32). Thus, it was surprising to observe a high level of expression of the foreign proteins when the live vector strains were grown under the aerobic conditions described. When bacteria were grown under putatively inducing, i.e., anaerobic, conditions and subjected to Western blot analysis, no increase in the level of expression of the fusion proteins

was apparent. A slight induction of the 27-kDa S1 protein was observed in *S. typhimurium* SL3261(pNFV-4) grown under anaerobic conditions (Fig. 4, lane C). These results could indicate promoter sensitivity to the local genetic environment. Studies to determine optimal promoter conditions for in vivo expression with these constructions are ongoing.

We attempted to determine if foreign protein expression during particular phases of growth varied. *S. typhi* CVD 908 derivatives harboring each of the constructions were sampled at 1-h intervals during the course of growth. The samples, normalized for cell number, showed no significant difference in protein expression as analyzed by Western blot (data not shown).

Mucosal immunization studies. Having established that the recombinant plasmids that we designed could result in a high level of expression of the encoded foreign antigens in *S. typhi* CVD 908 and in *S. typhimurium* SL3261, we proceeded to inoculate mice with the various strains to evaluate the immune responses to the heterologous antigens. In the first study, mice were inoculated orally with *S. typhi* CVD 908 or *S. typhimurium* SL3261 harboring each of the antigen constructions. Despite the administration of three spaced oral doses, each containing approximately 10^{10} CFU, the serum IgG responses to tetanus toxin and to pertussis toxin were meager in all groups (Fig. 5). After two oral doses, a modest serologic response to tetanus toxin was detected in mice vaccinated with *S. typhimurium* SL3261(pCFV-1), *S. typhimurium* (pCFV-2), and *S. typhi* CVD908(pCFV-2) (Fig. 5A and B), with two of five, two of five, and one of five mice, respectively, responding in each group. No response to pertussis toxin was elicited by oral inoculation with any strain.

Since ongoing studies in this laboratory have indicated the robust immunogenicity in mice of *S. typhi* live vector vaccines when administered by the intranasal route of mucosal immunization (11), we undertook a second study wherein mice were given three or four 10^{10} -CFU intranasal doses of attenuated *S. typhi* CVD 908 harboring each of the foreign antigen plasmid constructs. Sera tested for IgG antibody responses to pertussis toxin and to tetanus toxin confirmed the success of the intra-

FIG. 4. Western blot analysis of induced or uninduced cultures of *S. typhimurium* SL3261 derivatives probed with monoclonal antibody against pertussis toxin S1 subunit. Lanes B, D, F, and H include cultures grown with vigorous shaking and aeration overnight; lanes C, E, G, and I include cultures grown anaerobically in static broth overnight. Lane A, *B. pertussis* BP338; lanes B and C, SL3261(pNFV-4); lanes D and E, SL3261(pNFV-3); lanes F and G, SL3261(pCFV-1); and lanes H and I, SL3261(pCFV-2).

FIG. 5. Serum anti-tetanus toxin or anti-pertussis toxin IgG titers in mice in preimmune sera (horizontally striped bars) or following one (open bars), two (diagonally striped bars), three (solid bars), or four (shaded bar) doses of *Salmonella* derivatives. GMTs are indicated, and numbers atop bars are responders/number of mice tested. (A) Anti-tetanus toxin titers following oral inoculation with *S. typhimurium*; (B) anti-tetanus toxin titers following oral inoculation with *S. typhi* CVD 908 derivatives; (C) anti-tetanus toxin titers following intranasal inoculation with *S. typhi* CVD 908 derivatives; (D) anti-pertussis toxin titers following intranasal inoculation with *S. typhi* CVD 908 derivatives.

nasal route of immunization in eliciting serologic responses to the foreign antigens. After only a single intranasal inoculation, responses to the tetanus (FragC) moiety of the fusion proteins in two of five mice inoculated with CVD 908(pCFV-1) and an even greater response in three of four mice receiving CVD 908(pCFV-2) were observed (Fig. 5C). After two intranasal inoculations with *S. typhi* harboring either pCFV-1 or pCFV-2, all mice responded with high tetanus antitoxin titers, achieving GMTs of 2,512 and 15,849 respectively. It is of interest that intranasal immunization with *S. typhi* harboring pNFV-3, the fusion with S1 at the amino terminus of FragC, was not effective in eliciting tetanus antitoxin, as only one of four mice in this group responded.

A serum antibody response to pertussis toxin was observed in only one group of mice (Fig. 5D). After two intranasal inoculations with CVD 908(pCFV-2), two of three mice showed modest serum IgG responses to pertussis holotoxin, which were substantially boosted in two of three mice after a third dose. These responses were boosted even further, to quite high titers after four doses, with three of three mice responding. Not surprisingly, *S. typhi* harboring pNFV-4, in which S1 is expressed unfused and at a low level, failed to induce any serum IgG response in mice, even after three doses.

A control experiment to test the ability of killed *S. typhi* carrying the heterologous antigens to elicit an immune response following intranasal inoculation was performed. After

FIG. 6. GMTs of anti-tetanus toxin (A) or anti-pertussis toxin (B) serum IgG in mice parenterally inoculated with crude protein preparations. Strains from which protein preparations were made are indicated on the x axis. GMTs for preimmune sera (Bleed #1), sera following one subcutaneous inoculation (Bleed #2), sera following one boost by i.v. inoculation (Bleed #3), or sera following two boosts by i.v. inoculation (Bleed #4) are shown. Numbers atop bars are responders/number of mice tested.

two doses of intranasally administered nonviable CVD 908 harboring each plasmid construct, no serum antibody response against tetanus toxin or pertussis toxin was observed (Table 3). The mice that received these doses were additionally tested for serum antibody elicited against the vaccine vector itself. After two doses of formalin-killed *S. typhi*-harboring constructs, five of five mice in each group responded with a serum anti-LPS IgG response. The levels of response in these groups were lower than those in mice that received the same dose of live *S. typhi*.

Parenteral immunization studies. Since two of the three fusions tested were unable to elicit anti-pertussis toxin responses, we endeavored to determine if these antigens could elicit a response when administered via the more optimal parenteral route. Crude protein preparations of each fusion protein and S1 alone from CVD 908 strains harboring the con-

structions were prepared. The inoculation schedule consisted of administering one subcutaneous priming dose with Freund's complete adjuvant followed by boosting with i.v. inoculations. High serum IgG titers against tetanus toxin were evident after the initial subcutaneous dose of each fusion and were further elevated with each successive i.v. booster dose (Fig. 6A).

Significant rises in serum IgG antibodies to pertussis toxin were detected in four of four mice following a single subcutaneous inoculation with the pCFV-2-encoded protein (Fig. 6B). The titers in serum were boosted to high levels in this group following each additional dose. The pCFV-1-encoded fusion protein was able to elicit seroconversion in two of four mice following two doses. Neither pNFV-3 nor pNFV-4 preparations inoculated parenterally were able to elicit a consistent response to pertussis toxin in any mouse.

CHO cell assays for pertussis toxin neutralization. Pertussis toxin causes a characteristic clustering effect, when applied to CHO cells, which can be neutralized by specific antibodies (18). We tested the neutralization activity of sera from mice immunized intranasally with *S. typhi* CVD908(pCFV-2) or CVD 908(pNFV-3) and from mice immunized parenterally with crude preparations of pCFV-2- or pTETnir15-encoded protein. Following four intranasal doses of CVD 908(pCFV-2), sera from two of three mice exhibited pertussis toxin-neutralizing activity (Table 4). Intranasal inoculation with CVD 908(pNFV-3), which was unable to elicit anti-pertussis toxin serum IgG, correspondingly did not elicit neutralizing antibodies. Parenteral inoculation with three doses of a crude preparation of pCFV-2-encoded protein, which stimulated very high levels of pertussis antitoxin in serum, measured by ELISA, elicited high titers of neutralizing antibodies in three of three mice tested. The negative control group inoculated parenterally with a crude preparation of pTETnir15-encoded protein exhibited no CHO cell neutralizing activity.

TABLE 3. Antibody responses in serum in mice following two intranasal inoculations of live or formalin-fixed *S. typhi* derivatives

Vaccine construct ^a	Serum IgG GMT ^b		
	Anti-LPS	Anti-FragC	Anti-PTX ^c
CVD 908(pCFV-1)			
Live	ND ^d	2,512 (5/5)	10 (0/5)
Formalin killed	275 (5/5)	10 (0/5)	10 (0/5)
CVD 908(pCFV-2)			
Live	>2,560 (3/3)	15,849 (3/3)	63 (2/3)
Formalin killed	724 (5/5)	10 (0/5)	10 (0/5)
CVD 908(pTETnir15) (formalin killed)	479 (5/5)	10 (0/5)	10 (0/5)

^a *S. typhi* derivatives were inoculated at doses of approximately 10^{10} CFU.

^b The values indicated in parentheses are numbers of mice responding on day 38/number of mice tested.

^c PTX, pertussis toxin.

^d ND, not determined.

TABLE 4. Summary of IgG anti-pertussis toxin antibodies and neutralizing pertussis antitoxin titers

Vaccination group and mouse no.	Anti-pertussis toxin ELISA titer	CHO cell neutralization titer
Intranasal CVD 908(pCFV-2)		
A1	80	<25
A4	5,120	200
A5	640	50
Intranasal CVD 908(pNFV-3)		
C1	80	<25
C2	10	<25
Parenteral pCFV-2-encoded protein		
B1	10,240	400
B2	163,840	800
B3	81,920	ND ^b
B4	20,480	200
Parenteral pTET ^{nir} 15-encoded protein		
E1	10	<25
E2	10	<25

^a Shown are the results of ELISA for anti-pertussis antibodies and those of CHO cell assays for neutralizing pertussis antitoxin in sera of mice immunized intranasally with four doses of *S. typhi* live vector derivatives or parenterally with three doses of crude protein preparation. Parenteral inoculations consisted of a subcutaneous priming dose given with Freund's complete adjuvant followed by two i.v. booster doses.

^b ND, not determined because of background activity in mouse sera.

DISCUSSION

The stability and immunogenicity of tetanus toxin FragC when expressed in attenuated *Salmonella* strains were capitalized on to create a bivalent fusion protein with the pertussis toxin S1 subunit, which was immunogenic in mice when delivered via *S. typhi* vaccine strain CVD 908. Other examples in which FragC has been used to rescue an otherwise unstably expressed heterologous antigen exist. Khan et al. (26) expressed high levels of a protective epitope from the *Schistosoma* glutathione *S*-transferase antigen by fusion to FragC. Similarly, we used the FragC fusion technique to rescue expression of the binding domain of diphtheria toxin in *S. typhi* (14). Stabilization imparted by FragC may be due to protection of a peptide from proteolytic degradation, as polypeptides with abnormal structures or insoluble characteristics are degraded more rapidly (13).

In this study, we attempted to optimize antigen expression by engineering a variety of fusion protein conformations. Our vectors allowed insertion of the S1 antigen at the amino or carboxyl terminus of FragC. Although fusion proteins of both conformations were expressed at high levels in *S. typhi*, the optimal hybrid conformation was that directed from pCFV-2, the S1 truncate fused to the carboxyl terminus of FragC. This result is supported by the data from Antoine and Locht (1), who demonstrated that the hydrophobic nature of the carboxyl terminus of S1 had a destabilizing effect when expressed in the cytoplasm of a heterologous host strain. The pCFV-2-encoded fusion also proved to elicit the highest antibody responses in serum to both heterologous antigens. Inoculation of viable *S. typhi* expressing this fusion via intranasal delivery in CVD 908 or as a parenteral administration of crude protein induced markedly high levels of antibodies in serum which were able to neutralize pertussis toxin activity in CHO cells. This neutralization activity is consistent with findings from two other

groups who reported CHO cell neutralization activity following parenteral immunization with purified protein preparations of recombinant S1 molecules as a diphtheria toxin fragment A-S1 fusion (2) or as an S1-FragC fusion protein (3). Here, we present the first report of CHO cell neutralizing antibodies generated by an S1 construct delivered mucosally by a live vector vaccine.

In the case of the other fusions encoded by pNFV-3 and pCFV-1, antigen configuration was an important parameter in elicitation of antibody response. Whereas both hybrid proteins were expressed at high levels in CVD 908 and were recognized by monoclonal antibodies to both moieties on Western blots, fusions with S1 at the carboxyl terminus of FragC elicited better antibody responses to pertussis toxin and to tetanus toxin than the amino-terminal fusion protein, independent of the mode of administration. This could be the result of fusion stability or could reflect the importance of individual component configuration within the hybrid protein. In this case, FragC did not serve as a good immunogen unless it was the amino-terminal moiety.

Oral inoculation of mice with these *S. typhi* derivatives did not elicit consistent serum antibody response to the heterologous antigens, a result compatible with the experience of most other investigators who have fed *S. typhi* live vectors to mice. One notable exception is the report of Walker et al. (44), who observed an anti-S1 antibody response in the sera of mice orally inoculated with attenuated *S. typhi* Ty21a bearing a plasmid-encoded S1 construct. In contrast with the poor immunogenicity observed with our live vector constructs when given orally, we found high-titer serum antibody responses following intranasal administration. These results confirm recent studies in our laboratory demonstrating the utility of the intranasal route of immunization with *S. typhi* in the mouse model (11).

We have demonstrated that the *S. typhi* vaccine strains described in this work, when administered intranasally, must be live in order to deliver heterologous antigens to the host immune system; formalin-killed strains failed to elicit any antibody response to the guest antigens. These data suggest that a distinct interaction between live vaccine strain organisms and the intranasal inductive sites of the host mucosal immune system occurs. This information can be added to the growing body of knowledge concerning the utility of the intranasal route of immunization.

Successful intranasal immunization has also been achieved with soluble purified protein antigens or with particulate antigens but, in general, only if a powerful adjuvant (e.g., mutant *Escherichia coli* heat-labile toxin) was coadministered or if a special antigen delivery system (e.g., proteosomes or microcapsules) was used (6, 31, 38). In a comprehensive study involving pertussis antigens, Shahin et al. (38) inoculated mice intranasally with pertussis antigens as soluble proteins or in a microencapsulated form; microencapsulated antigen was clearly superior in generating a protective immune response.

Our strategy to utilize *S. typhi* as a live vector eliminates the need for an adjuvant. It also precludes the need for purification of individual protein antigens. One advantage of CVD 908 as a vaccine strain is the relative ease of genetically manipulating *S. typhi* to allow expression of multiple heterologous antigens. While we have succeeded in achieving expression and immunogenicity of one important pertussis antigen, the ultimate oral *S. typhi*-based pertussis vaccine will require that additional antigens such as filamentous hemagglutinin (16), pertactin (39), or adenylate cyclase toxin be expressed. Such a multivalent live vector is attainable by using CVD 908 as a vector.

Herein we report an early step towards the development of an *S. typhi*-based DTP vaccine that could be administered by

the oral or intranasal route. Inclusion of a diphtheria component, in addition to FragC and multiple pertussis antigens, could result in a multivalent live vector vaccine capable of protecting against four childhood diseases, typhoid, pertussis, tetanus, and diphtheria.

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