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Expression and Immunogenicity of a Mutant Diphtheria Toxin Molecule, CRM_{197}, and Its Fragments in *Salmonella typhi* Vaccine Strain CVD 908-*htrA*

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Mutant diphtheria toxin molecule CRM_{197} and fragments thereof were expressed in attenuated *Salmonella typhi* CVD 908-*htrA*, and the constructs were tested for their ability to induce serum antitoxin. Initially, expressed proteins were insoluble, and the constructs failed to induce neutralizing antitoxin. Soluble CRM_{197} was expressed at low levels by utilizing the hemolysin A secretion system from *Escherichia coli*.

The use of attenuated *Salmonella* strains as live vector vaccines to deliver foreign antigens to the mammalian immune system is an exciting area of vaccinology that has great potential. Previously, we utilized attenuated *Salmonella typhi* expressing fragment C of tetanus toxin and the S1 subunit of pertussis toxin to stimulate, respectively, serum antitoxin that neutralizes tetanus and pertussis toxins following mucosal (intranasal) immunization of mice (3, 7). The success of that approach led us to attempt the same with diphtheria toxin, the long-term goal being an *S. typhi*-based mucosally administered vaccine against diphtheria, pertussis, and tetanus.

Diphtheria toxin (DT), a 535-amino-acid protein encoded by *tox* of *Corynebacterium diphtheriae*, is secreted as a single molecule of 58,350 Da encompassing two functional subunits, subunit A, the catalytic domain responsible for the ADP-ribosylation activity of the toxin in eukaryotic cells, and subunit B, the *trans*-membrane and receptor-binding domains. Human antibodies raised by immunization with diphtheria toxoid react with both subunits A and B (4, 19), and monoclonal antibodies to both subunits can neutralize DT (5, 20, 23, 24). Nevertheless, most neutralizing antitoxin is anti-B subunit and inhibits binding of the toxin to its receptor. A neutralizing epitope has been described corresponding to a cysteine loop (residues 186 to 201) located between the A and B subunits (1).

Because of its potent toxicity, either a stable nontoxic mutant protein (i.e., a cross-reacting molecule, or CRM) or noncatalytic fragments would have to be expressed in *S. typhi*. The most extensively studied nontoxic mutant DT, CRM_{197}, which carries a glycine to glutamic acid substitution at residue 52 within the catalytic domain (8), can induce neutralizing antitoxin (11). We investigated attenuated *S. typhi* vaccine strain CVD 908-*htrA* (15, 22) as a live vector to deliver diphtheria antigens and induce protective antitoxin in animal models.

Initial attempts to express CRM_{197} holotoxin in CVD 908-*htrA*. Toward the goal of expressing relevant diphtheria toxin epitopes within CVD 908-*htrA*, we undertook two parallel approaches involving both expression of the full-length nontoxic mutant holotoxin CRM_{197} and expression of domains or fragments of CRM_{197} in an attempt to increase the levels of synthesis of neutralizing epitopes. With pβ197 as the template, we used PCR to synthesize three *Bgl*II-*Nhe*I cassettes encoding full-length unmodified CRM_{197} (creating pNO1), CRM_{197} into which an optimized ribosome binding site and start codon were engineered (creating pNO2), and CRM_{197} from which the signal sequence was removed (creating pNO3). These constructions are represented graphically in Fig. 1; all primers are summarized in Table 1, and plasmids are summarized in Table 2.

Expression of full-length CRM_{197} within CVD 908-*htrA* was examined by using Western immunoblot analysis of lysates prepared from strains grown anaerobically to induce optimum transcription from the P_{nir15} promoter. Expression of unmodified CRM_{197} within CVD 908-*htrA*(pNO1) was very low when probed with polyclonal antiserum specific for DT (Fig. 2). Expression of CRM_{197} increased with CVD 908-*htrA*(pNO2), wherein the ribosome binding site and initiation codon were optimized. The highest expression of CRM_{197} was detected with CVD 908-*htrA*(pNO3) from which the signal sequence was genetically removed and both the ribosome binding site and initiation codon were optimized.

BALB/c mice, 6 to 8 weeks of age, were immunized intranasally with 2×10^9 CFU of CVD 908-*htrA*(pNO3) on two occasions, 28 days apart (7). Twofold dilutions of sera collected on days 0, 14, and 42 were tested by enzyme-linked immunosorbent assay to detect antibodies to DT, tetanus toxin, and *S. typhi* O antigen (7). Antibodies against DT were not observed, despite the detection of a significant response against the bacterial vector (data not shown).

In a related experiment, three Hartley strain guinea pigs were immunized subcutaneously on days 1, 28, and 56 with 90 μl of crude extract of CVD 908-*htrA*(pNO3) mixed with 125 μl of Imject Alum (Pierce), in a total volume of 250 μl. Guinea pig sera collected on days 0, 10, 38, and 66 revealed a significant serum immunoglobulin G (IgG) ELISA response against DT. The baseline reciprocal geometric mean anti-DT titer (GMT) was <400, and the peak GMT of 25,600 was observed on day 66. These sera containing anti-DT were tested for neutralizing activity in the Vero cell neutralization assay. Briefly, serum samples and standards were diluted 1:2 in modified Eagle's medium supplemented with 2 mM glutamine and 0.5% fetal bovine serum. One hundred microliters per well was introduced into 96-well flat-bottom microtiter plates (Rainin)

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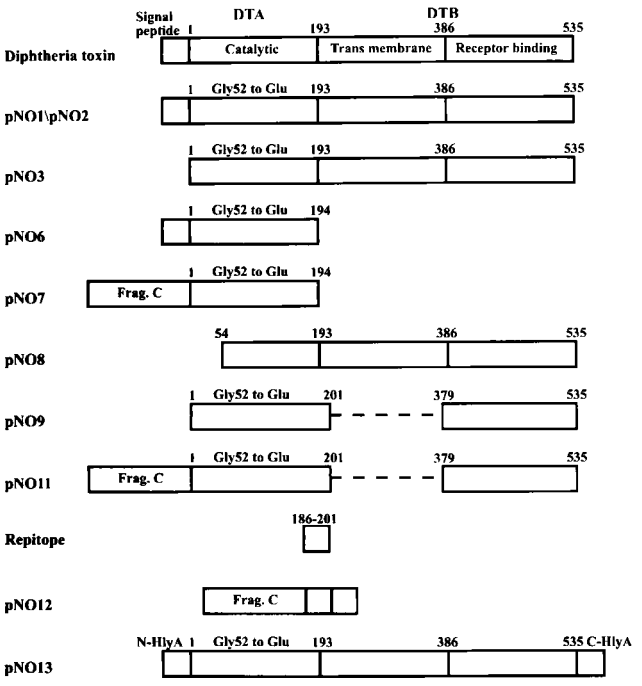

FIG. 1. Illustration of DT derivatives used in this study. The engineering of fragments encoding CRM_{197} or individual domains of this protein were carried out by PCR and Vent DNA polymerase (New England BioLabs, Inc., Beverly, Mass.) with the plasmid template p*B*197 which carries the promoter, signal sequence, and full-length mutant CRM_{197} structural gene. Primers used in the construction of the plasmids employed in this study are described in Table 1 and were designed by using the published sequence of the diphtheria *tox* gene encoded by corynebacteriophage β (10) (GenBank accession no. K01722). PCR products synthesized with Vent polymerase were then treated with *Taq* DNA polymerase to generate the 3′ deoxyadenosine necessary for direct cloning into the plasmid pGEM-T (Promega Corp., Madison, Wis.); recombinant plasmids were recovered by transformation into MAX Efficiency *E. coli* DH5α frozen competent cells (Gibco BRL, Gaithersburg, Md.). Restriction endonuclease sites incorporated into the primers were then used to subclone fragments into plasmids which were introduced into attenuated *S. typhi* live vector CVD 908-*htrA*, using electroporation as previously described (9). Detailed descriptions of the plasmids used in this work are listed in Table 2, and CRM_{197} derivatives generated are graphically represented. Most PCR products were subcloned into pTET*nir*15, either replacing the gene encoding the nontoxic fragment C of tetanus toxin or resulting in synthetic genes encoding protein fusions of fragment C and CRM_{197} or various subdomains. In an attempt to enhance the solubility of potentially relevant neutralizing epitopes of diphtheria toxin, a fragment encoding amino acids 186 to 201 of diphtheria toxin (1) was synthesized and fused in-frame as two copies to the carboxyl terminus of fragment C to create a repitope (14). In addition, we attempted to achieve secretion of CRM_{197} from CVD 908-*htrA* by inserting the open reading frame encoding CRM_{197} in-frame into the unique *Nsi*I site of a truncated version of *hlyA* encoding Hemolysin A within the plasmid pMOhly (13).

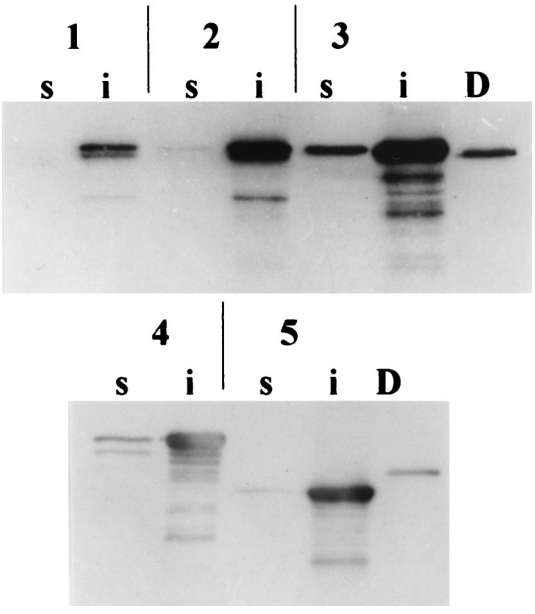

FIG. 2. Western immunoblot analysis of soluble (s) and insoluble (i) fractions from CVD 908-*htrA*-expressing CRM_{197} or its derivatives FC-DTA_{197} and *e*DTB_{197}. Membranes were probed with anti-DT antibodies. Lanes D, DT (5 pg); lane 1, pNO1; lane 2, pNO2; lane 3, pNO3; lane 4, pNO7; lane 5, pNO8. *E. coli* DH5α carrying recombinant plasmids was grown with Luria broth base (LB) medium (Gibco BRL) supplemented with 50 μg of carbenicillin (Sigma, St. Louis, Mo.)/ml. For expression studies, *S. typhi* CVD 908-*htrA* was streaked from frozen stocks onto LB agar supplemented with 0.0001% (wt/vol) 2,3-dihydroxybenzoic acid (DHB; Sigma) and 50 μg of carbenicillin/ml where appropriate. Isolated colonies were then inoculated into LB broth containing DHB and carbenicillin and incubated overnight at 37°C, 250 rpm. For expression under aerobic conditions, late-logarithmic or stationary-phase cultures were diluted 1:100 into fresh LB broth and again incubated overnight at 37°C, 250 rpm; for anaerobic induction experiments, Oxyrase solution (Oxyrase, Inc., Mansfield, Ohio) was added to identical LB broth cultures and incubated static at 37°C overnight. Selected CVD 908-*htrA* strains expressing significant levels of CRM_{197}-derived proteins were grown as previously described (7) for immunization of mice. The 150-ml cultures were grown to an optical density at 600 nm, ~1.0 were centrifuged, and bacterial pellets were resuspended in 3.5 ml of ice-cold sonication buffer (phosphate-buffered saline containing 100 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% Tween 20, and 20 mM β-mercaptoethanol) for solubility studies. Bacterial suspensions were disrupted by sonication for 5 cycles of 20 s on ice by using a model 550 sonic dismembrator (Fisher Scientific, Pittsburgh, Pa.) with a microtip and a power level of 5. Sonicates were centrifuged at 15,000 rpm for 30 min at 4°C, and supernatants representing the soluble fraction were removed; cell pellets were reconstituted with 3.5 ml of sonication buffer to represent the insoluble fraction. Proteins were then heat denatured after equal volumes of sample and lysis buffer were mixed, and proteins from 5 μl of each denatured sample were then separated by SDS-PAGE with 10% polyacrylamide gels. Separated proteins were detected either by staining with BLUPRINT Fast-PAGE Stain (Gibco BRL) or by being transferred to Immun-Lite blotting membrane (Bio-Rad Laboratories, Hercules, Calif.) for Western immunoblot analysis. CRM_{197}-derived proteins were detected by using polyclonal goat anti-DT serum (Biogenesis, Sandown, N.H.), and fragment C fusions were confirmed by using monoclonal mouse anti-fragment C antibodies (Boehringer Mannheim, Indianapolis, Ind.). Membranes were then incubated with horseradish peroxidase-conjugated rabbit anti-goat (Sigma) or peroxidase-conjugated goat anti-mouse IgG (Gibco BRL) as appropriate. Immunoblots were developed by chemiluminescence with an ECL Western blotting kit (Amersham Life Science Inc., Arlington Heights, Ill.), and signals were detected with X-OMAT XAR-5 film (Eastman Kodak Company, Rochester, N.Y.).

to which 37.5×10^{-3} limits of flocculation of diphtheria toxin was added; plates were incubated at room temperature for 1 h. A total of 10^4 Vero cells (ATCC no. CCL81) were added per well, and the plates were incubated at 37°C in 5% CO_2 for 96 h. Cell survival was quantitated by using neutral red at a concentration of 10 μg per well, and optical density was measured at 540 nm. None of the sera tested exhibited neutralizing activity.

We hypothesized that the lack of a serum immune response following the intranasal immunization of mice and the lack of neutralizing activity of the anti-DT antibodies raised by the subcutaneous immunization of guinea pigs might be due to incorrect folding or to reduced solubility of full-length CRM_{197} synthesized within CVD 908-*htrA*. Indeed, as shown in Fig. 2, the majority of CRM_{197} expressed within CVD 908-*htrA* (pNO1) and CVD 908-*htrA* (pNO2) is insoluble, although some soluble holotoxin is observed for CVD 908-*htrA* (pNO3). These results suggest that the insolubility of CRM_{197} expressed within CVD 908-*htrA* is not due to overexpression of the protein, since, as expression levels increased, the amount of apparently soluble CRM_{197} also increased (Fig. 2).

Expression of domains or fragments of CRM_{197} in CVD 908-*htrA*. Since the solubility and immunogenicity of full-length CRM_{197} expressed within CVD 908-*htrA* appeared

TABLE 1. Primers used in this work

Primer no.	Sequence
1	GCGCGCAGATCTAGCTAGCTTTCCCCATGTAACCAATCTATC
2	GCGCGCTAGCTTATCAGCTTTTGATTTCAAAAAATAGCGATAGC
3	GAGATCTTAATCATCCTAAGGAGGTATTCTGATGAGCAGAAAACTGTTTGCGTCAATC
4	AGATCTTAATCATCCTAAGGAGGTATTCTGATGGGCGCTGATGATGTTGTTGATTCTTCT
5	GCGCGGATCCTTATTATGATCGCCTGACACGATTTCCTGCACAGGCT
6	AAGCTTGGCGCTGATGATGTTGTTGATTCTTCTA
7	GCGCAGATCTTAATCATCCTAAGGAGGTATTCTGATGTATAGTACCGACAATAAATACGACGCTGCG
8	GCTAGCGGATCCTTATTAGCTCGAGCATGACAATGAGCTACCTACTGATCGC
9	CTCGAGCGCGTATTCTCCGGGGCATAAAACG
10	CGCATGCATGGGCCGGGGCCCATGAAAAACCTTGATTGTTGGGTC
11	GGATCCTCATTAGCTCGAGGGTACCCGCGGATCATGGTCGTTGGTCCAACCTTCATCGGTCGG
12	GCGTCGACTGGCGCTGATGATGTTGTTGATTCTTCTA
13	AGATCTTAATCATCCACAGGAGGATTTCTGATGTCGACTTGTGCAGGAAATCGTGTCAGGC
14	CTGCAGCTGGCGCTGATGATGTTGTTGATTC
15	ATGCATCGCTTTTTGATTTCAAAAAATAGCGATAGC

problematic, we expressed various domains of the holotoxin in an attempt to enhance the expression of soluble antigen. Initial attempts to express fragment A of CRM_{197} by simply replacing the *Bgl*II-*Nhe*I gene cassette encoding fragment C in pTET*nir*15 to create pNO6 were unsuccessful, probably due to proteolytic degradation (data not shown). We constructed another cassette encoding mature DTA_{197} without the signal sequence as a *Hin*dIII-*Bam*HI cassette, which was inserted into the expression vector pOG214 (9) to create pNO7, which now carries a synthetic gene encoding the protein fusion of mature DTA_{197} fused to the carboxyl terminus of fragment C and separated by a 4-amino-acid hinge region (Fig. 1). This approach was previously used to rescue expression of the receptor binding domain of CRM_{197} (9). In a related approach, we constructed pNO8 to express a truncated holotoxin in which the amino-terminal 53 amino acids including the catalytic site of the holotoxin were deleted; this construct therefore encodes an extended version of the DTB subunit, which we refer to here as *e*DTB_{197}. Both re-engineered genes were expressed at high levels in CVD 908-*htrA* when grown anaerobically, and the fusion proteins were readily detected both in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels stained for total protein (data not shown) and with anti-DT antibodies by Western immunoblot analysis (Fig. 2, lanes 4 and 5). However, the solubility of these protein fusions did not improve. Serum anti-DT was not detected in mice immunized intranasally with CVD 908-*htrA*(pNO7) or CVD 908-*htrA* (pNO8), and neutralizing antitoxin was not detected in guinea pigs immunized with extracts from these strains.

Since DT subunit B contains highly hydrophobic transmembrane domains which could contribute to the insolubility of CRM_{197} and its derivatives, a final derivative, designated

TABLE 2. Plasmids used in this work

Plasmid	Primers used	Description	Reference or source
pTET*nir*15		Derivative of pBR322 carrying the *toxC* gene, encoding fragment C, under control of the P_{nir15} promoter	18
p*B*197		Plasmid encoding CRM_{197}, a nontoxic mutant of DT carrying a substitution of glutamic acid for glycine at residue 52	8
pOG214		Derivative of pTET*nir*15 in which a 4-amino-acid hinge region was incorporated at the carboxyl terminus of fragment C	9
pNO1	1, 2	Derivative of pTET*nir*15 in which the gene encoding CRM_{197} replaced *toxC*	This work
pNO2	3, 2	Derivative of pNO1 in which the ribosome binding site and the initiation codon were optimized	This work
pNO3	4, 2	Derivative of pNO1 in which the signal peptide was removed and the ribosome binding site and initiation codon were optimized	This work
pNO6	1, 5	Derivative of pTET*nir*15 in which the gene encoding the mutated DTA of CRM_{197} (i.e., DTA_{197}) replaced *toxC*	This work
pNO7	6, 5	Derivative of pOG214 carrying a gene fusion in which the gene encoding DTA_{197} was fused in-frame to the 3′ terminus of *toxC*	This work
pNO8	7, 2	Derivative of pNO1 from which the sequence encoding the first 53 amino acids of CRM_{197} was removed	This work
pNO9	4, 8, 9, 2	Derivative of pNO3 in which the sequence encoding the *trans*-membrane domain was truncated to express *t*DT_{197}	This work
pNO10	10, 11	Derivative of pTET*nir*15 in which an 8-amino-acid hinge region was incorporated at the carboxyl terminus of fragment C (6)	This work
pNO11	12, 2	Derivative of pNO10 in which the gene encoding *t*DT_{197} was fused in-frame to the 3′ terminus of *toxC*	This work
pNO12	13, 8	Derivative of pNO10 in which a sequence encoding two tandem repeats of the 16-amino-acid epitope (186 to 201) of DT was fused in-frame to the 3′ terminus of *toxC*	This work
pMOhly		Plasmid containing a complete HlyA secretion system with an insertion site for foreign gene fusion within a truncated *hlyA*	12
pNO13	14, 15	Derivative of pMOhly in which the gene encoding CRM_{197} was inserted in-frame into *hlyA*	This work

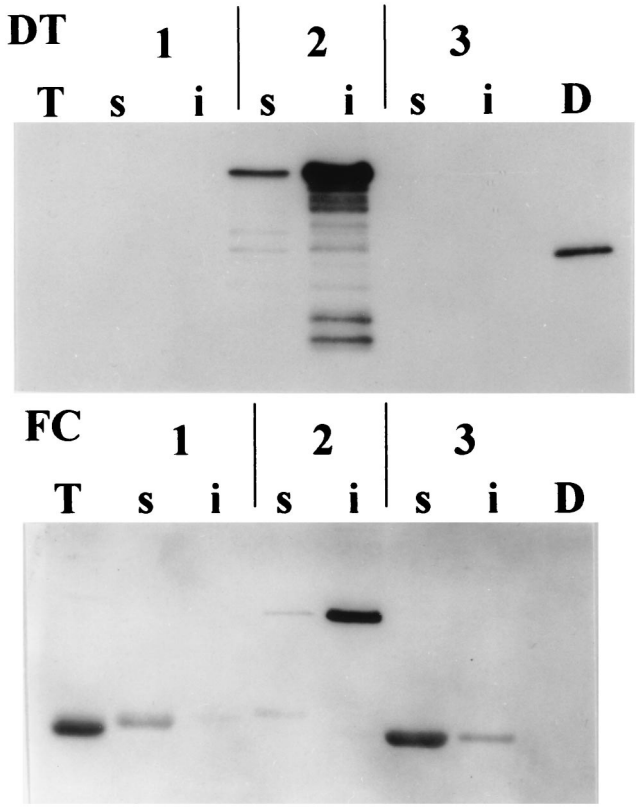

FIG. 3. Western immunoblot analysis of soluble (s) and insoluble (i) fractions of CVD 908-*htrA* expressing fragment C fused in-frame to a repitope of DT residues 186 to 201 or to $t\text{DT}_{197}$. Membranes were probed with anti-DT or anti-FC antibodies. Lanes: T, pTETnir15; 1, pNO12; 2, pNO11; 3, pNO10; D, DT (5 pg).

pNO9, was constructed encoding a mutant toxin in which the *trans*-membrane domain of CRM_{197} from residues 202 to 378 was removed and replaced with a unique *Xho*I site. We refer to this truncated DT holotoxin as $t\text{DT}_{197}$. Since expression of $t\text{DT}_{197}$ from pNO9 proved to be undetectable by using Western immunoblots, we also constructed the expression plasmid pNO11 (Fig. 1) which encodes a protein fusion in which $t\text{DT}_{197}$ was fused in-frame to the carboxyl terminus of fragment C and separated by an 8-amino-acid hinge region, previously reported to enhance expression of protein fusions involving the B subunit of the heat-labile enterotoxin from *Escherichia coli* (6). Although CVD 908-*htrA*(pNO11) expressed high levels of the fusion protein, detectable with antisera specific for both DT and fragment C, the majority of the product remained insoluble (Fig. 3, lane 2).

One final attempt to express soluble epitopes from DT involved construction of pNO12, expressing a repitope in which two tandem repeats of the hexamer peptide comprised of amino acids 186 to 201 of DT were fused to the carboxyl terminus of fragment C (Fig. 1) (1). The basic expression vector used in the construction of both pNO11 and pNO12 is designated pNO10 and is a derivative of pOG214 in which the 4-amino-acid Gly-Pro-Gly-Pro hinge region fused to the carboxyl terminus of fragment C was replaced by the 8-residue hinge His-Asp-Pro-Arg-Val-Pro-Ser-Thr. Cassettes encoding proteins to be fused in-frame to the carboxyl terminus of fragment C must be inserted into the unique *Xho*I site or must carry 5′-proximal *Sal*I or *Xho*I sites and 3′-proximal *Bgl*II, *Bam*HI, or *Nhe*I sites. Therefore, a repitope (14) consisting of

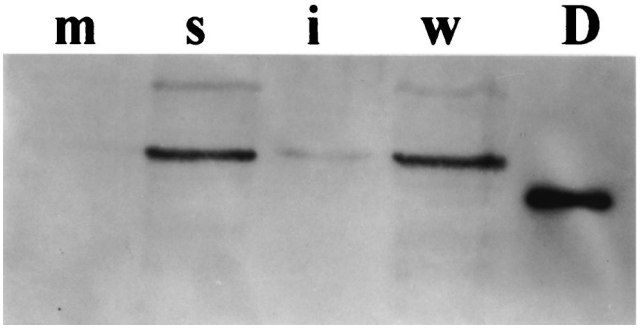

FIG. 4. Western immunoblot analysis of CVD 908-*htrA*(pNO13). Membranes were probed with anti-DT antibodies. Lanes: m, medium; s, soluble fraction; i, insoluble fraction; w, whole cell; D, DT (5 pg).

two copies of a 16-amino-acid loop region between cysteine residues 186 and 201 was constructed, and the DNA sequence encoding this repitope was inserted in-frame as a *Sal*I-*Xho*I cassette into the *Xho*I site of pNO10, creating pNO12. CVD 908-*htrA*(pNO12) expressed reasonable levels of soluble fusion product but was recognized in Western blots only by antibodies specific for fragment C (Fig. 3, lane 1). Mice immunized intranasally with CVD 908-*htrA*(pNO12) did not develop DT antibodies, and subcutaneous immunization of guinea pigs with extracts from CVD 908-*htrA*(pNO12) plus adjuvant did not elicit neutralizing anti-DT.

Expression of CRM_{197} by using the HlyA expression system. Since inclusion of the native signal sequence failed to drive expression of a soluble product within CVD 908-*htrA*(pNO1) and CVD 908-*htrA*(pNO2), we used an alternate secretion mechanism derived from *E. coli*, in which heterologous antigens are inserted in-frame into a truncated version of the hemolysin A protein and potentially secreted by the hemolysin secretion apparatus (13). For this purpose, the gene encoding mature CRM_{197} holotoxin was re-engineered to include the appropriate restriction sites and inserted in-frame as a *Pst*I-*Nsi*I cassette into the unique *Nsi*I site within the truncated *hlyA* gene of pMOhly, creating pNO13 (Fig. 1). In this case, the majority of the HlyA-CRM_{197} fusion product was soluble, although no product was detected in the medium (Fig. 4, lane s versus lanes m and i). However, the level of expression was low compared to that induced by pNO3. Despite this low level of expression, we examined the immunogenicity of this fusion protein in mice immunized intranasally with CVD 908-*htrA*(pNO13). No serum antibody response against DT was detected.

We have explored the use of CVD 908-*htrA* as a live vector to express CRM_{197} holotoxin, A subunit, B subunit, and epitope derivatives and to deliver these antigens to the host immune system for induction of a serum-neutralizing antitoxin response. Others have reported that immunization with CRM_{197} or a fusion protein comprised of a mutant DTA fused to the C180 peptide of the S1 subunit of pertussis toxin was able to elicit a neutralizing antibody response against DT (2, 11). The poor immunogenicity of our DT constructs is likely due to the insolubility of the protein products, an observation frequently made with recombinant proteins that are cytosolic or even periplasmic (17). Improperly folded insoluble protein products may fail to configure neutralizing epitopes and may expose nonrelevant epitopes that lead to a nonneutralizing antibody response. Immune responses induced after immunization with inclusion bodies may not correlate with the immune response induced by the corresponding soluble antigens (21). Overexpression of cytosolic, or even periplasmic, recombinant proteins in *E. coli* can lead to the formation of inclusion

bodies (17). This was not the case in our study, since CRM_{197} that included the signal sequence was insoluble, despite being expressed at relatively low levels by pNO1 or pNO2. Furthermore, the recombinant CRM_{197} remained insoluble even when the signal peptide was deleted in pNO3 and did not improve significantly when smaller domains of CRM_{197} were used. The results indicate that overexpression of the recombinant protein was not the major factor responsible for the insolubility.

In a distinct approach to achieve stable expression of an antigen that might elicit neutralizing anti-DT antibodies, we expressed a cysteine loop peptide (amino acids 186 to 201 of DT) that constitutes a putative neutralizing epitope (1). Audibert et al. parenterally immunized guinea pigs with synthetic peptides corresponding to this epitope, plus adjuvant, and elicited serum antibodies that protected against challenge with DT. This peptide, expressed as a repitope fused to the carboxyl terminus of tetanus toxin fragment C (14), was soluble even when expressed at high levels but was not recognized by anti-DT antibodies in Western blots, and animals immunized with this construct failed to develop neutralizing anti-DT. This result is supported by a recent study showing that antibodies raised against a linear peptide comprised of residues 168 to 220 of DT were poorly neutralizing in the Vero cell cytotoxicity assay (16).

The hemolysin A secretion system has been used to express foreign proteins in *Salmonella* (12, 13). Therefore, pursuing a final strategy, we adapted the Hemolysin A secretion system to express CRM_{197} in *S. typhi*. The hemolysin A secretion system includes genes encoding a highly truncated hemolysin A (the secreted protein) and hemolysins B, C, and D (accessory proteins that participate in the secretion mechanism). By cloning CRM_{197} in-frame within truncated hemolysin A (pNO13), we finally succeeded in producing predominantly soluble CRM_{197}. The level of expression, however, was markedly lower than that achieved with the earlier CRM_{197} constructs driven by P_{nir15}. Not surprisingly, mice immunized intranasally with CVD 908-*htrA* expressing these low levels of soluble CRM_{197} failed to manifest serologic responses against DT. In this case, it is likely that the low level of expression of HlyA-CRM_{197} precluded elicitation of an immune response by the live vector.

The next task is to improve the efficiency of the hemolysin A or another secretion system so that higher levels of expression of soluble CRM_{197} can be achieved. With greater expression of soluble mutant DT by *S. typhi* live vectors, it may be possible following mucosal immunization to stimulate serum antibodies capable of neutralizing DT. This would be a critical step toward an *S. typhi*-based mucosal diphtheria-pertussis-tetanus vaccine.

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