Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11261–11265, November 1994
Microbiology

Construction, expression, and immunogenicity of the *Schistosoma mansoni* P28 glutathione *S*-transferase as a genetic fusion to tetanus toxin fragment C in a live Aro attenuated vaccine strain of *Salmonella*

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Communicated by François Jacob, July 12, 1994 (received for review March 17, 1994)

ABSTRACT **A vector has been constructed to allow genetic fusions of guest antigens via a hinge domain to the C terminus of the highly immunogenic C fragment of tetanus toxin. A fusion has been constructed with the gene encoding the protective 28-kDa glutathione *S*-transferase (EC 2.5.1.18) from *Schistosoma mansoni*. The recombinant vector has been electroporated into the nonvirulent *Salmonella typhimurium aroA* live vaccine strain SL3261. The corresponding chimeric protein is stably expressed in a soluble form in *Salmonella* as evaluated by Western blotting with fragment C and glutathione *S*-transferase antisera. Mice immunized intravenously with a single dose of the live recombinant bacteria elicit antibodies to both fragment C and glutathione *S*-transferase as detected by enzyme-linked immunosorbent assays. Furthermore, all of the mice were solidly protected when challenged with lethal doses of either tetanus toxin or the virulent *Salmonella typhimurium* strain C5. Mice have also elicited antibodies to fragment C and glutathione *S*-transferase after oral immunization. It may be that a live trivalent vaccine against typhoid, tetanus, and schistosomiasis is feasible.**

Human schistosomiasis, a chronic and debilitating disease caused by trematodes of the genus *Schistosoma*, remains a major health problem with a prevalence of ≈200 million and some 500,000 deaths per year. Schistosomiasis in cattle also causes considerable economic loss. There is a search for vaccines for schistosomiasis, and significant progress has been made in the identification of protective antigens and the development of potential vaccine preparations (reviewed in ref. 1). The *Schistosoma mansoni* 28-kDa glutathione *S*-transferase (P28; EC 2.5.1.18) has displayed considerable promise as a candidate vaccine antigen. The recombinant protein is protective in experimental infections of mice, rats, hamsters, and baboons (2, 3). Vaccination of cattle with the glutathione *S*-transferase of *Schistosoma bovis* has been shown to protect against challenge infection (4).

The new generation of live oral *Salmonella* vaccines is showing promise as carriers for the delivery of heterologous antigens to the immune system. Recombinant salmonellae have been used to deliver antigens from viruses, bacteria, and parasites, eliciting secretory, humoral, and cell-mediated immune responses to the recombinant antigens, which protect from disease (5–8).

A major consideration in the development of combined *Salmonella* vaccines is obtaining a sufficiently high level of expression of the recombinant antigen in the *Salmonella* strain to trigger an immune response. However, unregulated high-level expression of foreign antigens can be toxic and affect cell viability, rendering the *Salmonella* carrier ineffective or causing loss of the recombinant DNA (reviewed in ref. 9). An elegant approach that has been applied in mycobacteria is to use heat-shock promoters that are inducible *in vivo* to express foreign antigens (10).

The latter approach has also met with considerable success in *Salmonella*, by using the *Escherichia coli* nitrite reductase promoter *nirB*, which is induced under anaerobiosis, to drive the expression of the atoxic but highly immunogenic fragment C of tetanus toxin (TetC) (11). A *Salmonella* Aro strain harboring this construct (pTETnir15) elicited very high antitetanus antibody responses in mice (11). The animals were protected against subsequent challenge with tetanus toxin following a single oral dose of the vaccine. Tetanus toxoid has been extensively used as an adjuvant for chemically coupled antigens (12). The potent immunogenicity of TetC in *Salmonella* suggests that it may be possible to exploit this character to promote the immune response to guest antigens. The genetic fusion of a guest antigen to a carrier protein is very attractive, as it allows a precise fusion of defined composition to be made. However, fusing two proteins together often leads to an incorrectly folded chimeric protein that no longer retains the properties of individual components. The B subunit of the *Vibrio cholerae* (CT-B) and *E. coli* (LT-B) enterotoxins are powerful mucosal immunogens. Genetic fusions to these subunits can alter the structure and properties of the carrier and hence their immunogenicity (13, 14). The successful use of LT-B as a fusion partner by incorporating a short flexible hinge region at the C terminus of LT-B into which guest peptides can be cloned has been reported (15, 16). The result is a fusion protein that retains many properties of the carrier and elicits an immune response to the guest antigen.

We describe the rational design of a TetC fusion vector and its use to express a C-terminal fusion with the full-length P28 protein. To promote the correct folding of the guest and also the carrier protein, a hinge region has been introduced at the

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Abbreviations: P28, *Schistosoma mansoni* 28-kDa glutathione *S*-transferase; TetC, tetanus toxin C fragment.
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3′ end of the TetC gene followed by cloning sites. Mice have been immunized with a single dose of the live recombinant salmonellae by intravenous and oral routes, and the ensuing immune responses are described.

MATERIALS AND METHODS

Plasmids, Oligonucleotides, the Polymerase Chain Reaction (PCR), and Bacterial Strains. The plasmid pTETnir15 directs the expression of TetC under the control of the *nirB* promoter (11). The TetC-hinge fusion vector pTECH1 was in part constructed from pTETnir15 by the PCR (17). PCR was performed by using the high-fidelity thermostable DNA polymerase from *Pyrococcus furiosus*, which possesses an associated 3′,5′-exonuclease proofreading activity (18). A TetC-hinge replacement cassette was synthesized by using the primers listed in Fig. 1. The amplification reaction was performed according to the manufacturer's instructions (Stratagene). Similarly, a P28 gene expression cassette was also produced by PCR with pUC19-P28 DNA (3) as template and the primers listed in Fig. 1.

The bacterial strains used were *E. coli* TG2 (*recA*; ref. 19), *Salmonella typhimurium* SL5338 (*galE* r^-m^+; ref. 20), SL3261 (*aroA*; ref. 21), and the mouse virulent wild-type *S. typhimurium* C5 (22). Bacteria were cultured in either L or YT broth and on L-agar with ampicillin (50 μg/ml) if appropriate. Plasmid DNA prepared in *E. coli* was first modified by transformation into SL5338 to increase the efficiency of electroporation into the SL3261 *aroA*(r^+m^+) vaccine (19).

SDS/PAGE and Western Blotting. Expression of the TetC fusions was tested by SDS/PAGE and Western blotting. Cells growing in mid-logarithmic phase, with antibiotic selection, were harvested by centrifugation and the proteins were fractionated by 10% SDS/PAGE. The proteins were transferred to a nitrocellulose membrane by electroblotting and allowed to react with either a polyclonal rabbit antiserum directed against TetC or the full-length P28 protein. The blots were then probed with goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (HRP) (Dako) and developed with 4-chloro-1-naphthol.

Inoculations, Viable Counts, and ELISA. Female BALB/c mice were purchased from Harlan Olac (Blackthorn, Bicester, U.K.) and used when at least 8 weeks of age. Bacteria were grown in YT broth supplemented with ampicillin (50 μg/ml) as required. For intravenous inoculation, stationary cultures were diluted in phosphate-buffered saline (PBS) and animals were given $\approx 10^6$ colony-forming units (cfu) in 0.2 ml by the lateral tail vein. For oral inoculation, bacteria were grown in shaken overnight cultures and concentrated by centrifugation, and animals under light ether anesthetic received 5×10^9 cfu in 0.2 ml intragastrically via a gavage tube. The inoculum doses were checked by viable counts on tryptic soy agar. For viable counts on organ homogenates, groups of three mice were sacrificed at intervals, the livers and spleen and (for orally inoculated mice) a pool of mesenteric lymph nodes were homogenized separately in 10 ml of distilled water in a Colworth stomacher (22), and viable counts were performed on tryptic soy agar supplemented with 50 μg of ampicillin per ml as required.

Groups of mice immunized intravenously or orally were bled from the tail at weekly intervals and the sera were stored individually. Anti-fragment C antibodies were measured by ELISA as described (23), using plates coated with 0.1 μg of TetC (Boehringer Mannheim) per well. Sera were diluted 1:20 in blocking buffer, 2% casein in PBS. Anti-mouse HRP conjugate was used according to the manufacturer's instructions (Dako). The plates were developed using 3,3′,3,3′-tetramethylbenzidine dihydrochloride (Sigma) according to the manufacturer's instructions. After 10 min at 37°C the reaction was stopped with 2 M H_2SO_4 and read at 450 nm. Anti-P28 antibodies were detected as above, coating the plates with 1 μg of recombinant P28 per well as described (24).

Tetanus Toxin and *Salmonella* Challenge. Mice were challenged with 50 LD_{50} doses of tetanus toxin as described (25). Each mouse was injected subcutaneously in a hind leg with 50 ng of tetanus toxin in a 0.2-ml solution of 50% bovine heart infusion/50% PBS, 28 days postimmunization. Mice that developed symptoms of tetanic paralysis were humanely euthanized and survivors were recorded after 4 days.

Mice were challenged orally with 10^9 cfu of the mouse virulent *S. typhimurium* strain C5, which has an oral LD_{50} of 10^6 cfu in BALB/c mice (21).

RESULTS

Design and Construction of the Expression Vectors. A vector for the expression of guest antigens as C-terminal fusions to TetC via a short intervening hinge region and driven by the *nirB* promoter, designated pTECH1, was constructed by modifying the existing pTETnir15 plasmid (11) by the following approach. Convenient restriction sites, preceded by a hinge region encoding a Gly-Pro-Gly-Pro motif, were introduced at the 3′ end of the TetC coding region by means of primers tailored with "add-on" adaptor sequences (Fig. 1) using the PCR (17). Essentially the strategy involved the amplification of a segment of DNA from the unique *Sac* II site within the TetC gene to the final codon (Fig. 2). The antisense primer in this amplification reaction was tailored with a 38-base 5′-adaptor sequence (Fig. 1). In general, adaptor sequences for cloning PCR products are <10 bp in length (26). This unusually long 38-base add-on sequence did not appear to affect the viability or specificity of the amplification reaction (data not shown). The product was gel-purified, digested with *Sac* II and *Bam*HI, and then cloned into the residual 2.8-kb pTETnir15 vector, which had been previously digested with the respective enzymes. This vector was designated pTECH1 (Fig. 2).

The hinge region was designed to promote the independent folding of the carrier and guest proteins by providing spatial and temporal separation between the domains. The same rationale was used for the design of an LTB fusion vector (15). The hinge region was synthesized to encode for a Gly-Pro repeat motif, as such regions are present in a number of different proteins and are thought to demarcate protein domains (27). Codons that are infrequently utilized in *E. coli*

A Primer 1. Sense TetC PCR primer (21mer):

*Sac*II

5′- AAA GAC TCC GCG GGC GAA GTT -3′

B Primer 2. Anti-sense TetC-Hinge PCR primer, add-on sequence underlined (64mer):

*Bam*HI STOP *Spe*I *Xba*I **Hinge Region** TetC sequence 3′-end

5′- CTAT GGA TCC TTA ACT AGT GAT TCT AGA **GGG CCC CGG CCC** GTC GTT GGT CCA ACC TTC ATC GGT -3′

C Primer 3. P28 sense PCR primer:

5′-TAGTCTAGAATGGCTGGCGAGCATATCAAG-3′

D Primer 4. P28 antisense PCR primer:

5′-TTAGGATCCTTAGAAGGGAGTTGCAGGCCT-3′

FIG. 1. DNA sequences of oligonucleotides utilized in construction of the pTECH vector and pTECH1-P28.

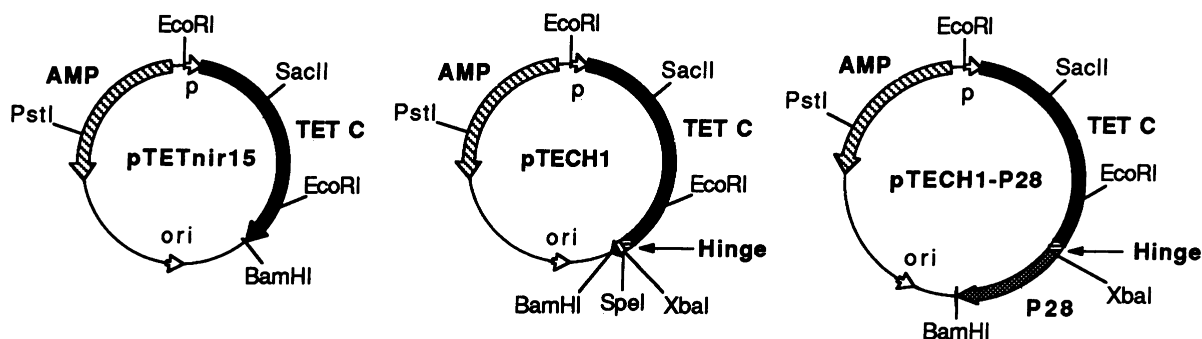

FIG. 2. Structure of the expression vectors constructed and utilized. The ampicillin-resistance gene is designated "AMP" and the *nirB* promoter is designated "p."

(28) and *Salmonella* were selected to encode for the hinge, as such rare codons are thought to cause ribosomal pausing during translation of the mRNA and allow for the correct folding of polypeptide domains (29). In addition, where possible, restriction enzymes were chosen for the cloning region that, when translated in the resulting fusion, does not encode for bulky side groups.

A P28 gene expression cassette was produced by PCR using as template P28 cDNA that had been cloned into pUC19. Oligonucleotide primers were designed to amplify the full-length P28 gene beginning with the start codon and terminating with the stop codon (Fig. 1). In addition, the sense and antisense primers were tailored with the restriction sites for *Xba* I and *Bam*HI, respectively. The product was gel-purified and digested with *Xba* I and *Bam*HI and then cloned into pTECH1, which had previously been digested with these enzymes and subsequently gel-purified (Fig. 2).

Analysis of Protein Expression. Expression of the TetC-P28 fusion protein was evaluated by SDS/PAGE and Western blotting of bacterial cells harboring the construct. The fusion protein remains soluble, reacts with antisera to TetC and P28, and is also of the expected molecular mass, 80 kDa, for a full-length fusion (Fig. 3). However, it has been estimated that the level of expression of the TetC-P28 fusion is ≈2-fold lower than that of TetC from pTECH1, which itself expresses TetC ≈3-fold less than the parental pTETnir15 vector (data not shown).

The fusion protein is stably expressed in a number of different genetic backgrounds, including *E. coli* TG2 and *S. typhimurium* SL5338 (data not shown) and also SL3261, as judged by SDS/PAGE and Western blotting (Fig. 3). Of interest, a band of 50 kDa that comigrates with the TetC-hinge protein alone and reacts exclusively with the anti-TetC sera is visible in a Western blot (Fig. 3, lanes 4). As the codon selection in the hinge region has been designed to be suboptimal, the rare codons may cause pauses during translation, which may lead to the premature termination of translation, thus accounting for the band.

The amino acid residues of glutathione *S*-transferases involved in binding glutathione are thought to be spatially separated in the primary structure of the polypeptide and brought together to form a glutathione binding pocket in the tertiary structure (30). To gauge whether the P28 component of the fusion has folded correctly to adopt a conformation capable of binding glutathione, we tested its ability to be affinity purified on a glutathione-agarose matrix. The results suggest that the TetC-P28 fusion protein can indeed bind to the matrix and the binding is reversible, as the fusion can be competitively eluted with free glutathione (data not shown).

Immunogenicity of Constructs. Viable counts performed on homogenates of liver, spleen, and lymph nodes of groups of mice inoculated intravenously and orally with SL3261, SL3261(pTECH1), and SL3261(pTECH1-P28) showed that the recombinant construct grew and persisted in the tissues in a manner very similar to that of the parent strain. Viable counts of SL3261(pTECH1-P28) on media with and without ampicillin were very similar for up to 11 days after inoculation, indicating that the plasmid was not being lost *in vivo* (data not shown).

Tail bleeds were taken at weeks 4 and 7 from all 16 mice. All mice immunized with SL3261(pTECH1) and SL3261-(pTECH1-P28) invoked a strong antibody response to TetC (Fig. 4). Only mice immunized with SL3261(pTECH1-P28) elicited antibodies to P28 and at week 4 all 16 mice were positive after a single immunization (Fig. 4). No anti-TetC or anti-P28 antibodies were detected in sera from mice immunized with SL3261 alone. A confirmatory experiment (data

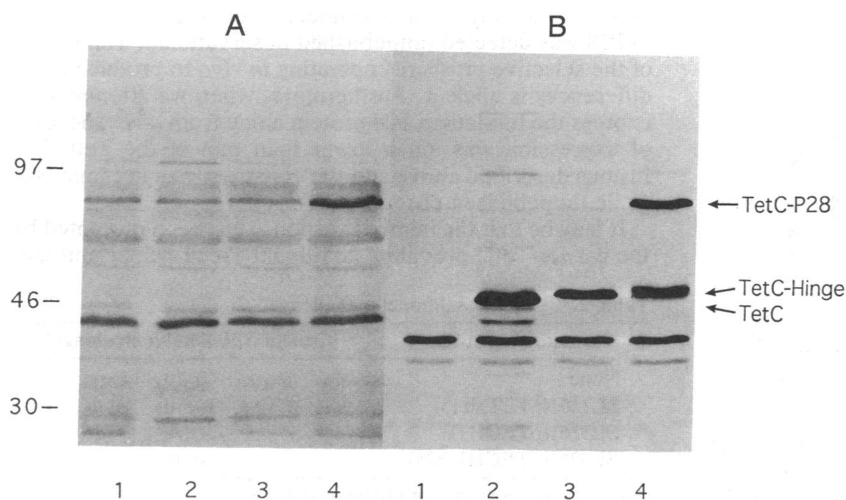

FIG. 3. Expression of TetC fusions as determined by SDS/PAGE and Western blotting. (*A*) Probed with a rabbit anti-P28 polyclonal antiserum. (*B*) Probed with a rabbit anti-TetC polyclonal antiserum. Lanes 1, SL3261 cells only; lanes 2, SL3261(pTETnir15); lanes 3, SL3261(pTECH1); and lanes 4, SL3261(pTECH1-P28). Molecular mass markers are indicated in kDa.

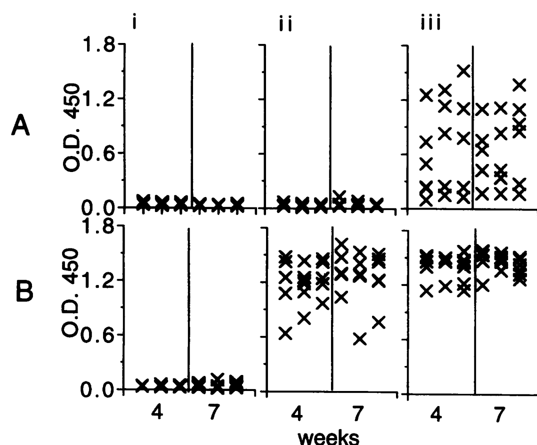

FIG. 4. Antibody responses against recombinant P28 (*A*) and TetC (*B*) as detected by ELISA in mice inoculated intravenously with SL3261 (i), SL3261(pTECH1) (ii), and SL3261(pTECH1-P28) (iii). Results are expressed as OD in individual mice at weeks 4 and 7 after immunization of 16 mice per group.

not shown) demonstrated antibody to TetC and P28 detectable by week 3.

Groups of 10 mice were immunized orally with SL3261, SL3261(pTECH1), and SL3261(pTECH1-P28) and were bled from weeks 3, 7, and 10. Mice immunized with the constructs expressing TetC made antibody responses to TetC (Fig. 5). From the mice immunized with SL3261(pTECH1-P28) approximately half of the mice elicited antibodies to P28. No anti-TetC or anti-P28 antibodies were detected in mice immunized with SL3261 alone.

Tetanus Toxin and *Salmonella* Challenge. It is possible that the addition of a hinge domain and a guest antigen to the C terminus of TetC would destroy or mask epitopes important in eliciting protective immune responses to tetanus toxin. To investigate this possibility mice immunized with SL3261-(pTECH1) and SL3261(pTECH1-P28) were challenged with 50 times the lethal dose of tetanus toxin. The experiment was performed twice; from the mice that were immunized with SL3261(pTECH1) 8/10 and 11/14 mice were protected. However, all of the mice, 10/10 and 14/14, that were immunized with SL3261(pTECH1-P28) were solidly protected against the lethal effects of tetanus toxin challenge (Table 1).

It cannot be excluded that the introduction of the constructs into the *Salmonella* strain SL3261 has had an adverse effect on the ability of the strain to protect against a virulent

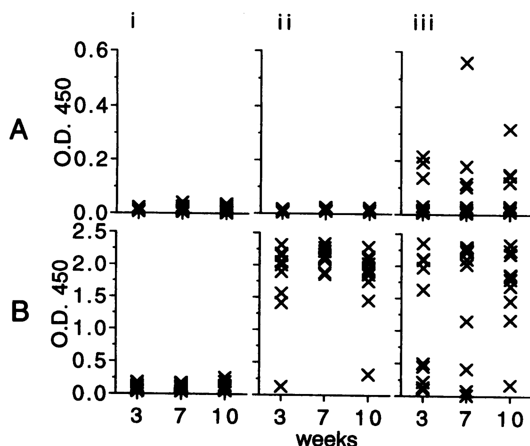

FIG. 5. Antibody responses against recombinant P28 (*A*) and TetC (*B*) as detected by ELISA from mice inoculated orally with SL3261 (i), SL3261(pTECH1) (ii), and SL3261(pTECH1-P28) (iii).

Table 1. Tetanus toxin challenge

Immunizing strain	Tetanus toxin challenge* Exp. 1	Exp. 2
SL3261	0/10	0/14
SL3261(pTETnir15)	10/10	14/14
SL3261(pTECH1)	8/10	11/14
SL3261(pTECH1-P28)	10/10	14/14

*No. of survivors/no. of challenged mice.

strain of *S. typhimurium*. Mice that had been immunized with SL3261(pTECH1-P28) and had also been challenged with tetanus toxin in the experiment described above (Table 1, experiment 1) were now challenged with 1000× LD_{50} virulent *Salmonella* strain C5, as were control groups of naive mice. In marked contrast to the naive mice, all of those that had been immunized with SL3261(pTECH1-P28) were completely protected (Table 2).

DISCUSSION

The present results indicate that expression of recombinant antigens in salmonellae as C-terminal fusions to TetC driven by the *nirB* promoter merits consideration as an effective strategy for constructing combined *Salmonella* vaccines. We describe the construction of genetic fusions between the nontoxic but highly immunogenic TetC and a guest protein, the full-length *S. mansoni* P28 antigen. Responses were observed to the full-length P28 and also to TetC. The method has allowed us to obtain the response to an immunogenic antigen from *S. mansoni* in a *Salmonella* carrier.

Tetanus toxoid has been extensively used as an adjuvant to promote the immune response to chemically linked epitopes (12). We have now shown that precise genetic fusions to TetC also result in immune responses to the guest antigens. Many heterologous genes expressed in bacteria are not produced in soluble, properly folded, or active forms and can accumulate as insoluble aggregates (31). However, the TetC fusions to the full-length P28 protein are all soluble and are expressed in *E. coli* and *S. typhimurium*. In addition, the TetC-P28 protein fusion was capable of being affinity purified by a glutathione-agarose matrix, suggesting that the P28 had folded correctly to adopt a conformation still capable of binding to its natural substrate.

The salmonellae expressing the recombinant antigens all persisted in the mouse tissues as well as the parental strain, and the plasmids were not lost *in vivo*. We have observed that P28 expressed from the constitutive *lac* promoter in pUC19 was stable *in vitro*, but the *Salmonella* carrier quickly lost the plasmid when the strain was injected into mice; no antibody to P28 was detected (unpublished observations). The nature of the selective pressures operating *in vivo* to produce these differences is unclear. Furthermore, when we attempted to express the full-length P28 protein alone from *nirB*, the level of expression was much lower than that of the TetC-P28 fusions described above and the construct was not immunogenic (unpublished observations).

It may be that the immune response has been promoted by the carrier TetC providing additional T-cell helper epitopes

Table 2. Virulent salmonella challenge

Immunizing strain	Virulent *Salmonella* challenge*
None	0/10
SL3261(pTETnir15)	10/10
SL3261(pTECH1)	8/8
SL3261(pTECH1-P28)	10/10

*No. of survivors/no. of challenged mice.

(32). By week 4 all of the mice immunized with SL3261 cells carrying pTECH1-P28 responded to TetC and also to the full-length P28 protein following a single intravenous inoculation. Mice immunized with only a single oral inoculation also responded to TetC and P28, although not all of the mice responded to P28. It is likely that the response to P28 could be improved by booster immunizations. Nevertheless, it is worth noting that mice immunized orally with salmonellae expressing the circumsporozoite protein have been protected against malaria despite the absence of detectable antibody responses (5).

It is conceivable that the addition of a hinge domain and a guest antigen to the C terminus of TetC has perhaps destroyed or masked epitopes important in eliciting protective immune responses to tetanus toxoid. To investigate this possibility, mice immunized with the recombinant salmonellae were challenged with 50 times the lethal dose of tetanus toxin. Not all of the mice that were immunized with SL3261(pTECH1) were protected; however, mice immunized with SL3261(pTECH1-P28) were solidly protected against the lethal effects of tetanus toxin challenge. In the case of pTECH1 it is possible that the introduction of a hinge domain and cloning region alone affects the folding of the TetC protein, perhaps destroying potentially protective epitopes. However, upon fusion to P28 this effect may be counteracted to restore the protection levels to 100%. Furthermore, the same mice were totally protected against challenge by a lethal dose of virulent *Salmonella*, suggesting that the constructs had not affected the ability of the host strain to elicit protective immunity.

One of the advantages of using salmonellae as delivery systems is precisely their ability to stimulate cell-mediated immunity, and the induction of T-cell responses to P28 requires further investigation. T-cell responses to recombinant influenza nucleoprotein (33) and cytotoxic T-cell responses to *Plasmodium* circumsporozoite antigens (7) have been described with the *Salmonella* delivery system. The ability of salmonellae to colonize precisely in the reticuloendothelial system may be a factor in their capacity to trigger humoral and cellular responses, which could be an advantage for eliciting protection against disease. We have investigated the ability of SL3261(pTECH1-P28) to protect mice against a challenge infection with *S. mansoni*, and preliminary results show that a significant reduction in worm burden is achieved after a single oral inoculation compared to control mice inoculated with the construct expressing TetC alone (unpublished data). Of great importance, the TetC-P28 fusion protein is stably expressed in the human live typhoid vaccine strain *S. typhi* Ty21A (unpublished observations). Live attenuated Aro^- human typhoid vaccines are presently undergoing promising trials in human volunteers (34). It may be that a live trivalent typhoid-tetanus-schistosome vaccine could be feasible.

We thank Dr. Gill R. Douce for assistance with some of the animal experiments. This investigation received financial support from the Wellcome Trust, the World Health Organization/United Nations Development Program (WHO/UNDP) Programme for Vaccine Development, and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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