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Influence of Preimmunization with Tetanus Toxoid on Immune Responses to Tetanus Toxin Fragment C-Guest Antigen Fusions in a *Salmonella* Vaccine Carrier

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We have previously described a new system for the delivery of recombinant antigens in live *Salmonella* vaccines as genetic fusions to the C terminus of fragment C of tetanus toxin (TetC) driven by the anaerobically inducible *nirB* promoter. It has been reported that preimmunization with tetanus toxoid (TT) can suppress the antibody response to peptides chemically coupled to TT (epitope-specific suppression) in both animals and humans, which could interfere with efficacy of the *Salmonella*-TetC delivery system. We report that preimmunization of BALB/c mice with TT in alum did not suppress the response to either of two protective antigens of *Schistosoma mansoni*, the full-length *S. mansoni* P28 glutathione *S*-transferase (P28) and a construct consisting of eight tandem copies of the protective peptide comprising amino acids 115 to 131 of P28. The guest antigens were expressed in the *aroA Salmonella typhimurium* SL3261 vaccine strain as fusions to TetC. Preimmunization with TT 10 weeks before administration of the recombinant salmonellae did not alter the antibody response to the full-length P28, whereas the response to the peptide comprising amino acids 115 to 131 was increased by preimmunization with TT, with the increase seen mainly in the immunoglobulin G1 isotype. The antitetanus response was increased by preimmunization with TT in all groups receiving salmonellae expressing TetC. The results could be important when one is considering the use of the *Salmonella*-TetC delivery system in populations preimmunized with TT.

Salmonellosis is a major human and veterinary health problem, and there is a need for effective *Salmonella* vaccines. Special attention has been paid to the development of live attenuated *Salmonella* vaccines, as conventional killed *Salmonella* vaccines given subcutaneously do not confer good protection and produce unacceptable side reactions (8, 9). Live attenuated *Salmonella* vaccines can be given orally; their superior protective capacity may be due to their ability to elicit cell-mediated immunity, which killed vaccines fail to do (15).

Salmonellae harboring precise, nonreverting deletions in genes of the aromatic pathway (Aro vaccines) are attenuated and confer solid protection from challenge with virulent organisms. Aro *Salmonella* vaccines are noninvasive in immunosuppressed hosts, probably because they require *para*-aminobenzoic acid, which is unavailable in mammalian tissues (33). Aro *Salmonella* vaccines are effective in mice (14), calves (23, 32), sheep (19), and chickens (3, 4); Aro *Salmonella typhi* vaccines (e.g., strain CVD908, an *S. typhi aroC aroD* mutant) are candidate human typhoid vaccines currently undergoing human trials with promising results (34).

The potent immunogenicity of live *Salmonella* vaccines makes them very attractive as delivery systems for heterologous antigens (15). Combined *Salmonella* vaccines have elicited humoral, secretory, and cell-mediated responses to recombinant antigens from viruses, bacteria, and parasites. They have conferred protection from malaria (26), streptococci (22), tetanus (2), *Francisella tularensis* (30, 31), schistosomiasis (17), and herpes simplex virus (our unpublished results) in experimental models.

We have described the construction of a novel vector (pTECH) that allows the expression of guest antigens in *Salmonella* vaccines as fusions to tetanus toxin fragment C (TetC) driven from the anaerobically inducible *nirB* promoter (16, 17). Guest antigens can be expressed either as full-length fusions or as multiple tandem copies of immunogenic epitopes (repitopes). An *aroA Salmonella typhimurium* vaccine expressing the protective *Schistosoma mansoni* 28-kDa glutathione *S*-transferase (P28) as a full-length fusion to TetC protected mice from *Salmonella* infection, tetanus, and schistosomiasis (17) following a single dose of the vaccine. The result suggests that a combined oral vaccine for typhoid, tetanus, and schistosomiasis may be feasible. A construct expressing multiple copies of a peptide comprising amino acids 115 to 131 (peptide 115-131) from the *S. mansoni* P28 as genetic fusions to TetC was also immunogenic when given intravenously (i.v.), the response improving with fusions of increasing numbers of copies of the peptide (16).

However, although the *nirB*-TetC *Salmonella* expression system is proving very successful experimentally, a possible drawback to its use in humans could be preexisting immunity either to the *Salmonella* carrier or to tetanus toxoid (TT). It has been reported that preexisting immunity against *Salmonella* infection did not interfere with the response to the binding subunit

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of *Escherichia coli* heat-labile enterotoxin (LT-B) delivered in a *Salmonella* carrier (1). On the other hand, although TT has been frequently used as a carrier to enhance the immune response to poorly immunogenic antigens or to overcome unresponsiveness to certain peptides (10, 35), a major drawback for its use could be the finding that the antibody response to an antigen coupled to a protein carrier can be suppressed in individuals previously immunized with the carrier.

This carrier-induced epitope-specific suppression was first described as a general regulatory process found among different hapten carrier systems and mouse strains. Preimmunization with a carrier can impair the antibody response, mostly for the immunoglobulin G2a (IgG2a) isotype, to a hapten coupled to the same carrier (11–13). The same phenomenon was observed with TT as a carrier, with a strong suppression of the IgG1 response against two different coupled antigens (28). The effect has been further found in mice (6, 24, 29) and humans (5). Possible mechanisms responsible for the observed suppression have been investigated (11, 25, 27, 29).

However, in some cases preimmunization with the carrier protein led to enhancement of the response to the coupled antigen rather than suppression (18). It has been suggested that the suppressive effect of preimmunization with TT could be avoided by using certain polypeptides rather than the whole toxoid protein as a carrier (6, 7). The question of whether the use of TetC as a carrier in *Salmonella* vaccines will induce this suppression needs to be addressed.

We investigated the effect of prior immunization with TT on the antibody response to antigens expressed as fusions to TetC in an *aroA S. typhimurium* vaccine. Two guest antigens were investigated: the full-length *S. mansoni* P28, and also the P28 peptide 115-131 as a fusion comprising eight tandem copies (octamer). No suppression was observed.

MATERIALS AND METHODS

Animals. Female BALB/c mice were purchased from Harlan Olac (Blackthorn, Bicester, England) and used when at least 8 weeks of age.

Bacteria. *S. typhimurium* SL3261, an *aroA* vaccine strain, has been described elsewhere (14).

Expression systems and combined vaccines. The expression vector pTECH1 allows expression of guest antigens in *Salmonella* vaccines as full length C-terminal fusions to TetC under the control of the anaerobically inducible *E. coli* nitrite reductase promoter (17). Plasmid pTECH2 is a derivative of pTECH1 specifically designed for the construction of fusions to TetC of repeating tandem copies (repitopes) of defined peptides (16). *S. typhimurium* SL3261(pTECH2), carrying the vector alone, expresses TetC. *S. typhimurium* SL3261(pTECH1-P28) expresses the full-length *S. mansoni* P28 glutathione *S*-transferase as a fusion to TetC (17). *S. typhimurium* SL3261(pTECH2-octamer) expresses eight tandem copies of the immunogenic peptide 115-131 of P28 as a fusion to TetC (16). The immunogenicity of these constructs has been described elsewhere (16, 17).

Immunization protocols. Bacteria were grown on LB broth supplemented with 50 μg of ampicillin per ml as required. For i.v. inoculation, stationary overnight cultures were diluted in phosphate-buffered saline (PBS), and animals were given approximately 5×10^6 CFU in 0.2 ml of PBS via a lateral tail vein. The inoculum doses were checked by viable counts on tryptic soy agar.

Groups of mice were preimmunized subcutaneously with 8 IU of alum-adsorbed TT (human use; Wellcome Diagnostics, Dartford, England). Ten weeks later, the same animals, and also naive controls, were inoculated i.v. with SL3261, SL3261(pTECH2), SL3261(pTECH2-octamer), or SL3261(pTECH1-P28).

Collection of sera. Animals were bled from the tail. Serum samples from all mice were taken beginning on week 3 and stored individually. Pooled sera from each group were also prepared.

Antibody responses. Antibody responses against TetC, P28, and peptide 115-131 were assessed by enzyme-linked immunosorbent assay (ELISA) as previously described (16, 17). Briefly, each well of 96-well microtiter plates (Nunc; GIBCO BRL, Life Technologies Ltd, Paisley, England) was coated overnight at room temperature with 1 μg of either recombinant P28 (17), a peptide 115-131–ovalbumin conjugate (36), or 0.1 μg of TetC (Boehringer Mannheim, Lewes, Sussex, England). Recombinant P28 and peptide conjugates were kindly supplied by R. Pierce, Lille, France. The plates were then blocked with 2% casein in PBS for 2 h at 37°C and washed three times with PBS–0.05% Tween 20. Sera from individual mice diluted 1:20 in blocking buffer were added, the mixture was incubated for 1.5 h at 37°C, and the plates were washed again. Peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako Ltd., High Wycombe, Buckinghamshire, England) was then added to the plates for an additional 1.5 h at 37°C, and then the plates were washed again. The substrate solution (3,3′,5,5′-tetramethylbenzidine dihydrochloride; Sigma, Poole, Dorset, England) was prepared as instructed by the manufacturer. The developing reactions were stopped after 15 min at 37°C with 2 M H_2SO_4. Optical density was determined at 450 nm with a microplate reader (Biotek Instruments Inc., ANACHEM, Luton, Bedfordshire, England).

Statistical significance of the differences in the antibody responses was evaluated by Student's *t* test and considered significant when *P* values of <0.05 were obtained.

Antibody titration. For antibody titration, serial twofold dilutions of pooled sera were assessed by ELISA as described above, using peroxidase-conjugated goat anti-mouse IgG (Sigma) or rabbit anti-mouse IgG1, IgG2a, or IgG2b (Zymed Laboratories Inc., Cambridge BioScience, Cambridge, England). Titers were expressed as the maximal dilution giving an absorbance threefold higher than that of sera from the control groups at the same dilution.

RESULTS

Eight groups of mice (12 or 18 mice per group) were defined as follows: four groups (groups 1, 3, 5, and 7) were immunized with TT, and the other four (groups 2, 4, 6, and 8) received no treatment. On day 51, all mice immunized with TT were tail bled and tested for anti-TetC antibodies; all were positive (results not shown). On day 70, pairs of groups with and without TT immunization were immunized with one dose of SL3261 (groups 1 and 2; 12 mice per group), SL3261 (pTECH2) (groups 3 and 4; 12 mice per group), SL3261 (pTECH1-P28) (groups 5 and 6; 18 mice per group), or SL3261(pTECH2-octamer) (groups 7 and 8; 18 mice per group). All mice were bled at weeks 3, 4, 6, and 8 after *Salmonella* immunization, and serum samples from individual mice were tested at 1:20 for antibody to TetC, P28, or peptide 115-131 of P28. Pooled sera from each group were used for titrations.

Effect of TT preimmunization on the antibody response against TetC. The anti-TetC antibody response was evaluated in all groups. Figure 1 shows the antibody response for each mouse at weeks 3 and 6. By week 3, there was a low response to TetC in all groups that were not preimmunized with TT but which received salmonellae expressing TetC (groups 4, 6, and 8). No anti-TetC response was detected in animals immunized with salmonellae alone (group 2). On the other hand, mice primed with TT and then immunized with salmonellae expressing TetC, either alone or fused with other antigens (groups 3, 5, and 7), showed a significant enhancement in the anti-TetC response ($P = 0.000002$, 0.005, and 0.000005, respectively) compared with the group which was primed with TT and then immunized with salmonellae alone (group 1). These differences were still significant at week 6 ($P = 0.00005$, 0.00008, and 0.000006 for groups 3, 5, and 7 respectively), when the anti-TetC response was higher in all groups. This result clearly shows that immunization with salmonellae expressing TetC boosts the anti-TetC response elicited by prior vaccination with TT in alum.

Effect of TT preimmunization on the antibody response to peptide 115-131 fused to TetC. The antibody responses against peptide 115-131 of *S. mansoni* P28 were compared in groups immunized with the *Salmonella* strain expressing the octameric fusion construct [SL3261(pTECH2-octamer)] and groups receiving SL3261 or SL3261(pTECH2). Analysis of the antibody response for individual serum samples (Fig. 2) shows that the group primed with TT (group 7) had a significant enhancement ($P = 0.008$) in the antipeptide response compared with the unprimed mice (group 8), which was seen as early as week 3 after immunization. The difference was still significant by week 4 ($P = 0.03$) but not by week 6. Thus, preimmunization with

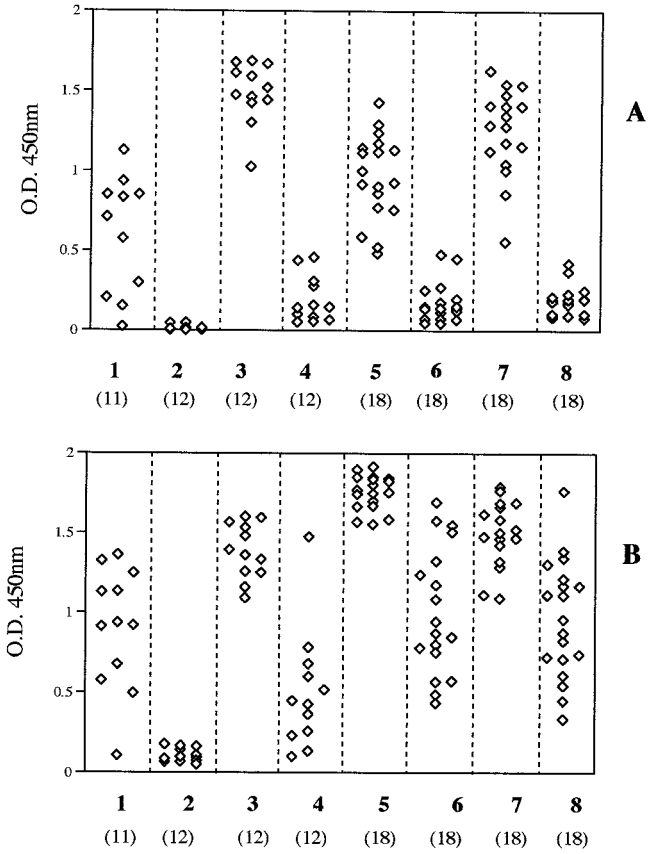

FIG. 1. Antibody response against recombinant fragment C as detected by ELISA in mice immunized with TT plus SL3261 (column 1), SL3261 only (column 2), TT plus SL3261(pTECH2) (column 3), SL3261(pTECH2) only (column 4), TT plus SL3261(pTECH1-P28) (column 5), SL3261(pTECH1-P28) only (column 6), TT plus SL3261(pTECH2-octamer) (column 7), and SL3261(pTECH2-octamer) only (column 8). Results are expressed as optical density (O.D.) in individual mice at weeks 3 (A) and 6 (B) after immunization. The numbers in parentheses indicate the number of mice in each group.

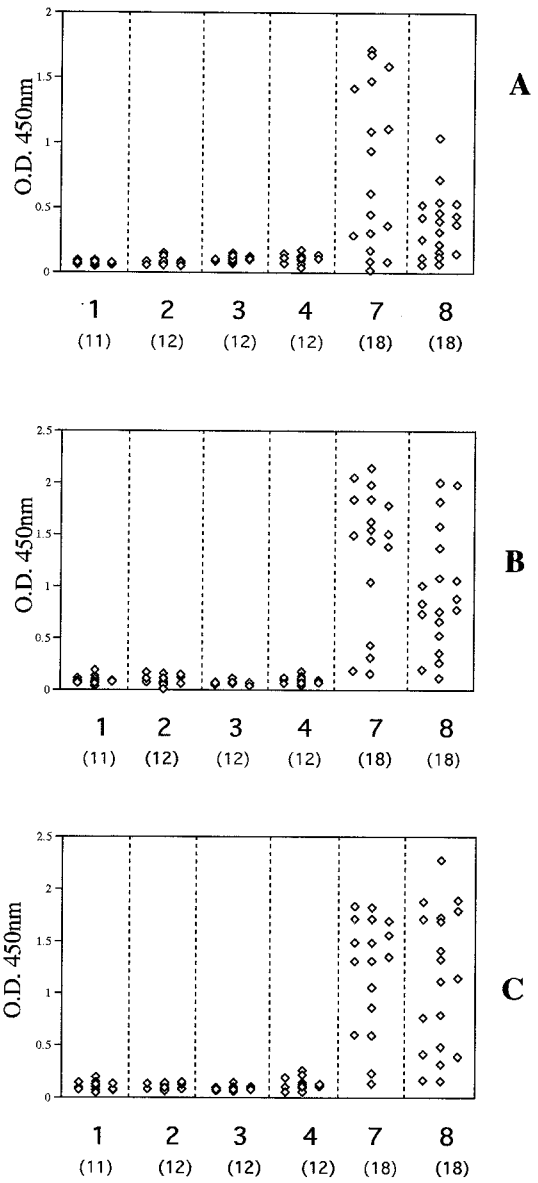

FIG. 2. Antibody response against peptide 115-131 of P28 coupled to ovalbumin as detected by ELISA in mice immunized with TT plus SL3261 (column 1), SL3261 only (column 2), TT plus SL3261(pTECH2) (column 3), SL3261 (pTECH2) only (column 4), TT plus SL3261(pTECH2-octamer) (column 7), and SL3261(pTECH2-octamer) only (column 8). Results are expressed as optical density (O.D.) in individual mice at weeks 3 (A), 4 (B), and 6 (C) after immunization. The numbers in parentheses indicate the number of mice in each group.

TT enhanced the response to the peptide fused to TetC in salmonellae.

Antipeptide antibody titers were compared in pooled sera from groups immunized with the octameric construct and in groups receiving salmonellae alone or salmonellae expressing TetC. Table 1 shows that there was a consistent fourfold increase in the antipeptide IgG titer in the TT-primed group, which persisted for the duration of the experiment. The IgM antipeptide response was very weak throughout (results not shown).

Results of titration of the IgG subclasses of the antipeptide antibody response are shown in Table 2. Mice receiving the *Salmonella*-octamer construct after TT vaccination showed a marked enhancement in the IgG1 response, which reached a 16-fold increase at week 4 compared with mice which were not preimmunized. There was only a twofold difference for the IgG2b and IgG2a isotypes.

Anti-P28 antibody response. Antibodies to recombinant P28 were evaluated in the groups immunized with the *Salmonella* strain expressing the full-length protein [SL3261(pTECH1-P28)]. Figure 3 and Table 3 show ELISA results for individual serum samples and IgG titers, respectively. Preimmunization with TT did not modify the anti-P28 antibody responses.

In summary, prior immunization with TT enhanced the antibody response to the multimeric peptide fused to TetC and delivered by a *Salmonella* vaccine strain. The response to full-length P28 was unaffected by preimmunization with TT. No suppression of the response to either P28 or the peptide was seen.

DISCUSSION

The results presented above show that preimmunization with TT did not suppress the response to two antigens expressed as fusions to TetC and delivered in a live attenuated *Salmonella* vaccine given i.v. The response to one of the guest antigens was actually increased by TT preimmunization. Sal-

TABLE 1. Effect of TT priming on the IgG anti-peptide 115-131 response upon immunization with salmonellae carrying pTECH2-octamer

Preimmunization	Antibody titer[a]			
	Wk 3	Wk 4	Wk 6	Wk 8
TT	320	1,280	2,560	1,280
None	80	320	640	320

[a] Anti-peptide 115-131 IgG titer for pooled sera as defined in Materials and Methods.

monellae expressing TetC boosted the antitetanus antibody levels in animals preimmunized with TT.

Combined *Salmonella* vaccines are showing great potential as antigen delivery systems. Expression of guest antigens as fusions to TetC by using the system that we have described is giving very promising results; it has allowed the expression of the recombinant antigens in salmonellae stably at a sufficiently high level to trigger the immune response. It is possible that the success of the *Salmonella*-TetC delivery system may be due in part to the known adjuvant effect of TT for chemically coupled antigens. Further, of the two universal human T-cell epitopes described for TT (20), one (p30) is represented in TetC. This could perhaps assist in overcoming major histocompatibility complex restrictions that could be associated with certain peptides, a major concern with peptide vaccines.

Nevertheless, the phenomenon of epitope-specific suppression through preimmunization with carrier could be a drawback to the use of this system in human vaccination programs, given that the majority of subjects would probably have been vaccinated against tetanus in early childhood.

Etlinger et al. (6) suggested that as the proposed mechanisms responsible for the suppression are B-cell clonal dominance of the carrier and the presence of suppressor T-cell epitopes in it, the problem of using TT as a carrier for epitopic vaccines in TT-vaccinated populations could be overcome by using as carriers small polypeptides from TT which include T-helper epitopes but lack suppressor T-cell epitopes and B-cell epitopes from TT. This approach would allow preservation of the carrier effect (due to the T-helper epitopes from the carrier) while avoiding suppression. This approach could have serious limitations for development of multivalent vaccines, including protection against tetanus toxin, since these short polypeptides may not necessarily generate protective immunity against tetanus toxin.

It has been demonstrated (27) that the induction of suppression can depend on the ratio between the concentrations of carrier and epitope on the conjugate: suppression induced with low concentrations of the conjugated molecule could be abrogated by using higher concentrations. It was therefore critical to determine whether the use of fusion proteins in which the relationship between the carrier and the fused molecule is 1:1 would induce a suppressive effect.

TABLE 2. Effect of TT priming on the IgG subclasses of anti-peptide 115-131 antibody titers upon immunization with salmonellae carrying pTECH2-octamer

Preimmunization	Antibody titer[a]								
	Wk 3			Wk 4			Wk 6		
	IgG1	IgG2a	IgG2b	IgG1	IgG2a	IgG2b	IgG1	IgG2a	IgG2b
TT	320	80	40	2,560	160	160	2,560	160	160
None	40	40	20	160	160	80	320	320	160

[a] Titer for pooled sera as defined in Materials and Methods.

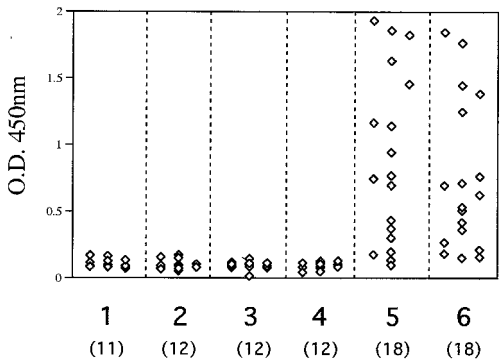

FIG. 3. Antibody response against recombinant P28 as detected by ELISA in mice immunized with TT plus SL3261 (column 1), SL3261 only (column 2), TT plus SL3261(pTECH2) (column 3), SL3261(pTECH2) only (column 4), TT plus SL3261(pTECH1-P28) (column 5), and SL3261(pTECH1-P28) only (column 6). Results are expressed as optical density (O.D.) in individual mice at week 6 after immunization. The numbers in parentheses indicate the number of mice in each group.

In this investigation, we studied the effect of TT preimmunization on the response to two *S. mansoni* antigens fused to fragment C, full-length P28 and peptide 115-131 from P28, both protective in experimental models (17, 36). The results showed that there was no suppression against either antigen in this experimental model.

The antibody response elicited by the TetC-octameric peptide fusion in the *Salmonella* carrier was actually enhanced by TT preimmunization. Although the individual serum responses showed that the difference seen at weeks 3 and 4 was no longer significant by week 6 (Fig. 2), the IgG antipeptide response in the pooled sera was consistently fourfold higher in the primed mice. Serum samples taken 6 months after immunization still showed this difference in total IgG and IgG1 isotypes between groups (results not shown).

It has been suggested (5, 18) that the effect of preimmunization with TT (enhancement or suppression) on the response to coupled antigens may also depend on factors such as the conditions of preimmunization, the nature of the coupled antigen, the given dose, and the nature of the adjuvant. However, it is noteworthy that the Ig subclass found to be elevated in cases of enhanced responses, both by ourselves in the case of peptide 115-131 and by others using very different experimental conditions, was mainly IgG1. Lise et al. (18) found that TT priming led to an enhanced IgG1 response against a *Plasmodium falciparum* sporozoite peptide coupled to TT and administered subcutaneously with two different adjuvants; Peeters et al. (21) reported an enhancement in the IgG1 response against a saccharide antigen in saccharide-TT conjugates. These results could be taken to suggest that if enhancement of the response does occur, the switch to one or another class of

TABLE 3. Effect of TT priming on the IgG anti-P28 response upon immunization with salmonellae carrying pTECH1-P28

Preimmunization	Antibody titer[a]		
	Wk 3	Wk 4	Wk 6
TT	80	160	320
None	80	160	160

[a] Anti-P28 IgG titer for pooled sera as defined in Materials and Methods.

antibody may somehow be directed by the nature of the carrier, even when different coupled antigens and immunization protocols are used.

The antibody response against the full-length *S. mansoni* P28 antigen was not influenced by preimmunization with TT, suggesting that extra helper activity due to T-cell epitopes supplied by TT is less important for the response against this protein when delivered by the *Salmonella*-TetC carrier system. Conversely, there was a very clear boost in the antitetanus antibody levels in animals preimmunized with TT and later given salmonellae expressing TetC, which would have obvious benefits if the present results applied to human immunization programs.

However, the experiments described here were performed with animals immunized with TT subcutaneously and later immunized with Aro salmonellae i.v., as we have done in our previous studies on mechanisms of immunity to salmonellae. The reason for the choice of this experimental model is that we have long experience with the immune responses obtained, and the main question of whether fragment C could exert suppression could be answered by considering the proposed mechanisms involved (clonal dominance and suppressor T-cell epitopes). Furthermore, all of the available evidence on epitope-specific suppression by TT involves parenteral administration of the antigens. Live Aro *Salmonella* vaccines are being developed with a view to use in humans by oral administration; i.v. immunization of humans is not considered. Nevertheless, the results presented here are highly encouraging, since they mean that TetC does not include suppressor epitopes and does not produce clonal dominance even against small epitopes (although in the last case, it is noteworthy that the actual epitope/carrier ratio was 8:1, leaving open the question of whether suppression could be seen when constructs with lower numbers of copies of the peptide were used). However, it remains to be seen whether the results obtained with the present experimental model in mice will also apply when this system is eventually used in humans vaccinated orally with *S. typhi* carrier vaccines.

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