Oral Vaccination of Mice against Tetanus by Use of a Live Attenuated *Salmonella* Carrier

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A *Salmonella typhimurium aroA* mutant has been used as a live carrier to immunize mice against tetanus. Plasmid pTETtac4, which expresses a 50-kilodalton fragment of tetanus toxin (fragment C) under the control of the *tac* promoter, was introduced into SL3261 *aroA*. When used as a live vaccine and administered orally or intravenously, this strain was able to induce protective immunity in mice against a lethal tetanus toxin challenge. When plasmid pTETtac2, which contains the *lacI* gene, was used, no immunity was obtained, indicating that the expression of fragment C was repressed in vivo. We believe that this is the first example of a successful oral vaccination that uses an attenuated bacterial carrier to deliver a protective antigen derived from tetanus toxin.

Vaccination programs against tetanus, which use formaldehyde-inactivated tetanus toxin purified from *Clostridium tetani*, are highly effective in the prevention of tetanus in Western countries (for a review, see reference 2). However, some countries are unable to implement fully these programs, resulting in significant annual mortality from neonatal tetanus (25). It is possible that the use of an effective oral tetanus vaccine may help to alleviate some of the problems associated with widespread vaccination in developing countries.

Tetanus toxin is a 1,315-amino-acid protein with a molecular mass of 150 kilodaltons which is produced by *C. tetani*. The toxin, as purified from a culture lysate, is cleaved, resulting in the nicked form, composed of the light chain (amino acid residues 2 to 457) disulfide bonded to the heavy chain (residues 458 to 1315) (7, 8). The toxin may be cleaved by papain to produce a 50-kilodalton peptide fragment (residues 865 to 1315), termed fragment C (14). Fragment C, because of its binding to gangliosides GD1b and GT1b (15, 26), is thought to be involved in the binding of the toxin to cell receptors prior to internalization. Fragment C is also immunogenic and will protect mice and guinea pigs against toxin challenge (9, 13, 14). The toxin gene has been cloned and sequenced (7, 8, 10), and we have recently obtained high-level expression of fragment C in *Escherichia coli* (18).

Recent studies on genetically defined attenuated *Salmonella* strains (4, 20, 21) have shown such strains to be promising candidates for the delivery of foreign antigens to the immune system (19). We report here the successful oral immunization against tetanus using a *Salmonella aroA* mutant as a carrier of fragment C.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Salmonella typhimurium* LB5010 *galE* ($r^- m^+$) and SL3261 *aroA his* have been described previously (3, 16). Plasmid pTETtac2 (18) carries the gene for tetanus toxin fragment C under the control of the *tac* promoter (1). Plasmid pTETtac4 was derived from

pTETtac2 by deletion of the *EcoRI* fragment carrying the *lacI* gene (Fig. 1a).

Growth conditions and immunization of mice. Bacteria were grown statically overnight in L broth which contained ampicillin when appropriate, centrifuged, and suspended in phosphate-buffered saline to approximately 5×10^{10} cells per ml for oral immunization and to approximately 2.5×10^6 cells per ml for intravenous immunization. Cell suspension (0.2 ml) was administered to 6- to 8-week-old male BALB/c mice by gavage tube for oral immunization and by tail injection for intravenous inoculation. During oral immunization, no technique was employed for the neutralization of gastric juices. Cell viability was determined by counting viable cells in L agar which contained ampicillin when appropriate. Sera were collected by tail bleeds on the days indicated. For immunization with heat-killed organisms, bacteria were incubated at 65°C for 2 h prior to injection.

Antibody ELISA. Sera were analyzed for the presence of anti-tetanus toxoid antibodies by enzyme-linked immunosorbent assay (ELISA) as described previously (9). The titers were measured as the A_{492} of 1/200 dilutions of the sera.

Tetanus toxin challenge. Mice were challenged with tetanus toxin by subcutaneous injection of 0.5 ml of toxin diluted in a 1:1 mixture of nutrient broth (Difco Laboratories, Detroit, Mich.) and borate-buffered saline (3.6 g of borax, 5.2 g of boric acid, and 9.1 g of sodium chloride in 1 liter of distilled water). Mice were observed for 4 days, and those alive after that time were scored as survivors.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting). Bacteria were grown in L broth containing 100 μ g of ampicillin per ml and were induced for the synthesis of fragment C by the addition of 60 μ g of isopropyl- β -D-thiogalactopyranoside per ml. The cells were centrifuged and suspended in sample buffer (17), and 10^7 cells were analyzed on a 10% gel by the method of Laemmli (17). Western blotting was carried out as described previously (10) by using the rat monoclonal antibody TT09 (24) and goat anti-rat immunoglobulin G-peroxidase conjugate (Bio-Rad, Watford, England).

RESULTS

Characterization of *S. typhimurium aroA* strains expressing fragment C. *E. coli* carrying plasmid pTETtac2 produces

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FIG. 1. Expression of fragment C in *S. typhimurium*. (a) Structure of plasmids pTETtac2 and pTETtac4. P_{tac} , *tac* promoter (1); ori, origin of replication; Ap^R , β -lactamase gene. —, Structural gene for fragment C (frag C). The arrows indicate the direction of transcription of the *lacI* and fragment C genes. (b and c) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of proteins synthesized by *S. typhimurium* SL3261(pTETtac2) and SL3261(pTETtac4). (b) Coomassie blue-stained gel; (c) Western blot. Lanes 1 and 2, pTETtac2; lanes 3 and 4, pTETtac4; lanes 5, 0.1 μ g of fragment C from *C. tetani*. The presence (+) and absence (–) of isopropyl- β -D-thiogalactopyranoside in the growth medium and fragment C (▶) are indicated.

fragment C of tetanus toxin at 3 to 4% total cell protein as a soluble cytoplasmic protein (18). The natural and recombinant molecules behave similarly on gel electrophoresis and have almost identical immunizing potentials in mice (18). In order to construct a *Salmonella* vaccine expressing tetanus fragment C, an intermediate strain, *S. typhimurium* LB5010, was transformed with plasmid pTETtac2 or pTETtac4. Both of these plasmids express fragment C. The 1.2-kilobase *EcoRI* fragment encoding the *lacI* gene is carried by pTETtac2 but not by pTETtac4 (Fig. 1a). Colonies expressing fragment C were detected by antibiotic selection followed by immunoblotting with anti-tetanus toxin fragment C sera. Bacteriophage P22 HT105/1 *int* (23) was used to transduce pTETtac2 and pTETtac4 from LB5010 into the smooth *Salmonella* vaccine strain SL3261. Strains containing these plasmids were examined for their expression of fragment C. SL3261(pTETtac2) produced fragment C at levels detectable by both Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels and Western blotting (Fig. 1b). However, this expression was dependent on the induction of the *tac* promoter by isopropyl- β -D-thiogalactopyranoside, as no fragment C could be detected in the absence of this inducer. In contrast, SL3261(pTETtac4), which does not carry a copy of the *lacI* gene on the plasmid, produced fragment C at a level similar to those of induced cultures of SL3261(pTETtac2), but as expected, expression was independent of the presence of the inducer.

In order to use these strains to vaccinate mice, the plasmid carrying the gene has to be inherited in the absence of antibiotic selection. Cultures of SL3261 carrying pTETtac2 or pTETtac4 were grown overnight in L broth without ampicillin and were plated out on L agar with and without ampicillin. No large differences between the viable counts of cultures plated with and without ampicillin were detected, indicating that the plasmids are inherited in a stable manner under these conditions (data not shown).

Immunization of mice by use of *Salmonella* strains carrying fragment C. Groups of 60 mice were inoculated either orally or intravenously with SL3261(pTETtac4) or with SL3261

alone, as described in Table 1. Control mice were injected subcutaneously with 10 μ g of fragment C purified from *E. coli*. Three weeks after immunization, sera were collected from 15 mice per group and analyzed by ELISA for antitetanus antibodies. All sera from mice injected intravenously with SL3261(pTETtac4) had detectable antitetanus antibodies, whereas the sera from mice inoculated orally with this strain did not (Fig. 2A). Mice vaccinated orally or intravenously with SL3261 had no detectable antibody response to fragment C. On day 28, 30 mice from each group were boosted by inoculation with a similar amount of organisms

TABLE 1. Immunization of mice against tetanus by using *S. typhimurium* SL3261(pTETtac4)

Vaccine	Dose ^a	No. of doses ^b	Route ^c	No. of mice (n = 10) surviving tetanus toxin challenge (LD ₅₀ s) ^d		
				500	50	5
SL3261(pTETtac4)	2.4×10^9	1	Oral	8	6	8
	2.4×10^9	2	Oral	10	10	10
SL3261	4.0×10^9	1	Oral	0	0	0
	4.0×10^9	2	Oral	0	0	0
SL3261(pTETtac4)	4.8×10^5	1	i.v.	10	10	10
	4.8×10^5	2	i.v.	10	10	10
SL3261	5.6×10^5	1	i.v.	0	0	0
	5.6×10^5	2	i.v.	0	0	0
Fragment C	10 μ g	1	s.c.	10	10	10
	10 μ g	2	s.c.	10	10	10

^a CFU or micrograms of protein (for fragment C) administered.

^b Doses were given on days 1 and 28 by the routes indicated.

^c i.v., Intravenous; s.c., subcutaneous.

^d Mice were challenged with the indicated amounts of tetanus toxin on day 28 (one dose of vaccine) or on day 59 (two doses of vaccine).

FIG. 2. Anti-tetanus toxoid titers of mouse sera. Sera from individual mice immunized with SL3261 alone, SL3261(pTETtac4), or pure fragment C were prepared as described in the text. (A) Titers after one dose of antigen; (B) titers after two doses. Abbreviations: 3261, SL3261 alone; TET4, SL3261(pTETtac4); C, fragment C; i.v., intravenous inoculation; O, oral inoculation.

by the same route as that of the primary immunization or, in the case of mice injected with purified fragment C, with an identical amount of protein given subcutaneously. ELISA analysis of the sera taken from these mice at day 35 showed the presence of antitetanus antibodies in the mice orally inoculated with SL3261(pTETtac4) and increased titers in the mice intravenously inoculated with SL3261(pTETtac4) (Fig. 2B). Mice boosted orally or intravenously with SL3261 alone failed to produce a detectable antibody response to tetanus toxoid (Fig. 2B).

Tetanus toxin challenge of immunized mice. The mice vaccinated with SL3261(pTETtac4), SL3261, or fragment C were tested for immunity to tetanus by challenge with one of three doses of tetanus toxin (500, 50, or 5 50% lethal doses [LD_{50} s]) (Table 1). Mice immunized orally with a single dose of SL3261(pTETtac4) showed partial protection against the toxin challenge, with some mice in each challenge group not surviving the challenge. Interestingly, the numbers of mice surviving the challenge were not related to the dose of the toxin challenge, as a few mice in each challenge group did not survive. All mice that received two doses of SL3261 (pTETtac4) survived the toxin challenge, as did all mice receiving one or two doses of this strain intravenously. As expected, all mice immunized with one or two doses of SL3261 alone died, and all mice immunized subcutaneously with purified fragment C survived (Table 1).

Effect of the *lac* repressor carried by SL3261(pTETtac2). SL3261(pTETtac2) and SL3261(pTETtac4) were compared for their ability to immunize mice against tetanus. Groups of 10 mice were inoculated either orally or intravenously with two doses of SL3261, SL3261(pTETtac2), or SL3261 (pTETtac4) (Table 2). Mice were challenged with 50 LD_{50} s of tetanus toxin on day 37. Table 2 shows complete protection of mice immunized with SL3261(pTETtac4), whereas SL3261(pTETtac2) totally failed to immunize the mice.

Use of heat-killed salmonellae carrying fragment C. In order to demonstrate that tetanus immunity was due to the presentation of fragment C by live salmonellae, mice were inoculated either orally or intravenously with two doses of heat-killed SL3261(pTETtac4) and were subsequently challenged with tetanus toxin. In contrast to injection of live

TABLE 2. Oral and intravenous immunization of mice by using *S. typhimurium* SL3261 carrying pTETtac2 or pTETtac4

Vaccine	Dose ^a	Route	No. of mice (n = 10) surviving tetanus toxin challenge ^b
SL3261(pTETtac2)	4.0×10^9	Oral	0
SL3261(pTETtac4)	2.3×10^9	Oral	10
SL3261	6.6×10^9	Oral	0
SL3261(pTETtac2)	3.0×10^5	i.v. ^c	0
SL3261(pTETtac4)	3.3×10^5	i.v.	10
SL3261	3.4×10^5	i.v.	0

^a Mice were immunized with the indicated CFU of organisms at days 0 and 21.

^b Mice were challenged with 50 LD_{50} s of tetanus toxin at day 37.

^c i.v., Intravenous.

salmonellae carrying pTETtac4, both oral and intravenous injections of equivalent numbers of heat-killed organisms totally failed to immunize the mice against tetanus (data not shown).

DISCUSSION

The data presented here demonstrate that SL3261 *aroA*, an attenuated *Salmonella* strain, can be used as a carrier of fragment C to induce protection against tetanus by oral and intravenous vaccination. We have used two recombinant plasmids, each containing the gene for fragment C under the control of the *tac* promoter. Oral immunization with two doses of SL3261(pTETtac4) gave complete protection against tetanus toxin challenge, whereas one dose gave partial protection. However, the numbers of survivors among the mice given one oral dose of vaccine did not correlate with the strength of the toxin challenge used. The similarity in the survival rates of the three challenge groups could indicate that when immunity is induced by this strain, it is always sufficient to induce protection against a high toxin challenge.

The use of heat-killed salmonellae carrying fragment C completely abolished the immunogenicity of fragment C, indicating the requirement of inoculation with live organisms for immunity. Since heat treatment could alter the antigenicity of fragment C, an alternate method of killing the cells such as UV irradiation could be employed.

There are a number of possible reasons why all animals were not protected against tetanus challenge by one oral dose. Using the oral vaccination techniques described in this report, we reproducibly obtain complete protection against salmonellosis, although it is still possible that oral presentation methods could be improved. Expression of foreign antigens such as fragment C by using multicopy plasmids in salmonellae could affect the immunogenicity of the strain. Such plasmids could place a metabolic burden on the bacteria, which might alter their in vivo behavior. Also, the fact that fragment C was expressed as an intracellular antigen may have affected presentation to the immune system. We are currently investigating the possibility of secreting fragment C to the periplasm or outer membrane of salmonellae.

The levels of antitetanus antibodies in individual mice were measured. All vaccines which protected mice induced detectable levels of antitetanus antibodies, with the exception of one oral dose of SL3261(pTETtac4). This is not surprising, because although antitetanus immunity is entirely

humoral and is dependent upon serum antibodies, the levels of antibody necessary for protection are small. Indeed, serum antibodies are not always detectable after a single dose of tetanus toxoid vaccine (11), and it is thought that in humans, the presence of detectable antibodies indicates protection against tetanus (for a review, see reference 2). The ELISA employed here for the measurement of tetanus antibodies is probably not sensitive enough to detect the presumptive low levels of antibody induced by a single oral dose of SL3261(pTETtac4).

The level of fragment C produced by the salmonellae appears to be critical for protection. The presence of the *lacI* gene on plasmid pTETtac2 completely prevented the strain carrying this plasmid from inducing immunity. Measurement by ELISA of the amount of fragment C produced in vitro by SL3261 carrying pTETtac2 and pTETtac4 has shown that the repression level is at least 100-fold (data not shown). If this were also the case in vivo, it would indicate a very low level of fragment C synthesis, which is insufficient to induce tetanus immunity.

Salmonella strains have been used as carriers of other bacterial and parasitic antigens (19, 22), but to our knowledge, this is the first example of successful vaccination against tetanus that uses a live carrier, and it is a first step in the development of an oral tetanus vaccine for human use. It is estimated that over 10^9 doses of tetanus vaccine are administered every year to the human population. However, in developing countries, over 1,000,000 people, mainly children in the early years of life, still die each year from tetanus (25). Several reports have outlined the potential of an effective oral vaccine against tetanus (2, 27), but to date there is still no such vaccine in use. In addition to the current *Salmonella typhi* *galE* oral vaccine (12), there are a number of *S. typhi* strains under development as human oral typhoid vaccines, including strains harboring mutations in the aromatic amino acid biosynthetic pathway (5, 6). Such strains could possibly be used as carriers of fragment C in the construction of an oral typhoid and tetanus vaccine which could offer protection against these two serious diseases.

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