Mutant of Subtilase Cytotoxin of Shiga Toxin-Producing Escherichia coli

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We have recently described a novel AB5 cytotoxin produced by certain Shiga toxin-producing Escherichia coli strains. The A subunit of this toxin is a subtilase-like serine protease, while the B pentamer mediates binding to host cell glycolipid receptors. The subtilase cytotoxin is lethal for mice, causing extensive microvascular thrombosis as well as necrosis in the brain, kidneys, and liver. In the present study, we have immunized mice with a purified derivative of the toxin with a Ser272 → Ala mutation in the A subunit which abolishes cytotoxicity. This elicited strong antibody responses, as judged by enzyme-linked immunosorbent assay, which conferred protection against intraperitoneal challenge with purified toxin. Immunized mice were also protected from weight loss resulting from oral challenge with an E. coli K-12 clone expressing the active surface.

Shiga toxin-producing Escherichia coli (STEC) is an important cause of gastrointestinal disease in humans, particularly since these infections may result in life-threatening sequelae such as hemolytic uremic syndrome (9, 12, 18). STEC produce one or both of two major types of Shiga toxin, designated Stx1 and Stx2; production of the latter is associated with an increased risk of developing hemolytic uremic syndrome (1, 10, 14). Other putative accessory virulence factors produced by subsets of STEC include the capacity to produce attaching and effacing lesions on intestinal mucosa, a property encoded by the locus for enterocyte effacement, as well as megaplasmid-encoded factors such as the enterohemolysin Ehx, an extracellular serine protease, EspP, and a putative adhesin, Saa (3, 5, 12, 16, 18, 19).

While there is circumstantial evidence that the accessory virulence factors referred to above contribute to pathogenesis of human gastrointestinal disease, the bulk of the pathology is believed to be directly attributable to the systemic effects of Shiga toxin, particularly damage to the microvascular endothelium (12, 18). For this reason, Shiga toxin is seen as an important target for vaccines being developed for prevention of STEC disease and its complications (18).

However, we have recently reported that some STEC strains produce an additional, previously undescribed AB5 cytotoxin, which is lethal for mice and also results in extensive microvascular damage, thrombosis, and necrosis in multiple organs, including the brain, kidneys, and liver (15). The new toxin has been named subtilase cytotoxin because its 35-kDa A subunit is a subtilase-like serine protease, EspP, and a putative adhesin, Saa (3, 5, 12, 16, 18, 19).

The overlapping pathology caused by SubAB in mice and Shiga toxin in humans and various animal models raises the intriguing question of whether the new toxin contributes to life-threatening disease in humans, either on its own or in concert with Shiga toxin. There is also the possibility that vaccines directed only at Shiga toxin may not provide adequate protection against STEC strains producing both toxins. Accordingly, in the present study, we sought to determine whether a derivative of SubAB with a mutation in a critical domain of the A subunit essential for its subtilase-like activity could be used as a vaccine antigen, to protect against the potentially fatal properties of the toxin.

Purification of the SubAB active-site mutant. Members of the subtilase family of serine proteases are characterized by the presence of three critical catalytic domains containing conserved Asp, His, and Ser residues, collectively referred to as the catalytic triad (20). In SubA, the conserved Ser is at position 272 of the primary amino acid sequence (i.e., including the signal peptide) and we have previously described construction of a derivative of pK184 containing a mutated subAB operon, such that this residue is replaced by Ala in the expressed protein. Lysates of Escherichia coli JM109 carrying this construct (pK184subA272B) were not cytotoxic for Vero cells, unlike those carrying the wild-type operon (pK184subAB) (15).

In order to purify the mutant toxin, the subAB coding region was amplified by high-fidelity PCR (Expand High Fidelity PCR kit; Roche Molecular Diagnostics, Germany) using the pK184subA272B DNA template and primers pETsubAF (5′-
TTGTAAGGATCCGGAGGAGCTTATGCTTAAG-3'; BamHI site italic) and pETSubBR (5’-ATTATTCGAGTGAGTGTGCTTCTTTTCCTGTCAGG-3'; XhoI site italic). The resultant PCR product was digested with BamHI and XhoI, ligated with similarly digested pET-23 (+) (Novagen, Madison, WI), and transformed into E. coli BL21(DE3) (Novagen). This results in isopropylthiogalactopyranoside (IPTG)-dependent production of both the SubA272 and SubB proteins (including their respective signal peptides), but with a His<sub>6</sub> tag fused to the C terminus of SubB. Correct insertion of the genes into the vector was confirmed by sequence analysis.

Cells were then grown in 1 liter LB broth (11) supplemented with 50 μg/ml ampicillin, and when the culture reached an A<sub>600</sub> of 0.5, the culture was induced with 5 mM IPTG and incubated for a further 3 h. Cells were harvested by centrifugation, resuspended in 20 ml loading buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0) and lysed in a French pressure cell. Cell debris was removed by centrifugation at 100,000 × g for 1 h at 4°C. The supernatant was then loaded onto a 2-ml column of ProBond Ni-nitrilotriacetic acid resin (Invitrogen, Carlsbad, CA), which had been preequilibrated with 20 ml loading buffer. The column was then washed with 20 ml wash buffer (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, pH 6.0). Bound proteins were then eluted with a 30-ml gradient of 0 to 500 mM imidazole in wash buffer; 3-ml fractions were collected and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and peak fractions were pooled, diafiltered against phosphate-buffered saline and stored in 50% glycerol at −15°C.

The final purified material (designated SubA<sub>272</sub>B) co-migrated with native SubAB on SDS-PAGE, exhibiting two protein bands with sizes of 35 kDa and 14 kDa, corresponding to that predicted for the A and B subunits, respectively (Fig. 1). Additional contaminating protein species were not detected when replicate gels were subjected to silver staining (result not shown). The purified SubA<sub>272</sub>B was also assayed for cytotoxicity for Vero cells, as described previously (15). No cytotoxic effect was observed after incubation of Vero cell monolayers with replicate gels, which were subjected to silver staining (result not shown). Bound proteins were then eluted with a 30-ml gradient of 0 to 500 mM imidazole in wash buffer; 3-ml fractions were collected and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and peak fractions were pooled, diafiltered against phosphate-buffered saline and stored in 50% glycerol at −15°C.

**Immunization of mice.** Animal experimentation was conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the Animal Ethics Committee of the University of Adelaide. Groups of 20 male BALB/c mice (4 weeks of age) were immunized intraperitoneally with three doses of either 10 μg of purified SubA<sub>272</sub>B (or phosphate-buffered saline placebo) in 0.1 ml phosphate-buffered saline containing 100 μg of alum adjuvant (Imjectalum; Pierce, Rockford, Illinois) at 10-day intervals. One week after the third dose, blood samples were collected from the retro-orbital plexus of eight mice selected at random from each group, and sera were stored at 4°C.

**ELISA.** Antibodies to SubAB were measured by enzyme-linked immunosorbent assay (ELISA) using 96-well Costar plates which were coated overnight at 4°C with 100 μl of 5 μg/ml purified native SubAB (15) in TBS (25 mM Tris-HCl, 132 mM NaCl, pH 7.5). Plates were then washed with TBS-0.05% Tween 20-0.02% bovine serum albumin (TBS-Tween-BSA) for 2 h at 37°C. Plates were washed again and then incubated for 4 h at 37°C with 100 μl serial dilutions of mouse serum in TBS-Tween-BSA, commencing at 1:200. Plates were then washed and incubated with goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate (enzyme immunoassay grade; Bio-Rad Laboratories, CA), diluted 1:15,000 in TBS-Tween-BSA for 2 h at 37°C. Plates were then washed and developed with 1 mg/ml p-nitrophenyl phosphate substrate (in 12.5 mM triethanolamine, 135 mM NaCl, 0.02% BSA, 1 mM MgCl<sub>2</sub>, 2.5 μM ZnCl<sub>2</sub>, pH 7.6) for 2 h at 37°C, after which absorbance at 410 nm was determined. Absorbance above background was plotted against serum dilution, and the ELISA titer was defined as the reciprocal of the serum dilution resulting in an A<sub>410</sub> reading of 0.2 above background. All but one of the immune mouse sera exhibited high anti-SubAB ELISA titers (>2,800), while no antibody response was detected in any of the control mouse sera (titers <200) (Fig. 2).

**Challenge of mice.** Twelve days after the final immunization, groups of 10 immunized and control mice were challenged by intraperitoneal injection of 5 μg of purified native SubAB (>25 times the lethal dose (15) in 0.1 ml phosphate-buffered saline. All control mice died with a median survival time of 4 days. However, eight of the 10 immunized mice survived and remained well for the duration of the experiment (15 days) (Fig. 3). One of the immunized mice died only 2 days after challenge, before any of the unprotected control mice. This particular mouse was the only immunized mouse tested that failed to mount a satisfactory anti-SubAB response (ELISA titer ~300) and may have been compromised in some way. The other immunized mouse that died on day 13 had not been
tested for antibody response. The difference in survival rate between the immunized and control groups was highly significant ($P < 0.005$, Fisher exact test).

We then examined whether parenteral immunization with purified SubAA$_{272}$B could also provide protection against active SubAB delivered via the gastrointestinal tract. The other 10 immunized and control mice were given oral streptomycin (5 mg/ml in drinking water) for 24 h before oral challenge with approximately 10$^8$ CFU of a streptomycin-resistant derivative of *E. coli* DH5α (DH5α SR) carrying pK184subAB. This clone expresses active SubAB toxin and results in significant weight loss when administered orally to 6-week-old mice (15). Drinking water was then supplemented with streptomycin (5 mg/ml) and kanamycin (100 µg/ml).

Mice were weighed daily, and the numbers of the recombinant bacteria in fecal samples from each group were monitored by plating on LB agar supplemented with 50 µg/ml streptomycin and 50 µg/ml kanamycin. There was no significant difference between the numbers of DH5α SR::pK184subAB in fecal pellets from either group on any given day; the recombinant clone was maintained at levels of 10$^8$ to 10$^9$ CFU/g for the first 8 days, after which numbers diminished slightly to approximately 10$^7$ CFU/g by day 15 (result not shown). However, there was a marked difference in weight gain between the two groups (Fig. 4). By day 9, the unprotected control group had gained on average only about 0.5 g per mouse, whereas the immunized group had gained over 2 g per mouse. From day 5 onwards, the difference in weight gain between the two groups was highly significant ($P$ values ranged from $<0.03$ to $<0.0001$, Student’s unpaired $t$ test, two-tailed).

**Discussion and conclusions.** Unlike many enteric infections, much of the pathology associated with STEC disease, including both gastrointestinal symptoms and life-threatening sequelae, is due to systemic effects of Shiga toxin, which can be blocked by neutralizing serum antibodies (17). Thus, parenteral immunization with chemical or genetic Shiga toxin toxoids is likely to be highly protective against the life-threatening complications of STEC disease. Indeed, several studies have shown that A subunit active-site mutants of Stx1 or Stx2 are capable of eliciting protective immune responses in animals (2, 4, 6, 7, 13). However, the recent discovery (15) that some STEC strains produce an additional potent AB$_5$ cytotoxin (SubAB) that has the potential to significantly augment clinical manifestations, or indeed to cause disease in its own right, raises the issue of whether immunity to Shiga toxin alone will be sufficient to prevent disease caused by such strains. The *subAB* genes are
not present in O157:H7 STEC strains (15), which are the commonest cause of severe STEC disease in North America as well as Japan and the United Kingdom (18). However, comprehensive studies of the prevalence of subAB in other STEC serotypes in these and other regions have yet to be conducted.

In the present study, we have shown that immunization of mice with a purified derivative of this novel toxin with a Ser272→Ala mutation in its catalytic A subunit (SubA A272B) is a protective immunogen in mice. Parenteral immunization elicited high serum anti-SubAB titers, as judged by ELISA, and mice were protected from intraperitoneal challenge with at least 25 times the lethal dose of native toxin. Moreover, unlike the controls, immunized mice continued to gain weight after oral challenge with an E. coli K-12 clone expressing the native toxin, indicating that parenteral immunization protects against the deleterious effects of toxin delivered via the gut.

SubA A272B should be safe to administer to humans, as it exhibits less than 0.1% of the cytotoxicity of the native toxin, indicating the crucial role of the Ser272 residue in the catalytic activity of the toxin (15). However, mutations at two other positions in SubA, Asp52 and His89 (amino acid numbers include the signal peptide), would also be predicted to have a major impact on cytotoxicity, as these residues also form part of the active site and are conserved in all known members of the subtilase family (20). Indeed, we have constructed Asp52→Ala and His89→Ala substitutions in SubA and demonstrated that both mutant holotoxins (designated SubA A52B and SubA A89B) have undetectable cytotoxicity for Vero cells (result not shown). An ideal SubAB-based vaccine antigen for use in humans might therefore include a combination of Ser272→Ala and additional mutations in SubA such as either or both Asp52→Ala and His89→Ala.

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REFERENCES

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