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Serological Responses to the B Subunit of Shiga-Like Toxin 1 and Its Peptide Fragments Indicate that the B Subunit Is a Vaccine Candidate To Counter the Action of the Toxin

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The B subunit of Shiga toxin and Shiga-like toxin 1 (SLT-1) and its fragments are potentially immunogenic and may generate protective humoral responses against the action of these toxins. We have analyzed the antibody response of rabbits immunized with pure B subunit of SLT-1 or synthetic fragments of the subunit. The immune response to the native B subunit was found to be largely directed at conformational epitopes. More importantly, rabbits immunized with the B subunit were protected from a lethal challenge with SLT-1, indicating that the B subunit represents an excellent vaccine candidate to counter the effects of Shiga toxin and SLT-1 in humans. Polyclonal antibodies against a synthetic peptide corresponding to residues 28 to 40 of the B subunit neutralized the cytotoxicity of SLT-1 towards Vero cells. This region is thus exposed in the native state of the B subunit. The sequence specificity of other antipeptide antisera also provides clues to the state of folding and assembly of the B subunit. Antisera to synthetic peptides representing the N- and C-terminal regions of the SLT-1 B subunit did not cross-react with native B subunit but strongly recognized denatured forms of the protein. Finally, the monoclonal antibody 13C4 was shown to bind to a discontinuous epitope expressed only on the native form of the protein. These immunological reagents can be used to probe the conformational state of the B subunit and the holotoxin as it relates to their functional properties.

Shiga toxin (ShT), produced by *Shigella dysenteriae* 1, and Shiga-like toxin 1 (SLT-1), elaborated by enterohemorrhagic strains of *Escherichia coli*, are closely related members of an expanding family of bacterial cytotoxins (19). Although the existence of ShT has been known for 80 years and that of SLT-1 has been known for over a decade, the role of these toxins in human disease remains poorly understood. Symptoms associated with human intestinal infection resulting from toxin-producing *Shigella* strains or *E. coli* include episodes of severe diarrhea and dysentery. Some cases of hemorrhagic colitis and hemolytic uremic syndrome, involving vascular damage in the colon and kidney, respectively, have also been correlated with the presence of Shiga-like toxin (12) and may arise as a direct consequence of the action of the toxin on endothelial tissue (6, 18, 20).

These toxins are composed of two polypeptide chains: a 32-kDa A subunit that differs by a single conservative amino acid substitution between ShT and SLT-1 and an identical 7.7-kDa B subunit which forms pentamers in both the holotoxin-associated form (3) and free form (20a). The B subunit binds to a cell surface glycolipid, globotriaosylceramide (14, 15). Following internalization, the A subunit is released into the cytoplasm, where it enzymatically inhibits protein synthesis through the inactivation of the eukaryotic 60S ribosomal subunit (21). It is believed that the effects of ShT and SLT-1 in humans and animal models reflect the mechanism of cytotoxicity towards susceptible cell lines as well as the differential tissue distribution and cell surface concentration of globotriaosylceramide between species (1).

Rabbit antisera raised against a toxoid form of ShT effectively neutralized the cytotoxic activity of the toxin for HeLa cell monolayers (3). Protective antibodies from the serum could be recovered by affinity chromatography with an immobilized B subunit affinity gel (3). In addition, several B subunit-specific monoclonal antibodies with toxin-neutralizing ability have been reported (3, 24). Recently, it was shown that synthetic peptides derived from the N-terminal (10) and C-terminal (9) regions of the B subunit sequence are able to elicit a protective B-cell response in mice.

Despite these encouraging results, there has been no report in the literature of the use of the B subunit alone as an immunogen. We believed that SLT-1 B would provide a safer alternative to the use of toxoid holotoxin and would circumvent the need for conjugation of peptides to carrier molecules. To this end, rabbits were immunized with purified B subunit, and their sera were analyzed for the presence of antibodies able to neutralize the action of the toxin. Using a panel of synthetic peptides covering overlapping regions of the B subunit, we determined that most of the B-cell response to the B subunit was directed at nonlinear epitopes. Finally, rabbit antiserum responses against synthetic peptides representing regions of SLT-1 B were investigated to assess their usefulness as sequence-specific probes for the exposure or flexibility of such regions in the native and unfolded forms of the B subunit as well as for their ability to neutralize the action of the toxin.

MATERIALS AND METHODS

Peptide synthesis. Overlapping hexapeptides were synthesized on polyethylene pins (Cambridge Research Biochemicals, Valley Stream, N.Y.) as described by Geysen et al. (8). Peptides were assembled on the pins in the C- to N-terminal direction by using 9-fluorenylmethyloxy-carbonyl-protected amino acids. At the completion of synthesis and deprotec-

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tion, the hexapeptides remained permanently coupled to the plastic pins. Peptides used for generation of antipeptide antibodies were synthesized on an Applied Biosystems peptide synthesizer by using standard *t*-butoxycarbonyl chemistry. Phenylacetamidomethyl resins were typically used for solid-phase peptide synthesis. When a C-terminal amide peptide was desired [SLT-1 B(1-25), SLT-1 B(20-30), and SLT-1 B(27-40)], a *p*-methylbenzhydrylamine resin support was used. For peptides other than SLT-1 B(1-25), the nonnative N-terminal residue was acetylated while the peptide was covalently attached to the resin. Peptides were cleaved from the resin and protecting groups were removed by using anhydrous hydrogen fluoride in the presence of anisole and dimethyl sulfide (9:1:0.2). Thiocresol was added during deprotection of cysteine-containing peptides. Crude peptides were desalted by Sephadex G-10 or reverse-phase chromatography.

Peptide composition and concentration were verified by amino acid analysis. The peptide spanning residues 28 to 40 was synthesized as a branched polymer as described by Tam (25). Briefly, a branched lysine peptide was generated by three successive couplings of *N*-α,*N*-ε-BisBoc-L-lysine to the resin support to yield eight free amino groups. Following the addition of two glycine spacer residues, the peptide was synthesized on the branched core, resulting in eight identical peptides being assembled on each polymer molecule. The molecular size of the final multiple-antigen peptide (MAP_{28-40}) was approximately 14 kDa, eliminating the need to conjugate the peptide to a carrier protein. Following cleavage from the resin, the multiple-antigen peptide was desalted by extensive dialysis against 20% (vol/vol) acetic acid, followed by 10, 1, and finally 0.1% (vol/vol) acetic acid in water. The MAP_{28-40} peptide was insoluble in solutions containing less than 10% acetic acid and was used for immunization of rabbits as a suspension as described below.

ELISA mapping of linear epitopes. Peptides synthesized on polyethylene pins were incubated in blocking buffer (1% [wt/vol] bovine serum albumin [BSA], 1% [wt/vol] ovalbumin, 0.1% [vol/vol] Tween 20 in 10 mM phosphate-buffered saline [PBS, pH 7.4]) for 1 h to prevent nonspecific absorption of antibodies. The pins were then incubated overnight at 4°C in wells containing 100-μl aliquots of antiserum diluted in blocking buffer. After four washes in PBS containing 0.05% (vol/vol) Tween 20, the pins were incubated for 1 h in wells containing 100 μl of goat anti-rabbit (or anti-mouse) immunoglobulin-peroxidase conjugate diluted 1:1,000 in blocking buffer. The pins were washed, and antibody binding was detected by incubation of the pins in enzyme-linked immunosorbent assay (ELISA) microtiter plates containing 100 μl of 0.05% (wt/vol) 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) dissolved in 0.1 M sodium phosphate–0.08 M citric acid (pH 4.0)–0.003% (vol/vol) hydrogen peroxide per well. The A_{405} was recorded with a Titertek Multiscan MCC/340 plate reader.

Purification of the B subunit. The B subunit of SLT-1 was purified from the overproducing *E. coli* strain pJLB122 by DEAE-Sephacel chromatography, chromatofocusing, and gel filtration with Sephadex G-50 as previously described (20a).

Preparation of peptide conjugates. Peptides were coupled unidirectionally to the carrier protein keyhole limpet hemocyanin (KLH) by using the thiol group of a unique cysteine residue present in each peptide. The heterobifunctional cross-linking agent *m*-maleimidobenzoyl-*N*-succinimide ester was used to prepare the peptide-KLH conjugates as described previously (22).

Immunization of rabbits. Two New Zealand White female rabbits (2 kg) were immunized with each immunogen. For the initial injection, each immunogen (0.5 to 1.0 mg) was dissolved in 1 ml of PBS and emulsified with an equal volume of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, Mo.). Four weeks later, rabbits were boosted with the same immunogen at an equivalent dose emulsified with Freund's incomplete adjuvant. Serum was collected 9 to 11 days later. Subsequent boosts were performed and sera were collected as described above. All injections were given subcutaneously at multiple sites in the dorsal region.

Purification of MAb 13C4. The hybridoma line 13C4 (ATCC CRL 1794 [24]) was grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and 1 mM sodium pyruvate in 1-liter spinner flasks (Bellco Glass Inc., Vineland, N.J.). Monoclonal antibody (MAb) 13C4 was purified from culture supernatants by affinity chromatography on Affigel-protein A (Bio-Rad Laboratories, Richmond, Calif.).

Carboxamidomethylation of the B subunit. The two cysteine residues of the reduced B subunit were covalently modified with iodoacetamide by the method of Crestfield et al. (2), as modified by Ludwig et al. (17).

Toxin neutralization assay. The ability of antipeptide and anti-B subunit antisera to inhibit the cytotoxicity of SLT-1 towards Vero cells was determined as described previously (13). The serum antitoxin titer was defined as the highest serial twofold dilution of serum able to protect a Vero cell monolayer from 1 CD_{50} of SLT-1 (1 CD_{50} is the dilution of purified toxin required to kill half the cells in a Vero cell monolayer [13]).

Detection of specific antibodies by ELISA. Purified B subunit was used to coat microtiter wells (1 μg per well) overnight. Wells were incubated with 2% (wt/vol) BSA in PBS for 1 h. Unbound protein was removed by washing the wells with PBS containing 0.05% (vol/vol) Tween 20. The antigen-coated wells were incubated with dilutions of preimmune or immune serum for 1 h at room temperature. Wells were washed and then incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin antibody. Following a final wash, antibody binding was detected with ABTS substrate as described above.

To determine the binding of antibodies to peptides or the B subunit in solution, a competition ELISA assay was used. Briefly, dilutions of serum were incubated with increasing concentrations of peptide or native B subunit prior to incubation with B subunit-coated wells. The ability of the test serum or antibody to bind the B subunit or peptide in solution was observed as a decrease in binding to the B subunit coated on the well. For all ELISAs, each point represents the average of absorbance measurements recorded for duplicate or triplicate wells. In all cases, the error associated with each point did not exceed 10% (typically less than 5%) of the average absorbance recorded.

Challenge of immunized rabbits with active SLT-1. Immunized rabbits whose serum displayed toxin neutralization activity in the Vero cell assay were injected intravascularly with 10 50% lethal doses (LD_{50}s) of SLT-1 purified as previously described (13). The LD_{50} in rabbits was defined as 2×10^3 CD_{50}s/ml/kg (21a). This dose corresponds to a challenge with 1 μg of SLT-1 per kg. Rabbits whose serum displayed no neutralization activity in the Vero cell assay were used as controls. Animals were observed postchallenge for clinical symptoms of SLT-1 toxicity: loose stool or diarrhea and/or flaccid paralysis.

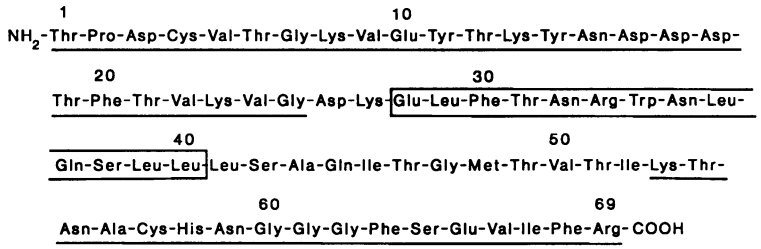

FIG. 1. Primary sequence of the B subunit of SLT-1. Sequences representing synthetic peptides used for immunization of rabbits are indicated: peptides 1-25 and 53-69 (underlined) were independently coupled to the carrier protein KLH. Peptide 28-40 (boxed) was synthesized as a branched polymer and is referred to as MAP_{28-40} in the text. In the native B subunit, Cys-4 and Cys-57 are joined by a disulfide bridge.

Preabsorption of antisera to the B subunit on peptide affinity columns. Synthetic peptides corresponding to B subunit residues 1 to 25 and 20 to 30 were covalently coupled to Affigel 15 and 10 (Bio-Rad), respectively. Antibodies directed to linear determinants were removed from the anti-B subunit antiserum by passing 100 μl of whole serum diluted in 10 ml of PBS through the peptide-bound affinity columns connected in series. Unbound serum proteins were collected in the column flowthrough and pooled with an additional column wash consisting of 20 ml of PBS. This preabsorbed serum was then concentrated to a known volume by ultrafiltration with a filter concentrator (Amicon Corp., Beverly, Mass.). Bound antibodies were eluted from each column independently with 5 ml of 0.2 M acetic acid, and the pH was immediately adjusted to 7 by addition of ammonium hydroxide. Eluents were lyophilized and resuspended in 1 ml of PBS. The eluted, preabsorbed, and whole sera were tested for peptide and B subunit binding by ELISA and for neutralization of SLT-1 as described above.

SDS-PAGE. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the method of Schägger and von Jagow was used for analysis of low-molecular-weight proteins (23). Protein samples were dissolved in sample buffer containing 4% (wt/vol) SDS, 5% (wt/vol) β-mercaptoethanol, 10% (wt/vol) glycerol, and 50 mM Tris (pH 6.8) and heated to 100°C for 3 min prior to loading and running the gel.

Western immunoblot analysis. Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes with a Polyblot transfer system (American Bionetics, Hayward, Calif.). Membranes were then incubated for 1 h in 2% (wt/vol) Carnation powdered milk in TBS (100 mM Tris-HCl, 0.15 M NaCl [pH 7.4]) to minimize the nonspecific binding of antibodies. Membranes were incubated with dilutions of antibody or antisera in TBS containing 0.2% (wt/vol) BSA or Carnation powdered milk for 1 to 2 h. After being washed with TBS, the membranes were incubated for 1 h with peroxidase-labeled goat anti-mouse or anti-rabbit antibody (Sigma) diluted 1:5,000 with TBS. The membranes were washed extensively, and antibody binding was detected by the method of Young (27). Briefly, 10 mg of 4-chloro-1-naphthol and 30 mg of 3,3'-diaminobenzidine tetrahydrochloride were dissolved together in 5 ml of methanol. This mixture was combined with 40 ml of PBS and 10 μl of 30% hydrogen peroxide and then incubated with the washed membranes. Color development was stopped by washing the membranes with distilled water.

RESULTS AND DISCUSSION

The analysis of B-cell responses to the B subunit of SLT-1 and its fragments was undertaken to identify regions of the molecule able to engender a protective immune response against the toxin and to generate a set of defined immunological probes that would allow us to assess the conformational state of the protein.

Probing linear regions of the SLT-1 B subunit. The B subunit of ShT and SLT-1 is composed of 69 amino acids. This short sequence codes for several functional domains: complementary regions that allow the monomer to form a pentamer, a glycolipid-binding domain, and a binding site for the catalytic A chain. Antipeptide antibodies which recognize linear determinants are useful site-specific probes of protein structure, since their binding site may be mapped to distinct regions of a protein. Information relating to the surface accessibility or flexibility of domains of the B subunit can thus be obtained (5, 7). We tested this approach by generating antipeptide antisera to synthetic peptides derived from the sequence of the B subunit. Initially, three peptides spanning the entire molecule were synthesized, for residues 1 to 25, 26 to 52, and 53 to 69 (peptides 1-25, 26-52, and 53-69, respectively). The central peptide (residues 26 to 52) proved to be highly insoluble and could not be efficiently coupled to a carrier molecule. A portion of this region encompassing residues 28 to 40 was subsequently synthesized for the generation of antipeptide antisera in rabbits. The positions of these peptides in the primary structure of the B subunit are illustrated in Fig. 1.

Antipeptide antibodies to regions 1 to 25 and 53 to 69 recognize unfolded forms of the SLT-1 B subunit. A strong antibody response was generated in rabbits injected with conjugates of peptides 1-25 and 53-69. Both antisera recognized SLT-1 B coated on ELISA wells (Fig. 2A and B) and on a Western immunoblot (see Fig. 4C, antiserum to peptide 1-25, as an example). When these antibodies were left to react with the B subunit in solution prior to binding to the subunit immobilized on the solid phase, little or no titratable reduction in the antibody signal occurred as monitored by ELISA (Fig. 2A and B). However, peptides corresponding to each epitope competed effectively in solution for binding to their respective antipeptide sera, as measured by the dose-dependent decrease in the ELISA signal observed (Fig. 2).

The lack of binding of these antipeptide antibodies to the B subunit in solution suggests that they recognize unfolded forms of the protein and explains their failure to neutralize the cytotoxic action of SLT-1 on Vero cells (Table 1). This

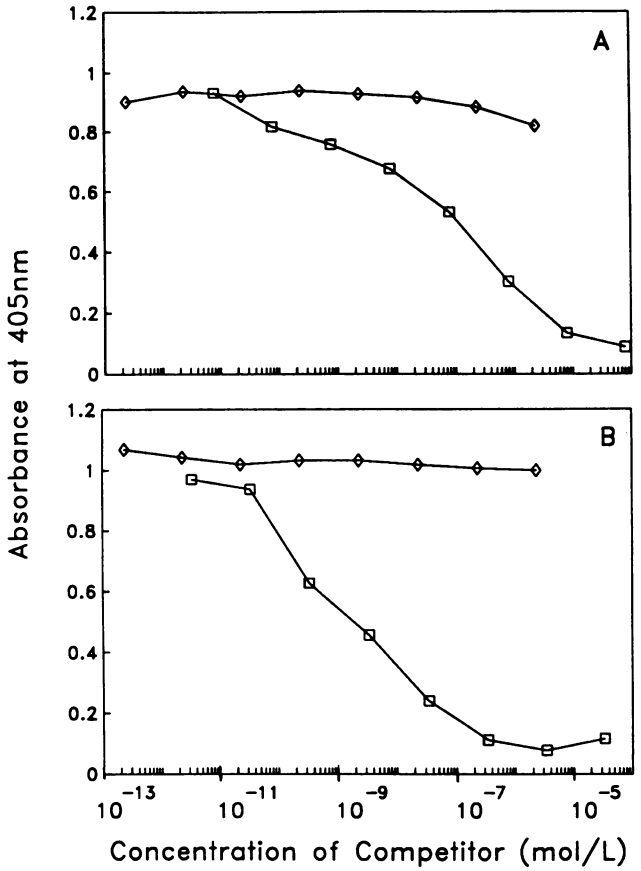

FIG. 2. Competition ELISA with antisera to the N- and C-terminal B subunit peptides. Antisera were incubated with the native B subunit or peptide fragment for 1 h and then allowed to bind to the B subunit coated on ELISA wells. (A) Antiserum to peptide 53-69 (dilution, 1:256) was incubated with the native B subunit (◇) or peptide 53-69 (□) prior to incubation in B subunit-coated ELISA wells. (B) Antiserum to peptide 1-25 (dilution, 1:1,000) was incubated with the B subunit (◇) or peptide 1-25 (□). All points represent the mean absorbance of duplicate wells. Preincubation of either antiserum with the heterologous peptide did not inhibit antibody binding to the B subunit coated on ELISA wells.

observation contrasts with the results of Harari and coworkers, who used peptides spanning residues 5 to 26 of ShT B subunit to generate a toxin-neutralizing antipeptide response in mice and rabbits (10). The nature of each immunogen used by both groups may explain these results. Harari et al. (10) generally used short peptides (<20 residues) which were coupled to a carrier molecule or cross-linked by using multiple sites within each peptide. We coupled peptide 1-25 to KLH by using the free sulfhydryl group of cysteine 4 so that the peptide was effectively presented to the immune system in one orientation only. The orientation of peptides coupled to a carrier protein such as KLH has been shown to influence the specificity and immunoreactivity of the antisera generated (4, 16).

Antisera to peptide 53-69 displayed weak affinity for the native B subunit at high concentrations in the competition ELISA (Fig. 2A) and showed no toxin neutralization ability. Antipeptide antisera to regions 54 to 67 and 57 to 67 of ShT B subunit have recently been shown to recognize the holotoxin in an ELISA, as in our case (9). However, immunization of mice with conjugates of these peptides resulted in

TABLE 1. Ability of anti-B subunit and antipeptide antisera to neutralize SLT-1 toxicity in vitro and in vivo

Antigen	No. of rabbits	Anti-SLT-1 neutralization titer[a]	Protective effect in vivo[b]
SLT-1 B(1-25)-KLH	2	0, 0	ND[c]
SLT-1 B(53-69)-KLH	2	0, 0	ND
SLT-1 B(MAP_{28-40})	2	128, 32	−, +
SLT-1 B[d]	2	102,400, 51,200	+, +
None	3	0, 0, 0	−, −, −

[a] Highest twofold dilution of serum able to protect a Vero cell monolayer from 1 CD_{50} of SLT-1. Each number represents the titer observed for individual rabbits in a minimum of two neutralization assays.

[b] Rabbits were challenged by intravascular injection of 10 LD_{50}s of SLT-1. Challenged animals developed fatal flaccid paralysis (−) or showed no clinical symptoms (+).

[c] ND, Not determined.

[d] Preabsorbed sera gave identical anti-SLT-1 neutralization titers.

partial protection of these animals against challenge with the toxin. Since we did observe that antiserum to our C-terminal conjugate recognized only a denatured form of the B subunit (i.e., it bound to the B subunit in the ELISA but did not recognize the B subunit in solution), we can only conclude that antiserum specificity differs significantly between investigators, reflecting differences in conjugation and immunization protocols as well as the type of assays performed. A likely possibility is that the residues constituting the major epitopes recognized by our antisera to regions of the N and C termini differ from those recognized by the antisera of Harari and his coworkers (9, 10). Thus, in our case, such residues as part of the native structure may adopt a conformation that lacks the exposure or the flexibility of the corresponding synthetic peptides coupled to a carrier protein (7), while neighboring residues remain available for binding a different antiserum in the folded state of the protein (9, 10). Both sets of data suggest that only a selected number of residues along the sequence between residues 13 to 26 and 54 to 67 are exposed in the native state of the toxin. A delineation of the exact residues recognized by the antisera of Harari and Arnon (9, 10) should allow more precise definition of which of those residues are exposed.

Rabbit antiserum raised against a synthetic peptide representing residues 28 to 40 of the B subunit can neutralize the cytotoxic action of SLT-1. To determine whether the central region of the B subunit is sufficient for the generation of toxin-neutralizing antisera, we synthesized the peptide representing the region from residues 28 to 40 of SLT-1 B and immunized rabbits with it. The peptide was synthesized in multiple copies on a branched lysine polymer as described by Tam (25) to avoid the need for a carrier protein. A further advantage of this technique is the generation of a chemically defined immunogen which may result in a more uniform antibody population. The resulting multiple-antigen peptide was designated MAP_{28-40}.

Antisera to the branched peptide recognized the B subunit immobilized on nitrocellulose, as determined by Western immunoblot analysis (results not shown), or in solution, as demonstrated by a competitive ELISA (Fig. 3A). Thus, the epitope is exposed in the native state of the molecule. To determine to which amino acid residues the anti-MAP_{28-40} response was directed, the rabbit sera were screened with a library of overlapping hexapeptides covering the entire sequence of SLT-1 B. The multiple-peptide synthesis method of Geysen et al. (8) was used to map the region. A typical profile for one of the immunized rabbits is shown in Fig. 3B.

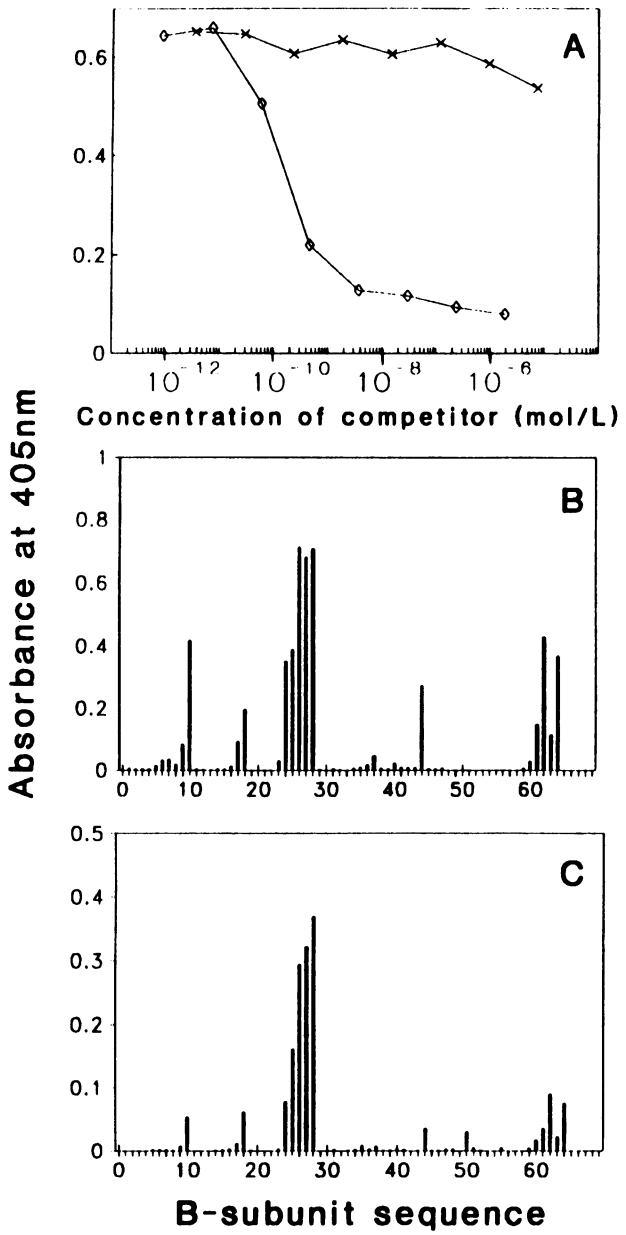

FIG. 3. Characterization of antiserum raised against the synthetic peptide MAP_{28-40}. (A) Competition ELISA with anti-MAP_{28-40} antiserum; antiserum at a dilution of 1:500 was incubated with the B subunit (◇) or BSA (×) prior to incubation with the B subunit coated on ELISA wells. All points represent the mean absorbance of duplicate wells. (B) Hexapeptide-binding profile of whole sera diluted 1:500. (C) Profile of serum which was preabsorbed on a peptide affinity column containing peptide 27-40. In panels B and C, each vertical bar represents a measure of antibody binding (mean A_{405} of duplicate pins) to a six-residue peptide starting at the amino acid position in the primary sequence of SLT-1 B indicated on the x axis.

We were initially surprised to find antibody binding to hexapeptides which were quite distant in sequence from the injected region. To determine whether these signals represented nonspecific binding of the antiserum to unrelated peptides, the antiserum was purified on an affinity column containing the peptide SLT-1 B(27-40). The peptide-binding profile of the purified antiserum is shown in Fig. 3C. It is clear that the bulk of the antibodies present are specific for the region encompassing residues 28 to 33, as indicated by strong antibody binding to hexamers 26 (Asp-Lys-**Glu-Leu-Phe-Thr**), 27 (Lys-**Glu-Leu-Phe-Thr-Asn**), and 28 (**Glu-Leu-Phe-Thr-Asn-Arg**) in Fig. 3C. The absence of antibody binding to hexamer 29 (**Leu-Phe-Thr-Asn-Arg-Trp**) indicates that Glu-28 is an essential residue composing the immunodominant epitope recognized by antiserum to MAP_{28-40}.

Since peptide 28-40 was synthesized on a branched polymeric core in the C- to N-terminal direction, it may be argued that the immune response has been directed towards the more exposed residues. However, preliminary results with antisera to the synthetic peptide SLT-1 B(26-40)(Cys-26) coupled to KLH through a nonnatural N-terminal cysteine gave epitope mapping profiles similar to the one shown in Fig. 3C (results not shown). These results suggest that residues 28 to 33 constitute an immunodominant epitope. Residues present in the sequence of the MAP_{28-40} peptide are highlighted in boldface letters above. The weakly positive hexapeptides found elsewhere in the sequence (Fig. 3B) have some partial sequence homologies with this hexapeptide, a fact that may explain the low level of cross-reactivity observed.

The anti-MAP_{28-40} antisera (both whole sera and affinity-purified antibodies) also neutralized SLT-1 in the Vero cell assay, demonstrating that this linear region is able to generate a toxin-neutralizing immune response (Table 1). The two immunized rabbits were then challenged with an intravascular injection of 10 LD_{50}s of pure SLT-1 in an effort to evaluate the protective nature of the immune response. One animal showed no clinical symptoms, while the other rabbit developed the severe paralysis characteristic of SLT-1 toxemia, which was also observed in all three control rabbits tested (Table 1). This region differs from the ones previously selected from hydrophilicity plots as being potentially immunogenic (9, 10). Future animal studies will clarify the potential of this segment as a peptide vaccine.

MAb 13C4 recognizes a conformationally restricted epitope located on the native B subunit. To complement the panel of antipeptide antibody probes which recognize native and unfolded forms of the B subunit, we investigated the nature of the epitope recognized by MAb 13C4 (24). This antibody is specific for the B subunit of ShT and SLT-1 and can be readily purified from hybridoma supernatants. The cell line ATCC CRL 1794 was initially derived from mice immunized with a toxoid form of the holotoxin (24).

Since this MAb is able to neutralize the action of the toxin, the antigenic site is probably exposed on the multimeric form of the B subunit. We probed the entire sequence of the B subunit in an effort to locate linear determinants recognized by MAb 13C4. Screening a library of hexapeptides (see Materials and Methods section) covering the protein sequence did not reveal any unique linear epitope, suggesting that the major determinant is probably conformational in nature (results not shown). To further establish that such a conformational epitope did exist, we investigated the extent of reactivity of MAb 13C4 with native and denatured forms of the B subunit. Western blot analysis showed that MAb 13C4 recognition of the B subunit was significantly stronger following SDS-PAGE of the protein under nonreducing conditions (Fig. 4B, lane 2) than after parallel experiments performed in the presence of the reducing agent β-mercaptoethanol (Fig. 4B, lane 3).

To confirm that antibody binding was dependent on the integrity of the single disulfide bridge of SLT-1 B, we

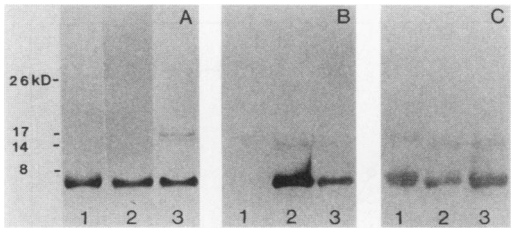

FIG. 4. Western blot analysis of MAb 13C4 recognition of the B subunit. Carboxamidomethylated B subunit (lanes 1), B subunit denatured in nonreducing sample buffer (lanes 2), and B subunit denatured in the presence of β-mercaptoethanol (lanes 3) were analyzed by SDS-PAGE, followed by Coomassie blue staining (A) or Western blotting with MAb 13C4 (B) or with antiserum to peptide 1-25 (C).

chemically modified the cysteine side chains of SLT-1 B with iodoacetamide to prevent reoxidation. MAb 13C4 was unable to bind to the derivatized B subunit on Western blots (Fig. 4B, lane 1). A control set of Western blots made with the antipeptide antiserum directed against region 1 to 25 of SLT-1 B indicated that all three presentations of the B subunit gave equivalent responses under similar conditions (Fig. 4C, lanes 1 to 3). Antibody binding to the B subunit on a Western blot following SDS-PAGE under reducing conditions is probably due to partial reoxidation and folding of the protein bound to nitrocellulose. The binding of MAb 13C4 to native B subunit in solution was also demonstrated by competition ELISA measuring the residual antibody response that resulted from the competitive removal of antibody in solution in the presence of increasing amounts of B subunit (results not shown). In summary, MAb 13C4 clearly recognizes a discontinuous epitope defined by the proper folding of the B subunit.

Investigating determinants of the B subunit recognized by anti-SLT-1 B antiserum. A toxoid form of whole ShT has been used in the past as an immunogen and shown to produce a protective antibody response to ShT in rabbits (3). Specific antibodies to the B subunit were affinity purified from this serum and shown to neutralize the cytotoxic activity of ShT towards HeLa cells (3). A recent analysis of the hydrophilic regions of the B subunit suggested that peptides derived from the N- and C-terminal regions may elicit a neutralizing immune response to the whole toxin. This approach has yielded peptide vaccine candidates (9, 10).

We suspected that the B subunit alone would constitute a safer and more practical immunogen than detoxified holotoxin and would be more immunogenic than peptides, generating a broader immune response to both linear and conformational determinants. The recent establishment of a B subunit overexpression system has facilitated this approach (20a).

We have looked directly at the linear determinants recognized by rabbit antisera raised against SLT-1 B by screening our library of hexapeptides spanning the primary sequence of SLT-1 B. Sera from two rabbits immunized with SLT-1 B showed similar linear epitope profiles located in the N-terminal region of the B subunit (Fig. 5A and B). The immune response was further analyzed by indirectly determining the presence of discontinuous epitopes recognized by antibodies present in the immune sera. Antibodies directed at linear epitopes of SLT-1 B were immunoabsorbed by affinity chromatography. Synthetic peptides covering regions 1 to 25

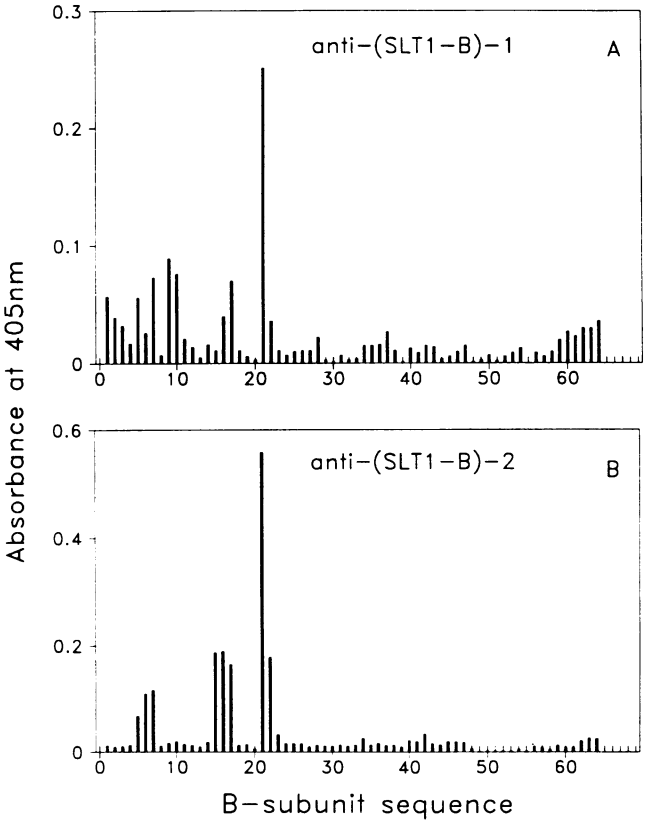

FIG. 5. Linear epitope profile of two rabbit antisera raised against the B subunit of SLT-1 B (A and B). Both sera were screened at a dilution of 1:500. Linear epitopes detected in both cases are situated in the N-terminal domain of the B subunit. Each vertical bar represents a measure of antibody binding (mean A_{405} of duplicate pins) to a six-residue peptide starting at the amino acid position in the primary sequence of SLT-1 B indicated on the *x* axis.

and 20 to 30 of the B subunit were independently coupled to activated gel supports (see Materials and Methods section). Removal of peptide-binding antibodies was monitored by ELISA. Whole sera were able to bind SLT-1 B, peptide 1-25, and peptide 20-30 coated on ELISA wells (Fig. 6A and C). However, preabsorbed sera lost most of their antipeptide response while showing no significant reduction in B subunit binding (Fig. 6B and D). These results suggest that most antibodies were directed at nonlinear epitopes. Preabsorbed serum showed no reduction in neutralization titer in the Vero cell assay (Table 1). Furthermore, antibodies which were retained on the peptide affinity columns were eluted and found to possess no detectable neutralization ability, although they could still bind to the peptides coated on ELISA wells (results not shown). Thus, the neutralizing immune response engendered against the B subunit in rabbits is directed at nonlinear determinants.

Active immunization with the B subunit generates a protective immune response to SLT-1. The whole and preabsorbed sera from both rabbits immunized with SLT-1 B had equivalent neutralization titers in the Vero cell assay (Table 1). Compared with the MAP_{28-40} antiserum, these titers are high, similar to those obtained with toxoid SLT-1 as an immunogen (21a). Interestingly, neither of the two rabbit

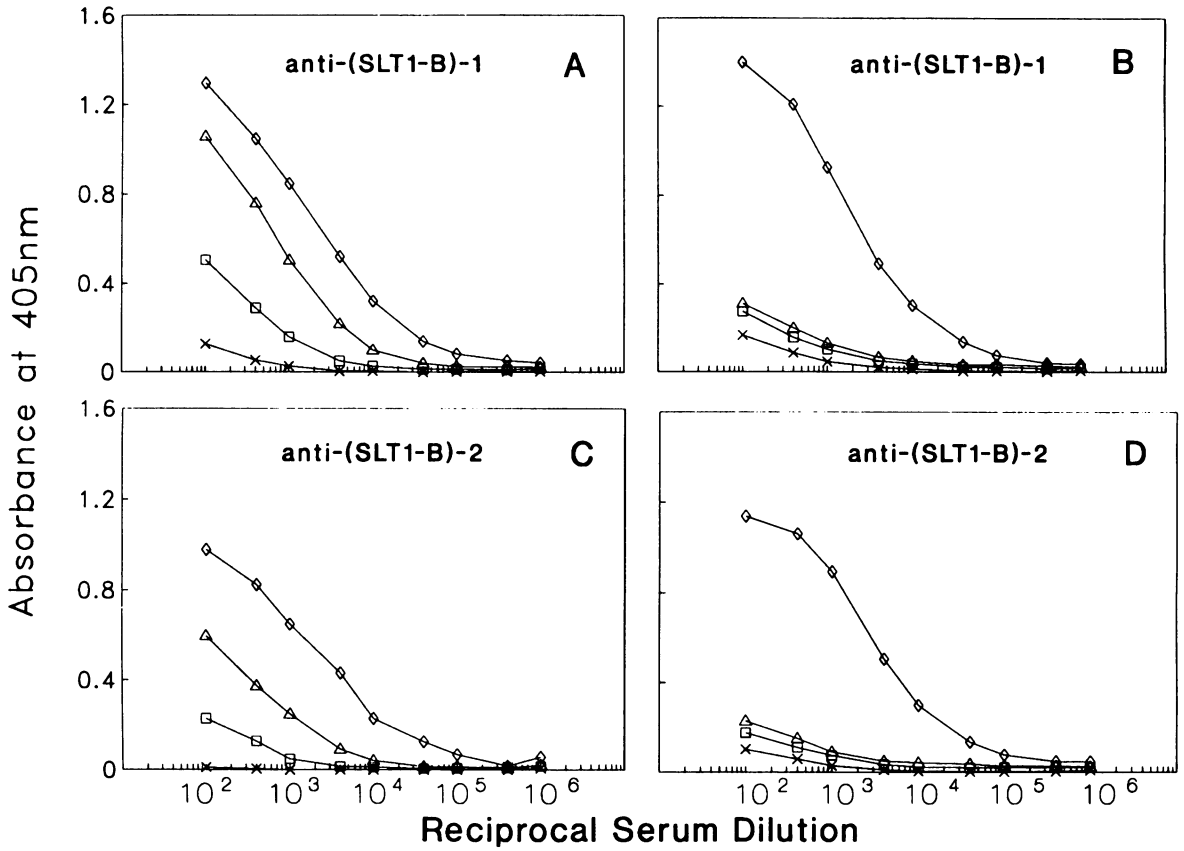

FIG. 6. Removal of peptide-specific antibodies from anti-B subunit antisera. (A and C) Titration of antibody response present in whole sera from each of two rabbits ([SLT-1 B]-1 and [SLT-1 B]-2) immunized with the B subunit; binding to peptide 1-25 (△), peptide 20-30 (□), B subunit (◇), and BSA (×) coated on 96-well plates. (B and D) Binding of each antiserum following preabsorption on affinity columns containing peptides 1-25 and 20-30. All points represent the mean A_{405} of triplicate wells.

antisera displayed any neutralizing activity against SLT-2 in the Vero cell assay despite their high neutralizing titers against SLT-1. SLT-2 has 57% amino acid sequence homology with SLT-1, and both B subunits have identical glycolipid receptor specificity (11, 26).

Neither of the immunized rabbits showed any signs of paralysis or diarrhea after a challenge with 10 LD_{50}s of SLT-1 (Table 1). At this dose, a nonimmune animal shows definite symptoms. In light of our preabsorption experiments, it is clear that the B subunit is an immunogen different from the N- and C-terminal peptides reported earlier by Harari et al. (9, 10) as vaccine candidates. In our case, the majority of the immune response is directed at nonlinear determinants and the response observed clearly suggests its use as a vaccine candidate against the effects of ShT and SLT-1 in humans.

In conclusion, the B subunit of ShT and SLT-1 is an immunogen able to mount a protective immune response in rabbits against a challenge with the toxin. The reactivity of antipeptide antibodies raised against regions of the subunit indicates that in the native B subunit, the N- and C-terminal domains probably adopt a conformationally restricted geometry while still being partially exposed. Furthermore, the central region, particularly the hexapeptide 28 to 33 of SLT-1 B, is exposed and may represent a site in proximity to the receptor-binding domain.

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