INFECTION AND IMMUNITY, June 1988, p. 1618–1624
0019-9567/88/061618-07$02.00/0

Vol. 56, No. 6

Synthetic Peptides of Shiga Toxin B Subunit Induce Antibodies Which Neutralize Its Biological Activity

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Received 10 November 1987/Accepted 29 February 1988

Shiga toxin B chain, the binding subunit of Shiga toxin, was recently purified; and the amino acid sequence of this 7,716-dalton polypeptide was determined (N. G. Seidah, A. Donohue-Rolfe, C. Lazure, F. Auclair, G. T. Keusch, and M. Chretien, J. Biol. Chem. 261:13928–13931, 1986). In the present study, synthetic peptides corresponding to three overlapping sequences from the N-terminal region of this subunit were prepared. The peptides synthesized consisted of residues 5 to 18, 13 to 26, and 7 to 26. This region coincides with the major peak of hydrophilicity and surface area residues predicted from a computer analysis. For the purpose of immunization, the peptides either were conjugated with a protein or synthetic carrier or were polymerized with glutaraldehyde. Antisera against these peptide derivatives raised in rabbits reacted not only with the respective homologous peptide but also to a comparable extent with the intact Shiga toxin. The anti-peptide antisera effectively neutralized the various biological activities of the Shiga toxin, namely, cytotoxicity to HeLa cells, enterotoxic activity (the fluid secretion into ligated ileal loops in rats), and neurotoxicity in mice. Furthermore, active immunization with the peptide conjugates was found to protect mice against the lethal effect of Shiga toxin.

Vaccination against *Shigella* species for the prevention of shigellosis presents a number of problems, and as a consequence, no efficient shigella vaccine is available as yet. The pathogenesis of these infections is dependent on the epithelial cell invasive properties of the organism (8), a property that is under the polygenic control of both chromosomal and plasmid genes (11, 34). Hence, recent attempts to develop shigella vaccines have been based on the expression of virulence-associated membrane antigens (10, 28). The various species of *Shigella* are also known to produce a potent protein toxin which experimentally reproduces the major features of the infection (5, 20). Neutralizing antibodies against this toxin have been detected in sera of convalescing patients with shigellosis (19). Nevertheless, the role of the toxin in the pathogenesis of this disease is rather controversial (22). In the present study we explored the possibility that a prospective vaccine could be based on peptides of the shigella toxin.

Shiga toxin, the toxin isolated from *Shigella dysenteriae* 1 strains, is one of the most potent of the lethal microbial toxins. It was initially classified as a neurotoxin because of the limb paralysis and death it causes when parenterally administered to sensitive animals. In addition, it is cytotoxic to certain tissue culture cells and exerts enterotoxic activity when applied to intestinal ileal loops in vivo (18). This toxin has been purified and shown to consist of A and B subunits with M_rs of 32,000 and 6,500, respectively (3). Recently, the complete amino acid sequence of the B chain was determined, and the molecular weight of this subunit was found to be 7,716 (29). Incidentally, this sequence is identical to that of the B subunit of the Shiga-like *Escherichia coli* toxin, as deduced recently from its DNA sequence (2). Antibodies raised against the B subunit neutralize the cytotoxic effects of the toxin in HeLa cell monolayers and inhibit the binding of labeled toxin to these cells (3). These data indicate that the B subunit is involved in the binding of shigella toxin to the cell surface of HeLa cells, as well as to the intestinal cell microvillus membrane, from which a glycolipid receptor has been recently isolated and identified (14).

In recent years it has been demonstrated that synthetic peptides corresponding to fragments of some bacterial toxins, when attached to appropriate carriers, can induce antibodies that are reactive with the native toxin and neutralize its biological activity. This has been shown for diphtheria toxin (1) and cholera toxin (16). In the latter case the antibodies elicited by a 15-amino-acid-residue fragment of the B chain of the toxin also neutralized the heat-labile toxin of enterotoxigenic *E. coli*, which has a sequence that is highly homologous to that of cholera toxin (15). Here we report the successful induction of neutralizing antibodies and protective immunity against Shiga toxin by the use of synthetic peptide antigens.

MATERIALS AND METHODS

Animals. SPD rats (age, 8 to 12 weeks) and New Zealand rabbits were obtained from the breeding house of the Weizmann Institute of Science (Rehovot, Israel). C57/BL mice (age, 4 to 5 weeks) were obtained from Jackson Laboratory (Bar Harbor, Maine).

Shiga toxin. Shiga toxin was purified from a total cell lysate of *Shigella dysenteriae* 1 by column chromatography on blue Sepharose followed by chromatofocusing, and final chromatography on Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, Calif.), as described previously (3). The A and B subunits of the toxin were separated, after dissociation in 5% formic acid (21), as described previously (3).

Carriers. The following carriers were used for conjugation of the peptides: (i) tetanus toxoid (M_r, 150,000) obtained from RAFA Laboratories (Jerusalem, Israel) and purified as a 15 to 30% saturation cut of $(NH_4)_2SO_4$ precipitation, followed by column chromatography on Sephadex G-100; (ii) a linear copolymer (M_r, 60,000) of D-glutamic acid and D-lysine in a 60:40 ratio (pDGDL); and (ii) a branched copolymer of L-tyrosine, L-glutamic acid, L-alanine, and

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L-lysine [(T,G)-A--L]. Both polymers were purchased from Bio-Yeda (Rehovot, Israel). All other reagents were of analytical grade or the best grade available.

Peptide synthesis. Peptides were synthesized by the solid-phase method of Merrifield (24), as modified by Stewart and Young (30), using *tert*-butyloxycarbonyl derivatives of amino acids with their appropriate protecting groups (obtained from J. Jacobson, Weizmann Institute, or from Peptide Institute Inc., Osaka, Japan). The side chain-protecting groups were *o*-benzyl ether for threonine, dichlorobenzyl ether for tyrosine, carbobenzoxy ether for lysine, benzyl ester for aspartic and glutamic acids, and *p*-nitrophenyl ester for asparagine. The initial amino acid was coupled to the resin by esterification of the relevant amino acid to chloromethylated resin (polystyrene–1% divinylbenzene). The progress of synthesis in the coupling of each of the subsequent amino acid *tert*-butyloxycarbonyl derivatives was monitored by ninhydrin analysis. Two cycles of coupling were performed whenever the coupling reaction was <99% complete. When the synthesis was completed, the protecting groups were removed and the peptides were cleaved from the resin at 0°C with anhydrous hydrogen fluoride containing 10% anisole and 1% 1,2-ethanedithiol as scavengers. Crude peptides recovered after cleavage from the resin were purified on a Sephadex G-15 column. The purity of the peptides was established by amino acid analysis, using a D-500 amino acid analyzer (Durrum).

Conjugation of peptides to the carriers. 1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) was used as a coupling reagent, and conjugation was performed as described previously (25). In addition to the conjugates, a polymer of each peptide was prepared by using a 0.1% glutaraldehyde solution as the polymerizing agent (1). The molecular weight of the resulting polymer was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Immunization procedures. (i) Preparation of anti-Shiga toxin. Purified Shiga toxin was converted to toxoid by treatment with Formalin, and the toxoid which contained <1% of the original cytotoxic activity was used for immunization of rabbits in complete Freund adjuvant, as described previously (3).

(ii) Preparation of anti-peptide antibodies. Rabbits were immunized by multisite intradermal injections of 1 mg of the appropriate conjugate in complete Freund adjuvant, followed by booster injections done by a previously described schedule (25).

(iii) Immunization of mice. Mice were immunized by injecting 100 μg of the conjugate in complete Freund adjuvant into their footpads; this was followed by two booster injections in incomplete adjuvant.

Enzyme-linked immunosorbent assay. A micro-enzyme-linked immunosorbent assay (6) (ELISA) was performed on antigen-coated (1 μg per well) flat-bottom microtiter plates (precoated with 0.2% glutaraldehyde whenever peptides were used as the antigen). This was done by adding a threefold serial dilution of the test serum and then by adding a β-galactosidase conjugate of protein A (Amersham Corp., Arlington Heights, Ill.). After the addition of the substrate *o*-nitrophenyl-β-D-galactopyranoside, the optical density was read in an automatic reader (Titertek Multiskan; Flow Laboratories, Inc., McLean, Va.) at 405 nm. The titer of each antiserum was defined as the dilution which led to 50% binding.

Immunoblotting. Shiga toxin, which was separated into subunits on a 5 to 15% sodium dodecyl sulfate-polyacrylamide gel, was transferred to a nitrocellulose sheet (32). To reduce nonspecific binding of the antiserum, the blot was incubated for 1 h with 9 mM Tris hydrochloride buffer (pH 7.4) containing 0.9% NaCl and 3% (wt/vol) bovine serum albumin. The blot was cut into strips, which were than incubated for 3 h at room temperature with a 1:50 dilution of the various antisera. After a thorough washing, the strips were incubated for 2 h with ^{125}I-labeled protein A (1.5×10^7 cpm/μg). The washed and dried blots were autoradiographed.

Cytotoxicity assay. Cytotoxicity to HeLa cells was assessed as the extent of HeLa cell detachment from microtiter plates (9). HeLa cells (line H-229) were maintained at 35°C in growth medium consisting of Eagle minimum essential medium containing Earle salts supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 180 μg of both penicillin and streptomycin per ml in a 5% CO_2 atmosphere. To establish monolayers, 2.5×10^4 freshly trypsinized cells suspended in 0.1 ml of growth medium were dispensed into 96-well microtiter plates (Costar, Cambridge, Mass.) and allowed to attach overnight. Serial dilutions of toxin were added, and the plates were incubated for an additional 18 h. The endpoint of the assay was determined by fixing the cells and staining them with crystal violet in a 2% formaldehyde solution. Stained cell monolayers were dissolved in 50% ethanol containing 1% sodium dodecyl sulfate. The A_{595} of the extracts was read with a microtiter plate colorimeter. The toxin dilution resulting in 50% cell detachment, namely, 50% dye uptake, was chosen as the endpoint for the assay.

Inhibition of cytotoxicty was determined by incubating serial dilutions of rabbit anti-peptide sera at 37°C for 1 h in the presence of an equal volume of a 10-ng/ml Shiga toxin solution. Duplicate 0.1-ml samples of these toxin-antitoxin mixtures, which contained 500 pg of toxin (ca. 2 × 50% cell detachment units), were incubated with HeLa cell monolayers for the toxicity assay as described above.

For calculation of the extent of inhibition, HeLa cell mortality resulting from the addition of 500 pg of toxin per well (which usually caused about 60% mortality) was taken as zero inhibition (100% toxicity) and the value for identically prepared cells incubated in the absence of toxin was taken as 100% inhibition (0% toxicity).

Ligated ileal loop assay. The ligated ileal loop assay was carried out by a slight modification of the method of Duhamel and colleagues (4). Briefly, SPD rats were starved for 24 h and then anesthetized with ether; their abdomens were opened, and the small intestine was ligated in 2- to 3-cm loops. The loops (4 to 8 per animal) were injected with 1 to 10 μg of Shiga toxin in phosphate-buffered saline (PBS). Each sample was injected into duplicate loops. Control loops

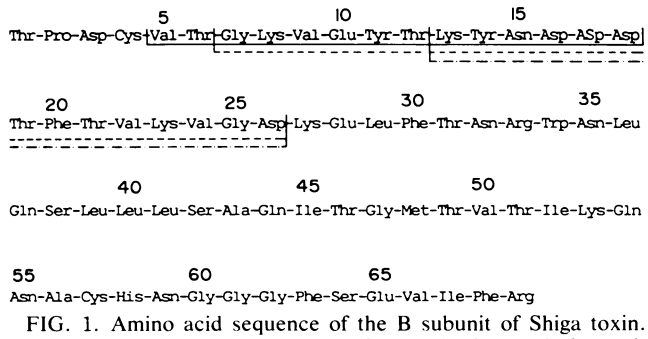

FIG. 1. Amino acid sequence of the B subunit of Shiga toxin. The regions of the protein selected for synthesis are designated. Symbols: ——, peptide 5-18; – – –, peptide 7-26; –·–·–, peptide 13-26.

TABLE 1. Amino acid composition the three synthetic peptides

Residue	Peptide[a]		
	5–18	13–26	7–26
Asp	3.73 (4)	4.82 (5)	4.32 (5)
Thr	1.76 (2)	1.94 (2)	2.74 (3)
Ser			1.02 (1)
Glu	1.08 (1)		1.10 (1)
Pro			
Gly	1.00 (1)	1.00 (1)	2.00 (2)
Ala			
Val	1.81 (2)	1.86 (2)	2.69 (3)
Met			
Ile			
Leu			
Tyr	1.76 (2)	0.91 (1)	1.73 (2)
His			
Lys	1.92 (2)	1.99 (2)	1.89 (2)
Arg			
Trp			
Phe		1.01 (1)	0.82 (1)
Cys			

[a] Values were calculated based on a value of 1.00 for the glycine residue. Values in parentheses represent expected values.

were injected with the same volume of PBS alone. The abdomens were then closed with metal clips. Food and water were withheld and animals were sacrificed after 12 h. Fluid accumulation per centimeter of loop was determined by measuring the length and weight of each loop.

For inhibition assays, the toxin was preincubated for 1 h at 37°C with a 1:25 dilution of the anti-peptide sera in PBS before it was injected into the loops. There were both negative (PBS alone) and positive (Shiga toxin alone) controls for each treatment.

Neurotoxic activity. Neurotoxicity was assayed after intraperitoneal injection of various amounts (0.1 to 0.5 μg per mouse) of toxin in 200 μl of PBS into C57/BL mice (5 to 10 mice per group). Cumulative death was recorded at 3, 5, and 7 days after toxin injection. The 50% lethal doses (LD_{50}s) were calculated by the method of Reed and Muench (26). The observed LD_{50} was 8.5 μg/kg.

Neutralization of neurotoxicity was determined by incubating the toxin with a 1:10 dilution of the tested antiserum for 30 min at 37°C, before the intraperitoneal injection of the mixture into the mice. Injections of toxin alone and PBS alone were used as positive and negative controls, respectively.

RESULTS

Synthesis of antigenic peptides of the Shiga toxin B subunit. To identify the regions to be synthesized, computerized plots of the hydrophilicity pattern of the B subunits and the regions containing high amounts of surface residues were derived from the amino acid sequence (29), as described by Hopp and Woods (12, 13). We found that the N-terminal region contains a peak in which both high hydrophilicity and high amounts of surface residues coincide. Three peptides were synthesized; these were related to sequences 5 to 18, 13 to 26, and 7 to 26 of the B-chain molecule, which are referred to as peptides 5-18, 13-26, and 7-26, respectively (Fig. 1). In all the peptides Thr replaced Lys in position 13 because of an error in the sequence at the time of synthesis. The peptides were purified by gel filtration on Sephadex G-15 and analyzed for their amino acid content. The results (Table 1) show good agreement between the expected and observed values of the various amino acid residues.

Preparation and characterization of conjugates. Each of the three peptides was conjugated to tetanus toxoid and to the synthetic copolymer pDGDL. Two of the peptides were conjugated also to the branched synthetic polymer (T,G)-A--L. The contents of the coupled peptide in each conjugate was determined by amino acid analysis, in comparison with the composition of the carrier alone. The characteristics of the various conjugates are listed in Table 2. The number of moles of peptide attached per mole of carrier ranged between 20 and 38.

Antibodies to the synthetic peptides. Each of the conjugates described above and polymers of the peptides were used for immunization of four rabbits, and all the antisera were evaluated by ELISA for their interaction with the respective homologous peptide and the intact Shiga toxin. The titer ranges of the various antisera with the intact toxin are given in Table 2. For each peptide the serum of the rabbit manifesting the highest reactivity with the homologous peptide and cross-reactivity with intact toxin was used in all further studies. In the case of the anti-5-18 peptide, the highest titer was obtained in a rabbit immunized with the conjugate, with pDGDL used as a carrier, whereas in the cases of peptides 13-26 and 7-26, the highest responses were induced by the glutaraldehyde polymers of the peptides.

TABLE 2. Characterization of conjugates

Peptide	Carrier	Coupling reagent	Content (mol/mol) of peptide	Titer[a] of antiserum (range)
5-18	Tetanus toxoid	EDCI[b]	36	1:90–1:270
	(T,G)-A--L	EDCI	38	ND[c]
	Poly-D-Glu-D-Lys	EDCI	26	1:210–1:810
	Polymerized	Glutaraldehyde	M_r, 60,000	1:30–1:60
7-26	Tetatanus toxoid	EDCI	35	1:200–1:810
	Poly-D-Glu-D-Lys	EDCI	20	1:90–1:270
	Polymerized	Glutaraldehyde	M_r, 60,000	1:220–1:810
13-26	Tetanus toxoid	EDCI	34	1:270
	(T,G)A--L	EDCI	33	1:60–1:150
	Poly-D-Glu-D-Lys	EDCI	20	1:20–1:90
	Polymerized	Glutaraldehyde	M_r, 80,000	1:180–1:810

[a] Dilution of serum leading to 50% binding in ELISA in a reaction with the intact Shiga toxin.
[b] EDCI, 1-Ethyl-3(3'-dimethylaminopropyl)carbodiimide hydrochloride.
[c] ND, Not done.

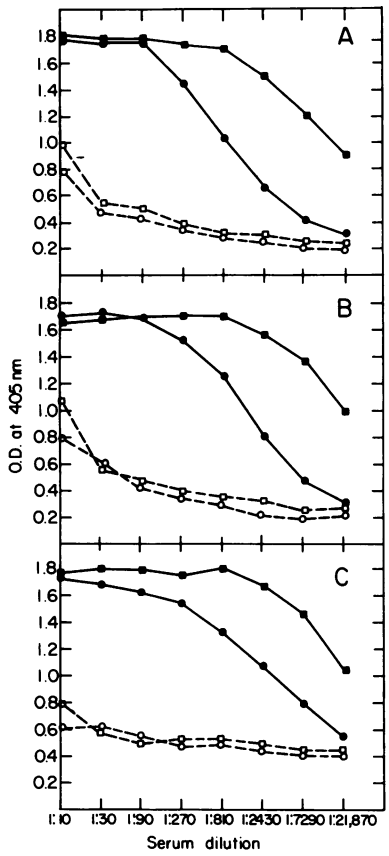

FIG. 2. Interaction (ELISA) of the antisera against the conjugate 5-18–pDGDL (A) and against the polymers of peptides 13-26 (B) and 7-26 (C) with the respective homologous peptide (■) and the intact Shiga toxin (●). The analogous controls with the preimmune sera are indicated by broken lines for reactivity with the respective peptide (□) and the Shiga toxin (○). OD, Optical density.

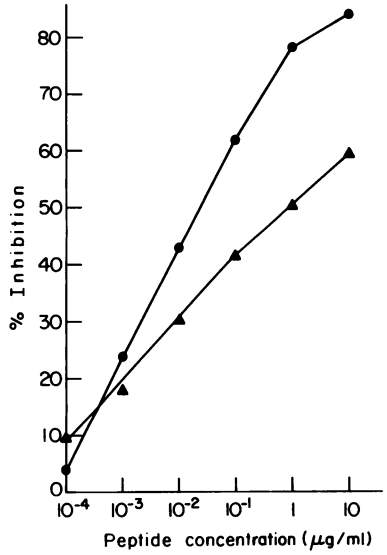

FIG. 3. Inhibition of the ELISA reaction between anti-5-18–pDGDL serum (1:150 dilution) and the homologous peptide by increasing amounts of peptide 13-18 (▲) and the homologous peptide 5-18 (●).

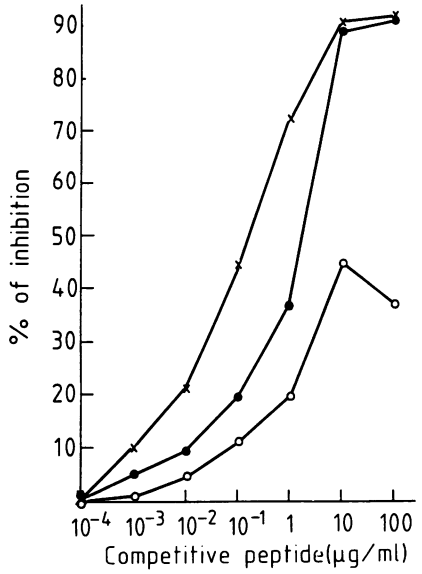

FIG. 4. Inhibition of the homologous ELISA reaction of peptide 7-26 by peptides 5-18 (○) and 13-26 (●) and the homologous peptides 7-26 (×).

Conjugates with (T,G)-A--L induced antisera with lower titers and were not evaluated further.

(i) ELISA. The reactivity of the antisera against the three peptides assayed by ELISA against both the homologous peptide and the intact Shiga toxin is illustrated in Fig. 2. Each of the antisera reacted with the respective homologous peptide with a titer of approximately 1:10,000 and showed a high cross-reactivity with the intact Shiga toxin, giving a titer of 1:1,000 or higher. The specificity of the homologous reaction was analyzed by inhibition reactions. Peptide 13-18 efficiently inhibited the homologous reaction of peptide 5-18 (Fig. 3), indicating that specificity of this reaction is largely toward the C-terminal, shorter segment of this peptide. Similarly, the homologous reaction of peptide 7-26 was inhibited mainly by the peptide 13-26 region, with the 5-18 segment having less of an effect (Fig. 4). Both peptides overlapped in the peptide 13-18 segment, which apparently dictates the specificity of this region.

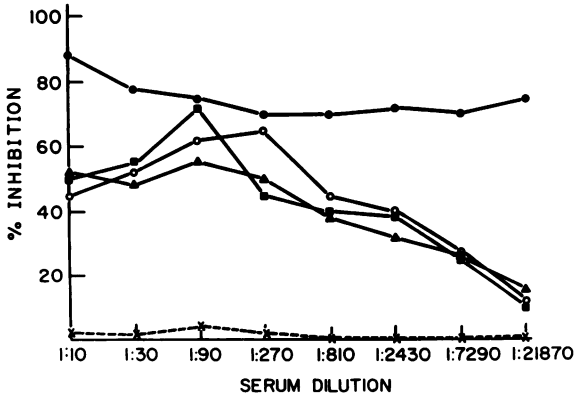

FIG. 5. Inhibition of the cytotoxic effect of Shiga toxin (500 pg per well) on HeLa cells by antisera against Shiga toxin (●), peptide 5-18–pDGDL (■), peptide 13-26 polymer (○), peptide 7-26 polymer (▲), and normal serum (×).

TABLE 3. Neutralization of the enterotoxic effect of Shiga toxin by anti-peptide sera

Antiserum[a]	Wt (g/1-cm loop) (mean ± SD) with[b]:			% Inhibition
	PBS	Toxin only	Toxin-antiserum	
Anti-peptide 5–18 (6)	0.21 ± 0.02	0.63 ± 0.05	0.27 ± 0.02	83
Anti-peptide 13–26 (4)	0.25 ± 0.03	0.40 ± 0.05	0.30 ± 0.06	67
Anti-peptide 7–26 (2)	0.23 ± 0.02	0.58 ± 0.08	0.30 ± 0.01	80
Anti-Shiga toxin (4)	0.20 ± 0.02	0.50 ± 0.05	0.23 ± 0.05	90
Normal serum (4)	0.20 ± 0.02	0.62 ± 0.05	0.54 ± 0.05	19

[a] Values in parentheses are the number of tested loops per treatment.
[b] Intestinal ligated loops, 2 cm long, were injected with 200 μl of PBS, toxin (35 μg/ml), or a mixture of the toxin and a 1:25 dilution of the respective antisera.

(ii) Immunoblotting. The interaction of the anti-peptide sera with the intact Shiga toxin was also determined by immunoblotting by using ^{125}I-labeled protein A. The results (not shown) indicated that both A and B subunits of the toxin were recognized by antiserum against the intact Shiga toxin. The antisera against the synthetic peptides, on the other hand, did not recognize the A subunit, but all reacted strongly with the B subunit.

Neutralization of biological activity of the toxin. The cytotoxicity of the Shiga toxin toward HeLa cells was efficiently inhibited by prior incubation (for 1 h at 37°C) with the antisera against the three synthetic peptides (Fig. 5). The most effective inhibition was brought about by antiserum against intact Shiga toxin, which even at a dilution of <1:20,000 led to >80% reduction of the toxic effect. Antisera against the three synthetic peptides inhibited up to 70% of the cytotoxic activity of the Shiga toxin toward the HeLa cells, but only at the higher concentrations in serum.

The enterotoxic activity of Shiga toxin, as measured by fluid secretion into ligated ileal loops of rats, was also effectively inhibited by the antisera against the three peptides. Antibodies against all these peptides were effective (Table 3). Anti-peptide 5-18 showed the highest activity, exhibiting essentially the same level of neutralization as anti-Shiga holotoxin.

Similar results were obtained when the effect of the anti-peptide sera on the neurotoxic activity of Shiga toxin in C57/BL mice was evaluated (Table 4). In most experiments, mice exposed to approximately 2 LD_{50}s of Shiga toxin (0.35 μg per mouse) died within 7 days. Thus, in nine different experiments, the mortality level in control groups injected with the toxin alone was about 80%. However, prior incubation of the toxin with a 1:10 dilution of the antisera against the various peptides led to neutralization of its toxic effect, resulting in increased survival of the mice. When the effect of the anti-peptide antisera was compared with that of antiserum against the intact Shiga toxin (taken as 100%), relative neutralization values of up to 60% were observed, with the highest effectivity exhibited by the antiserum against peptide 7-26.

Anti-toxin effect of immunization with the peptide conjugates. Not only were antisera induced by the three peptides capable of neutralizing the various activities of the toxin, but active immunization of mice with the conjugates of the three peptides led to the development of partial protection against the lethal effect of the Shiga toxin. In nonimmunized mice 96% mortality was reached 7 days after they were exposed to the lethal dose (2 LD_{50}s) of the toxin (0.35 μg in C57/BL mice) (Table 5). In contrast, in mice immunized with the conjugates of the three peptides and exposed to equal doses of toxin, the mortality rate was much lower. The best protection was obtained by immunization with a polymer of peptide 13-26. In this case, mortality was only 20%, with 80% long-term survival; and in two of five experiments no mortality was observed at all.

DISCUSSION

The suitability of the use of synthetic peptides for the induction of antibodies with neutralizing activity against disease-causing agents has been demonstrated previously for several bacterial and viral systems, including at least two bacterial toxins, namely, diphtheria toxin (1) and cholera toxin (16). In the last few years considerable effort has been devoted to understanding the nature of protein antigenicity, to allow a more reliable prediction method for choosing sequences suitable for the production of chemically synthesized immunogens (33). A number of computer programs have been suggested for the prediction of regions with high exposure, based on polarity scales, in which high local hydrophilicity (12, 27), flexibility (17, 35), or surface location (13) along the primary sequence of the protein has been evaluated. In the present study we used a topological analysis of the Shiga toxin B-subunit molecule based on the program of Hopp and Woods (13) and focused on regions in which peaks of high hydrophilicity and high surface exposure coincided.

The three peptides synthesized in the present study corresponded to overlapping sequences in the N-terminal region of the B subunit, but they contained a threonine residue

TABLE 4. Neutralization of neurotoxic activity of shiga toxin by anti-peptide

Antiserum	No. of expts	Total no. of mice	% Mortality on[a]:			% Relative neutralization
			Day 3	Day 5	Day 7	
None	9	100	20 ± 11.0	65 ± 8.8	79 ± 7.0	0
5–18	5	70	9 ± 5.5	50 ± 4.5	67 ± 7.7	18
13–26	6	80	12 ± 8.3	26 ± 11.5	43 ± 18	55
7–26	2	30	0 ± 0	20 ± 0	40 ± 0	60
Shiga toxin	3	30	0 ± 0	13 ± 4.5	14 ± 3.5	100

[a] Values represent mean ± standard error.

TABLE 5. Protective immunization against the neurotoxicity of Shiga toxin

Immunizing agent	No. of expts	Total no. of mice	% Mortality on[a]:			% Protection
			Day 3	Day 5	Day 7	
None	9	100	15 ± 4.4	75 ± 8.0	96 ± 3.3	0
5–18 conjugates	7	90	19 ± 7.6	58 ± 7.8	59 ± 7.3	38
13–26 derivatives (all)	9	120	9 ± 4.8	33 ± 8.8	40 ± 9.4	58
13–26 polymer	5	60	4 ± 4.0	16 ± 7.4	20 ± 8.9	79
13–26 TT[b]	4	50	15 ± 9.5	55 ± 9.5	65 ± 5.0	32
7–26 conjugates	3	40	13 ± 13.3	53 ± 12.6	60 ± 11.5	37

[a] Values represent mean ± standard error.
[b] TT, Tetanus toxoid.

(instead of the correct lysine) at position 13. It is worth noting that, notwithstanding this Thr → Lys switch, the antibodies induced by these peptides recognized a structure very similar to that assumed by the corresponding regions in the intact toxin. This is indicated by the high cross-reactivity of the antisera with the toxin in the ELISA and the immunoblot test, and even more so by the considerable neutralization capacity of several biological effects of the Shiga toxin exerted by the antibodies elicited by the synthetic peptides. The anti-peptide antibodies (at a dilution of 1:100) led to about 70% inhibition of HeLa cell cytotoxicity, almost equal to that effected by antibodies against whole Shiga toxin in a similar dilution. The higher inhibitory capacity of whole Shiga toxin was evident only at higher dilutions.

Anti-peptide antisera also neutralized the enterotoxic and neurotoxic activities of the toxin, albeit not as effectively as the anti-holotoxin did (Tables 3 and 4). Although the mechanism of neurotoxicity is unclear, cytotoxicity and enterotoxicity are believed to follow binding of the B subunit to an N-linked glycoprotein on HeLa cells (3) or to a glycolipid (Gb3) on the intestinal cell membrane (14), respectively. It is not known yet whether the N terminus of the toxin B subunit represents the binding epitope for both of these receptors or whether the antibody binding inhibits receptor interactions by steric effects. It was previously observed that a B-chain-specific monoclonal antibody produced the same effect (3).

The most promising aspect of the results of this study is the protective efficacy against the lethal neurotoxic effect of Shiga toxin that is brought about by active immunization with the peptide conjugates (Table 5). We consider these results to be important for the rational design of peptide vaccines. It should be borne in mind, however, that although our long-term goal is the development of an effective vaccine for Shiga toxin-producing organisms, to which the present observations are relevant, there are several reasons to remain cautious. First, the actual role of the toxin in the pathogenesis of shigellosis is uncertain. Second, protection by natural infection or experimental live oral vaccine strains in this disease is species and type specific, indicating the importance of general antibacterial mechanisms. Third, parenteral immunization of rhesus monkeys with Formalin-treated Shiga toxin, which resulted in high toxin-neutralizing antibody titers in serum, failed to protect them from oral challenge with virulent shigellae (23). Fourth, the presence of antitoxin antibody to Shiga toxin in serum has not protected human volunteers experimentally challenged with live virulent *Shigella* organisms (M. M. Levine and G. T. Keusch, unpublished data). Thus, it seems likely that if antitoxin immunity is to be effective, it must be expressed at the mucosal surface of the gut. Preliminary data from trials with cholera B-subunit immunogens, which suggest that this is possible (31), are encouraging, so work with Shiga toxin synthetic peptide antigens should be continued.

It has been suggested that somatic antigens of *Shigella* species which are associated with its virulence could serve as the basis for a genetically engineered live vaccine for oral use (7). The availability of a synthetic peptide(s) that has no toxic effect of its own and that, yet, can elicit protection against the damaging and lethal effects of the toxin could offer an important additional ingredient that should be considered in the design of an efficient recombinant *Shigella* vaccine.

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