PATHOGENESIS OF SHIGELLA DIARRHEA

IX. Simplified High Yield Purification of Shigella Toxin and Characterization of Subunit Composition and Function by the Use of Subunit-specific Monoclonal and Polyclonal Antibodies

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For over 80 years now, *Shigella dysenteriae* 1 has been known to produce one of the most potent of the lethal microbial toxins. It was originally called Shiga toxin (after the discoverer of the organism, K. Shiga) and classified as a neurotoxin because it results in a delayed-onset limb paralysis terminating in death when parenterally administered to sensitive animals (reviewed in reference 1). Shigella toxin is also cytotoxic to certain tissue culture cells, as well as enterotoxic (results in fluid secretion) when applied to intestinal mucosa (2–5). Biochemical and immunological evidence indicate that the three biological activities are the properties of the same molecule (3, 5). Its role in the pathogenesis of shigellosis has always been controversial, in part because other species of the genus could not be shown to produce the same toxin. This argument is no longer valid, for both *S. flexneri* and *S. sonnei* have been found to produce shigella toxin under appropriate in vitro conditions, and convalescent patients develop an antibody that neutralizes the *dysenteriae* 1 toxin (6–8). Pathogenic bacteria of other genera have also been found to produce a similar toxin that is neutralized by antibody to shigella toxin. These organisms include a variety of *E. coli* serotypes including human enteropathogenic strains, the causative strain of human hemorrhagic colitis (*Escherichia coli* 0:155), the noninvasive rabbit pathogen RDEC-1, human *Salmonella* strains, and even *Vibrio cholerae* (9–11). The cross-reactive toxin has been dubbed "Shiga-like toxin." Since these *E. coli* strains do not produce the well-known LT or ST toxins and since a mutant strain of *V. cholerae* deleted of the gene for the production of the ADP-ribosyl transferase enzyme subunit A of cholera toxin (12) causes diarrhea in humans, the shigella (or Shiga-like) toxin may well be a critical virulence factor in diarrheal disease.

Because of these observations, there is great interest in this toxin and the immunologically related products of other organisms. Shigella toxin has recently (9–15) been purified and partially characterized by several laboratories. The

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toxin consists of two separate polypeptide chains, a larger A chain and a smaller B chain. These polypeptides associate noncovalently to form a complex consisting of one A chain and multiple B chains, although the precise stoichiometry is not certain. While it is known that the A chain inhibits protein synthesis in vitro, apparently by catalytically inactivating the eukaryotic 60S ribosomal subunit (16), the presumed function of the B subunit to mediate the binding of toxin to glycoprotein receptors (17) at the target cell surface has not been directly demonstrated. In fact, isolated shigella toxin B chains do not bind to toxin-sensitive cell lines (14, and our unpublished observations).

In the present study, we report the characterization of shigella toxin obtained by a rapid, high yield purification scheme. In addition, we present studies with subunit-specific, polyclonal and monoclonal toxin-neutralizing antibodies which indicate that the B subunit is involved in toxin binding to cell surface receptors. This method and the reagents developed should help to better define the nature of the Shiga-like toxin of *E. coli*, *Salmonella* sp., and *V. cholerae*.

Materials and Methods

Materials. Casamino acids and Freund's complete and incomplete adjuvants were obtained from Difco Laboratories, Detroit, MI. Blue Sepharose CL-6B, Sephadex G-25, cyanogen bromide–activated Sepharose 4B, protein A, Polybuffer exchanger 94 and Polybuffer 96 were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Bio-Gel P-60, all reagents for sodium dodecyl sulfate (SDS)[1] polyacrylamide gel electrophoresis, and Zeta-Probe paper were from Bio-Rad Laboratories, Richmond, CA. Pristane was obtained from Aldrich Chemical Company, Milwaukee, WI. Chloramine T, Na metabisulfite, rabbit hemoglobin, ovalbumin, human transferrin, and bovine serum albumin were obtained from Sigma Chemical Company, St. Louis, MO. Fixed *Staphylococcus aureus* (IgGsorb) was purchased from The Enzyme Center, Malden, MA, and dimethyl pimelimidate (DMP) was from Pierce Chemical Company, Rockford, IL. $Na^{125}I$ (17.4 Ci/μg) was purchased from New England Nuclear, Boston, MA. All tissue culture media were from Gibco Laboratories, Grand Island, NY.

Bacterial Strain and Culture Conditions. *S. dysenteriae* 1 strain 60R was used for all studies reported. This strain is a noninvasive, avirulent, toxigenic rough mutant originally isolated by Dubos and Geiger (18). The culture medium used was a modified syncase broth (19) containing 1% casamino acids, 0.004% tryptophan, and 0.2% glucose. This medium contained an optimal concentration of iron (~0.1 μg Fe^{+++} per ml) for maximal toxin production in culture (1).

Toxin Purification. A 1% overnight bacterial inoculum was added to the medium and the cultures were grown aerobically with shaking at 300 rpm at 37°C. Bacterial cultures were harvested in early stationary growth (A_{600} = 3–3.5) and chilled to 4°C for the remainder of the isolation procedure. Cells were pelleted by centrifugation at 10,000 *g* for 10 min and washed twice by resuspension in 10 mM Tris-HCl, pH 7.4, followed by centrifugation. The final cell pellet was suspended in wash buffer to 1/50th of the original culture volume and lysed by sonication using a Branson Sonifier (model 184; Branson Sonic Power Co., Danbury, CT) until >95% lysis was achieved, measured by following the absorbance at 600 nm. Unbroken cells were then pelleted by centrifugation for 20 min at 5,000 *g* in a Sorvall GSA rotor (DuPont Instruments, Wilmington, DE).

The crude cell lysate was applied at room temperature to a column (2.5 × 50 cm) containing Cibacron Blue F3G-A coupled to Sepharose CL-6B (Blue Sepharose), equilibrated with 10 mM Tris-HCl, pH 7.4. The flow-through was continuously recycled for 12 h, when the column was washed with 10 column volumes of 10 mM Tris-HCl, pH 7.4.

[1] *Abbreviations used in this paper:* DMP, dimethyl pimelimidate; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline, pH 7.4.

Bound material was then eluted with the same buffer containing 0.5 M NaCl. Fractions from the salt elution containing protein were detected by absorbance, pooled, and dialyzed against 25 mM Tris-acetate, pH 8.3. The material was then applied to a column (0.9 × 20 cm) of Polybuffer exchanger 94 equilibrated with 25 mM Tris-acetate, pH 8.3. Elution of bound material was initiated with a degassed solution of Polybuffer 96 diluted 1:13 in water and adjusted to pH 6.0 with acetic acid. Fractions of 1.5 ml were collected, the pH determined, and cytotoxin activity assayed in HeLa cells after adjustment of pH to 7.4.

Fractions containing cytotoxin were pooled, transferred to a dialysis bag, and incubated with dry polyethylene glycol (M_r 20,000) at room temperature to reduce the volume to 2 ml. The concentrated cytotoxin was then applied to a column (1.5 × 100 cm) of Bio-Gel P-60 equilibrated with 20 mM ammonium bicarbonate. Cytotoxin was eluted in the same buffer, and the toxin containing fractions were pooled and lyophilized.

Separation of A and B Subunits. Shigella toxin A and B subunits were separated by a modification of the procedure of Lai et al. (20). 100–200 μg of purified toxin containing tracer amounts of ^{125}I-labeled toxin were dissolved in 0.2 ml of 5% formic acid and applied to a Bio-Gel P-60 column (0.9 × 18 cm). Fractions of 0.4 ml were eluted with 5% formic acid at a flow rate of 2 ml/h. Fractions containing the A and B subunits were identified by SDS-polyacrylamide gel electrophoresis, and were separately pooled and lyophilized. To resuspend the lyophilized subunits, we initially solubilized them in 10 mM Tris-HCl, pH 7.4, containing 8 M urea. The solutions were then transferred to dialysis tubing and dialyzed against the same buffer. The concentration of urea in the dialysis buffer was gradually diluted to 0.8 M over 4 h by the addition of urea-free 10 mM Tris-HCl, pH 7.4. The subunits were then dialyzed against the latter buffer.

Radioiodination. Protein was radiolabeled with ^{125}I using a modification of the chloramine T procedure (21). One mCi of carrier-free Na^{125}I was added to 10–20 μg of toxin in 150 μl of 0.1 M sodium phosphate, pH 7.4. 20 μl of a 2.5 mg/ml solution of chloramine T was added and, after a 20 s incubation, 20 μl of a 5 mg/ml solution of sodium metabisulfite was added. Rabbit hemoglobin (100 μg) was added as a carrier protein and bound and unbound label were separated on a 10 ml Sephadex G-25 column.

Protein Determinations. For protein determinations we used the Bio-Rad assay kit II (Bio-Rad Laboratories) with bovine serum albumin as standard.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in 15-cm slab gels, 1.5 mm thick. The SDS gel system described by Dharmalingam and Goldberg (22) was used. Gels containing ^{125}I-labeled protein were dried and exposed at −70°C to Kodak XRP X-omat film with an intensifying screen.

Cross-linking. Protein samples were cross-linked with DMP using a modification of the procedure of Brew et al (23). Protein solutions were dialyzed against 0.2 M triethanolamine, pH 8.5. DMP-HCl was dissolved in 0.2 M triethanolamine buffer to a concentration of 10 mg/ml, and the pH was adjusted to 8.5. 30 μl of protein solution containing up to 10 μg of protein and 6 μl of DMP was incubated at 37°C for 1 h. The reaction was terminated by the addition of glycine to a final concentration of 0.2 M.

Analysis of Amino Acid Composition. Samples containing ~10 μg of the purified toxin protein were hydrolyzed in 6 N HCl *in vacuo* for 24 h at 110°C and analyzed using a D-500 amino acid analyzer (Durrum Instrument, Sunnyvale, CA). No attempt was made to analyze for either tryptophan or cysteine.

Development of Polyclonal Rabbit Antiserum to Shigella Toxin. To neutralize the lethal neurotoxin activity, purified shigella toxin was converted to a toxoid by formalin treatment. Samples of toxin containing 100 μg of protein were treated for 3 d at 37°C with 0.1 M sodium phosphate, pH 8, containing 1% formalin. The protein was then dialyzed against phosphate-buffered saline (PBS). The toxoid contained <1% of the original cytotoxin activity. Initially, 100 μg of toxoid protein in 0.5 ml PBS was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into a New Zealand White rabbit at four sites. 5 wk after the initial immunization a booster injection of 50 μg toxoid in Freund's incomplete adjuvant was administered. Identical booster doses were given at 5-wk intervals. Bleedings for antiserum were performed 8–10 d after each booster injection. Antiserum was stored at −70°C.

Isolation of Anti–Subunit B Rabbit Antibodies. Cyanogen bromide–activated Sepharose 4B (0.2 g) was washed on a sintered glass filter with four 25-ml aliquots of 1 mM HCl. The washed gel was added to 50 μg of toxin subunit B dissolved in 0.1 M sodium bicarbonate, pH 8.3, containing 0.5 M NaCl. The suspension was placed on a rocking platform and incubated at 4°C overnight. To block remaining active groups on the Sepharose, the gel was transferred to 5 ml of 0.2 M glycine, pH 8.0. After a second overnight incubation at 4°C, the gel was washed five times, alternately with 0.1 M acetate, pH 4.0, containing 0.5 M NaCl, and 0.1 M sodium bicarbonate, pH 8.3, containing 0.5 M NaCl. The subunit B–Sepharose 4B gel (0.5 ml wet volume) was suspended in 0.1 M sodium bicarbonate, pH 8.3 and poured into a 0.5-cm diam column.

To minimize nonspecific binding of the immune serum, 3 ml of nonimmune rabbit serum was applied to the column of subunit B–Sepharose 4B. After passage of the nonimmune serum, 0.5 ml of rabbit antitoxin serum was applied to the column. The immune serum was allowed to interact with the gel matrix for 1 h at room temperature. The column was then washed with 50 ml of 0.1 M sodium bicarbonate, pH 8.0, containing 0.5 M NaCl. To dissociate anti–subunit B antibodies from the gel-protein complex, the column was washed with 50 mM glycine-HCl buffer, pH 2.7. The column effluent was collected in 0.5-ml fractions and immediately neutralized by the addition of Tris base. The elution of antitoxin antibody was measured by the ability of the fractions to immunoprecipitate 1 ng of ^{125}I-labeled shigella toxin (~30,000 cpm). Antibody-toxin complexes were immunoprecipitated using fixed protein A–positive *Staphylococcus aureus* by the procedure of Kessler (24).

Production and Screening of Monoclonal Antitoxin Antibodies. 4–8-wk-old female BALB/c mice were injected subcutaneously with 20 μg of toxoid in complete Freund's adjuvant. 1 mo later they were boosted subcutaneously with 20 μg of toxoid in incomplete Freund's adjuvant. 3 wk later, the mice were intravenously boosted with the same dose of toxoid in PBS via the tail vein. 4 d later mice were sacrificed by cervical dislocation and spleen cells were prepared and fused to P3x63Ag8.653 myeloma cells at a ratio of 2:1 by the procedure of Kohler and Milstein (25). Fused cells from one animal's spleen were distributed among five 24-well plastic trays containing BALB/c spleen cells as a feeder layer. After 2 wk in culture, supernatants were analyzed for toxin-specific antibody by immunoprecipitation of ^{125}I-toxin. Strongly positive cultures were expanded and cloned twice by limiting dilution. Ascites fluid was prepared from stable hybridoma cell lines by injecting 5×10^6 cells intraperitoneally into BALB/c mice that had been primed 2 wk earlier by intraperitoneal injection of 0.5 ml of Pristane.

Western Blot Analysis of Antibodies. The subunit specificity of the antitoxin antibodies was analyzed using Western blotting (26). Toxin (20–50 μg) was subjected to SDS-polyacrylamide gel electrophoresis in 15% acrylamide slab gels. The dissociated protein subunits were then electrophoretically transferred (2 h at 300 mA) to a Zeta-Probe filter. The buffer used in the transfer was 15.6 mM Tris–120 mM glycine, pH 8.3. The filter was then incubated for 16 h at 50°C in 0.1 M Tris–HCl, pH 7.4, 0.9% NaCl (Tris-saline) containing 10% bovine serum albumin (BSA). Next, the filter was incubated overnight at 4°C with a 1:100 dilution of either hybridoma ascitic fluid or rabbit serum, and then washed twice by rocking for 10 min in Tris-saline. In the case of two non–protein A-precipitating monoclonal antibodies, 5D2 and 5B2, the filter was incubated for 2 h at room temperature with a 1:100 dilution of rabbit anti-mouse IgG serum and then washed for two 10-min periods with Tris-saline. To visualize antibody bound to antigen, the blot was incubated for 2 h at room temperature in Tris-saline containing 5% BSA and 5×10^5 cpm/ml of ^{125}I-labeled protein A. After this incubation, the filter was washed extensively in Tris-saline, air-dried, wrapped in plastic wrap, and developed by autoradiography.

Assay for Toxin and Antitoxin Activities. Cytotoxin was assessed with a cytolethal assay using HeLa cells in monolayer culture in microtiter plates as previously described (27). Anticytotoxin was determined by a neutralization assay and percent inhibition was calculated from the dose-response curve of cytotoxin on HeLa cell monolayers, as previously described (6). Enterotoxicity was assayed in ligated rabbit ileal loops as the fluid volume/length ratio response (28), and neurotoxicity was measured as the LD_{50} in

20-g Swiss-Webster mice after intraperitoneal injection of dilutions of toxin in 10 mM Tris-HCl, pH 7.4 (2).

Results

Shigella Toxin Purification. Toxin was purified from a total cell lysate of washed early stationary phase *S. dysenteriae* 1, strain 60R. As a first step, we used chromatography on a column containing Blue Sepharose and salt (0.5 M) elution as described by Olsnes et al. (14). This step gave an initial 8–12-fold purification of toxin with a recovery of 80–90% of starting activity. Next, toxin was subjected to chromatofocusing, using a pH gradient of 8.3–6. The peak of cytotoxic activity appeared at an eluate pH of 7.0–7.1 (Fig. 1), and resulted in a 100-fold purification with a 50–60% yield of activity. The third and final step of this purification was molecular sieve chromatography on Bio-Gel P-60 to remove minor protein contaminants as well as the ampholytes present in the chromatofocusing elution buffer.

The yield and the increase in specific activity for each step in the purification is shown in Table I. 0.8 mg of toxin was obtained from a 3 liter culture, representing a final yield of ~50% of initial cytotoxic activity. Fig. 2 shows an SDS–polyacrylamide gel electrophoresis analysis of the peptides obtained during each step of the purification scheme. After the Bio-Gel P-60 step, two major bands were observed (Fig. 2*E*), corresponding to molecular weights of 32,000 and <10,000. These two peptides have been found by others and have been referred to as the toxin A and B subunits, respectively (14). In addition to the major subunits, a third minor peptide (M_r 28,000), corresponding to the A_1 fragment of the A subunit described by others (14, 15) was observed when the SDS gels were run under reducing conditions (Fig. 2*E*).

Purified shigella toxin was cytotoxic to HeLa cells (3.4×10^7 TC_{50} per

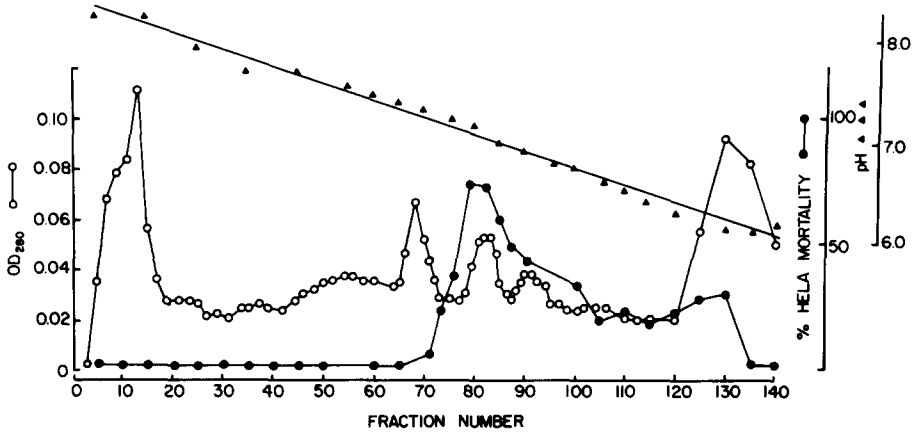

FIGURE 1. Chromatofocusing of partially purified shigella toxin. Fractions from the Blue Sepharose salt elution containing protein were dialyzed against equilibration buffer and were chromatofocused as described in Materials and Methods. The column flow rate was 9 ml/h and 1.5-ml fractions were collected. HeLa cell cytotoxicity was measured using a 10^{-4} dilution of the fractions. Tubes 75–88 containing the peak of cytotoxin activity were pooled, concentrated, and subjected to molecular sieve chromatography.

TABLE I

Purification of Shigella Toxin from a Whole Cell Lysate of S. dysenteriae 1

Purification step	Total protein	Cytotoxin specific activity (TC_{50})	Increase in specific activity (fold)	Yield
	mg			%
Cell lysate	2,300	2.5×10^4	—	100
Blue Sepharose eluate	165	3.0×10^5	12	86
Chromatofocusing, pH 7.1 peak	1	3.1×10^7	1,240	54
Bio-Gel P-60	0.8	3.4×10^7	1,360	47

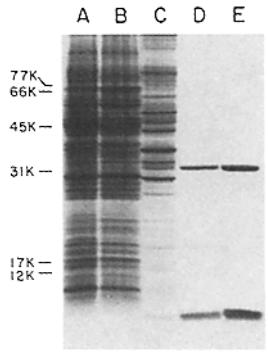

FIGURE 2. SDS-polyacrylamide gel electrophoresis of the different stages of toxin purification. All samples were dissolved in SDS sample buffer containing β-mercaptoethanol. Samples were heated in boiling water for 10 min before applying to a 15% acrylamide gel. (*A*) Whole cell lysate of *S. dysenteriae* 1; (*B*) Blue Sepharose flow-through; (*C*) Blue Sepharose salt eluate; (*D*) pI 7.1 region from the chromatofocusing step; (*E*) the final product after chromatography on Bio-Gel P-60. The molecular weight standards (LKB) were transferrin (M_r 77,000), bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000), myoglobin (M_r 17,000), and cytochrome *c* (M_r 12,000).

milligram protein [tissue culture LD_{50} ratio]) and also possessed both neurotoxin and enterotoxin activities. In six rabbits tested, injection of >150 ng of purified toxin into ligated ileal loops resulted in maximal fluid accumulation of 1.8 ml/cm of ileum. A half-maximal response was obtained with 30 ng toxin. During the purification, the ratio of cytotoxicity (TC_{50}) to enterotoxicity (maximum secretory dose) remained constant at ~1,000:1. Intraperitoneal injection of 10 ng of the purified toxin into mice resulted in the characteristic hind limb paralysis and neurotoxic death in 7 of 12 mice tested.

Subunit Composition. To determine whether the two observed bands associated with one another and to investigate the actual subunit composition of the native toxin, we carried out chemical cross-linking experiments. We used the bifunctional reagent DMP to form covalent complexes of the subunits, using ^{125}I-labeled toxin (~30,000 cpm/ng) to amplify the sensitivity of the detection procedure. The cross-linked products were separated by SDS–polyacrylamide gel electrophoresis and detected by autoradiography. In addition to the 32,000 and <10,000 mol wt peptides, a series of new species appeared (Fig. 3). The molecular weights of these peptides and their probable subunit composition are

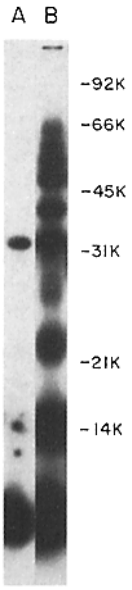

FIGURE 3. SDS-polyacrylamide gel electrophoresis of cross-linked and non–cross-linked shigella toxin. ^{125}I-labeled toxin was cross-linked as described in Materials and Methods in the absence of reducing agents. An autoradiogram of a 15% acrylamide gel is shown. (*A*) ^{125}I-shigella toxin; (*B*) cross-linked iodinated toxin. The molecular weight markers (Bio Rad) were phosphorylase b (M_r 92,000), bovine serum albumin (M_r 66,000, ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000)), soybean trypsin inhibitor (M_r 21,000), and lysozyme (M_r 14,000).

TABLE II
Molecular Weights and Tentative Composition of Cross-linked Complexes of Shigella Toxin

Estimated M_r	Most probable subunit composition
64,000	A + 5B
57,000	A + 4B
51,000	A + 3B
45,000	A + 2B
39,500	A + 1B
32,000	A
27,000	4B
22,500	3B
14,000	2B
<10,000	B

presented in Table II. The lower molecular weight peptides are approximate multiples of 6,500, consistent with their being cross-linked dimers, trimers, and tetramers of the B subunit. Peptides >32,000 mol wt appear to be composed of the M_r 32,000 A subunit plus multiples of 6,500, suggesting that they were derived by crosslinking the A subunit with the monomer, dimer, trimer, tetramer, and pentamer of the smaller B subunit. We were unable to detect a separate band corresponding to a B pentamer, probably because of the nearly identical

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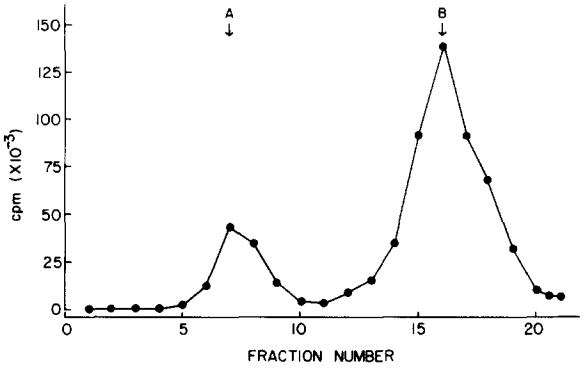

FIGURE 4. Separation of toxin A and B subunits. ^{125}I-labeled toxin (6×10^5 cpm) was treated with 5% formic acid and the dissociated subunits were separated on a Bio-Gel P-60 column as described in Materials and Methods.

TABLE III

Amino Acid Compositions of the Subunits from Shigella Toxin

Amino acid	Subunit A* (residues/mol)	Subunit B‡ (residues/mol)
Asp/Asn	33	10
Thr	28	6
Ser	30	2
Glu/Gln	16	5
Pro	7	1
Gly	25	6
Ala	21	2
Val	22	5
Met	7	1
Ile	16	3
Leu	29	5
Tyr	7	2
Phe	16	4
His	8	1
Lys	4	5
Arg	24	2

* Based on estimated M_r of 32,000.

‡ Determined by normalizing the values of methionine, histidine, and proline to one residue per mole.

molecular weights of such a complex and the A subunit. We conclude that the toxin A subunit (M_r 32,000) associates with five B subunits (~M_r 6,500) to produce the holotoxin of 64,000 mol wt.

Separation of A and B Subunits. Although quantities <100 ng/ml of ^{125}I-labeled toxin could be dissociated into A and B subunits with 10 M urea, dissociation was incomplete when higher concentrations of toxin (1–2 mg/ml) were used. However, an alternative procedure, treatment with 5% formic acid, was successful. Fig. 4 shows the gel filtration profile of toxin dissociated in this manner. Neither the isolated A nor B subunits bound to HeLa cells, and they failed to cause measurable HeLa cell cytotoxicity (data not shown). The subunits

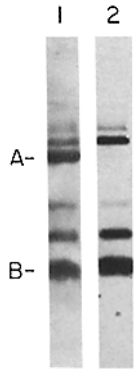

FIGURE 5. Immunoblots of shigella toxin demonstrating subunit specificity of the rabbit polyclonal antibodies. Shigella toxin (10 μg/lane) was dissolved in SDS sample buffer without reducing agents. To increase the probability of detecting an antibody reaction with nondissociated toxin A-B or B-B complexes, the samples were applied to a 15% acrylamide gel without prior heating. The protein transfer and antibody treatment were performed as described in Materials and Methods. The immune reactions were detected by ^{125}I-labeled protein A and subsequent autoradiography. Lane *1*, rabbit polyclonal antitoxin antibody; lane *2*, purified rabbit anti–B subunit antibody eluted from an subunit B–Sepharose 4B column prepared from the antibody in lane *A* as described in Materials and Methods.

TABLE IV

Immunoglobulin Class and Immunoprecipitation Titer of Subunit-specific Shigella Toxin Antibodies

Antibody	Immunoglobulin class*	Subunit specificity	Immunoprecipitation titer‡
Monoclonal 5B2	IgG1	A	1:800
Monoclonal 5D2	IgG1	A	1:1600
Monoclonal 4D3	IgG1	B	1:6400
Rabbit antitoxin	—	A + B	1:40000
Rabbit anti–subunit B	—	B	1:1600

* Immunoglobulin class was determined by Ouchterlony immunodiffusions in agarose using mouse isotype-specific antisera.

‡ Immunoprecipitation titer was determined by measuring the dilution of antibody from which 10 μl immunoprecipitates >80% of added ^{125}I-labeled toxin (3×10^4 cpm).

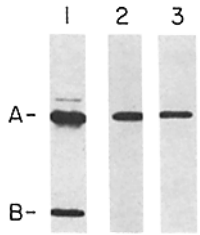

FIGURE 6. Immunoblots of shigella toxin demonstrating subunit specificity of two antitoxin monoclonal antibodies. The procedure was identical to that described in the legend to Fig. 5, except that toxin samples were heated in a boiling water bath for 10 min prior to application to the SDS-polyacrylamide gel. Lane *1*, rabbit polyclonal antibody; lane *2*, mouse 5B2 monoclonal antibody; lane *3*, mouse 5D2 monoclonal antibody.

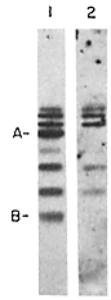

FIGURE 7. Immunoblots of cross-linked shigella toxin demonstrating subunit specificity of the mouse monoclonal antibody 4D3. Conditions were identical to those in Fig. 6 except that toxin was cross-linked with DMP before SDS–polyacrylamide gel electrophoresis. Lane *1*, rabbit polyclonal antibody; lane *2*, mouse 4D3 monoclonal antibody.

TABLE V

Neutralization of Cytotoxin by Polyclonal and Monoclonal Antibodies

Sample	Source of antibody	Subunit specificity of antibody	HeLa cell mortality	Percent toxin neutralization
			%	
Medium	—	—	0	—
Toxin	—	—	59.2	—
Toxin	Rabbit antitoxin	A + B	34.1	>99
Toxin	Rabbit anti–subunit B	B	30.0	>99
Toxin	Monoclonal 5B2	A	34.0	>99
Toxin	Monoclonal 5D2	A	29.7	>99
Toxin	Monoclonal 4D3	B	11.8	>99

Toxin, 1 ng/ml in McCoy's 5a modified medium, was preincubated at 37°C for 1 h with a 1:100 dilution of rabbit polyclonal antitoxin, a 1:10 dilution of anti–subunit B rabbit antibody, or a 1:10 dilution of ascites fluid from the hybridomas 5B2, 5D2, or 4D3. After preincubation, 0.2 ml of the antibody-toxin mixture was inoculated onto HeLa cell monolayers (20,000 cells) in duplicate wells of a 96-well tissue culture plate. After overnight incubation, cells remaining attached to the wells were counted and percentage mortality was calculated. The addition of a 1:100 dilution of normal rabbit serum or a 1:10 dilution of an unrelated ascites fluid had no toxin-neutralizing activity.

obtained in this fashion were subjected to amino acid analysis (Table III). No amino sugars were present in either subunit.

Subunit Specificity of Shigella Toxin Antibodies. To eliminate the lethal effects of the toxin, we immunized animals with formalin toxoid. We obtained high titer polyclonal rabbit antiserum capable of immunoprecipitating toxin A and B subunits (Fig. 5 and Table IV). B subunit–specific polyclonal antibody was purified from this rabbit serum by affinity chromatography as described in Materials and Methods. The immunoblot analysis with this purified antibody (Fig. 5) demonstrates that it binds only to complexes containing the B subunit.

We isolated three monoclonal neutralizing antibodies against shigella toxin. Two (5D2 and 5B2) showed specificity by immunoblot analysis for the toxin A chain (Fig. 6). The third monoclonal, 4D3, did not react with either the A or monomeric B subunit. However, when the toxin subunits were cross-linked and subjected to immunoblot analysis using the 4D3 antibody, the monoclonal reacted with all B subunit–containing complexes but not with the B monomer

TABLE VI
Ability of Antibodies to Neutralize Prebound Cytotoxin

Sample	Source of antibody	Subunit specificity of antibody	HeLa cell mortality	Percent toxin neutralization
			%	
Medium	—	—	0	—
Toxin	—	—	55	—
Toxin	Rabbit antitoxin	A + B	31.8	>99
Toxin	Rabbit anti–subunit B	B	55	0
Toxin	Monoclonal 5B2	A	45.5	90
Toxin	Monoclonal 5D2	A	36.6	98
Toxin	Monoclonal 4D3	B	54.7	6

Toxin, antibody, and HeLa cell monolayers were all prechilled to 4°C. 0.2 ml of a 1 ng/ml solution of toxin was inoculated on HeLa cell monolayers in duplicate wells of a 96-well tissue culture plate. After a 1 h incubation at 4°C, the cell supernatants were removed and the cells washed three times with cold McCoy's medium. After removal of unbound toxin, 0.2 ml of each antibody diluted 1:10 in the culture medium was added to the wells. The cells were incubated at 4°C for 1 h and then transferred to 37°C. The following day, the cells remaining attached to the wells were counted and the percentage mortality was calculated. No cytotoxin-neutralizing activity was detected with either normal rabbit serum or with an unrelated ascites fluid.

TABLE VII
Effect of Antibodies on Toxin Binding to HeLa Cell Monolayers

Sample	Antibody subunit specificity	cpm/10^5 cells*	Percent inhibition
Toxin	—	38,525	—
Toxin + rabbit antitoxin	A + B	2,480	94
Toxin + rabbit anti–subunit B	B	1,352	96
Toxin + 5B2	A	14,895	61
Toxin + 5D2	A	18,530	52
Toxin + 4D3	B	855	98

^{125}I-toxin (2×10^4 cpm, 1 ng) in McCoy's culture medium was incubated at 37°C for 1 h with a 1:100 dilution of rabbit polyclonal antitoxin serum, with 1:10 dilution of the rabbit antisubunit B antibody or with a 1:10 dilution of the monoclonal antibodies. The toxin antibody mixtures (0.2 ml) were then applied at 4°C to HeLa cell monolayers in duplicate wells of a 96-well tissue culture plate. The monolayers were incubated for 1 h at 4°C and then were washed twice with culture medium and three times with phosphate-buffered saline. The monolayers were then trypsinized and the number of cells per monolayer (~20,000) and radioactivity measured.
* Corrected for the nonspecific binding of toxin to empty wells.

(Fig. 7). The immunoglobulin class and immunoprecipitating titers of these antibodies are shown in Table IV.

Neutralization of Cytotoxicity by Antibody. The ability of the polyclonal and monoclonal antibodies to neutralize cytotoxin activity was investigated in two ways. In one set of experiments, toxin was preincubated at 37°C with antibody and then added to HeLa cell monolayers. Cytotoxicity was measured after an overnight (16 h) incubation. All of the antibodies significantly (25–47%) reduced the observed HeLa cell mortality (Table V). This represents neutralization of >99% of added toxin activity, based on the dose-response curve in HeLa cells

(21). The B subunit–specific monoclonal, 4D3, was the most effective neutralizing antibody under these conditions.

In the second set of experiments, toxin was added to cells prechilled to 4°C. After a 1 h incubation at 4°C to permit binding but not internalization of toxin, antibody was added and incubated for 1 h in the cold. The cells were then warmed to 37°C and cytotoxicity was measured. Under these conditions, the polyclonal rabbit antitoxin and both A-specific monoclonals, 5D2 and 5B2, were significantly neutralizing (Table VI). In contrast, neither of the two B-specific antibodies, the monoclonal 4D3 and the purified rabbit anti–subunit B polyclonal, were able to neutralize the prebound toxin. The effect of these antibodies on binding of ^{125}I-toxin to HeLa cell monolayers is shown in Table VII. All B subunit–specific reagents inhibited the binding of toxin to the HeLa cell by 94% or more. In contrast, the two antibodies with A subunit specificity, 5B2 and 5D2, were much less effective, reducing toxin binding by ~50%.

Discussion

In this paper we present a simple, rapid, high-yielding purification scheme for shigella toxin. In addition to cytotoxin activity, the purified toxin also possessed potent neurotoxin and enterotoxin properties. The cytotoxin and enterotoxin activities copurified, as determined by the ratio of their specific activities. This adds to the evidence that one toxin molecule is responsible for all three biological activities.

On SDS-polyacrylamide gels, the purified toxin consisted of two major components, an A chain of 32,000 mol wt and a B chain of <10,000 mol wt. To investigate the subunit stoichiometry, we used the bivalent reagent, DMP, to cross-link the toxin subunits. Analysis of the cross-linked complexes by SDS–polyacrylamide gel electrophoresis revealed four distinct complexes of molecular weight less than that of the isolated A chain. These are consistent with the position of monomeric, dimeric, trimeric, and tetrameric forms of the B chain, with molecular weights that were approximate multiples of 6,500. Five discrete complexes of >32,000 mol wt were present, also in molecular weight increments of ~6,500. These clearly represent the formation of A-B, A-2B, A-3B, A-4B, and A-5B complexes. Since a complex of five cross-linked B monomers would be expected to have a molecular weight of 32,500 (5 × 6,500), it is unlikely that it would have been distinguished on SDS gels, being obscured by the A chain itself at M_r 32,000. On this basis, we propose that shigella toxin is composed of one A subunit linked to five B subunits, with a molecular weight of 64,000.

The proposed 1A-5B structure differs slightly from that reported by others. Olsnes et al. (14) suggested that shigella toxin consists of one A subunit (M_r 30,500) and six or seven B subunits (M_r 5,000). O'Brien and LaVeck (9) have reported that the toxin is composed of one A subunit (M_r 31,500) and six or possibly more B subunits (M_r 4,000). These discrepancies are probably due to an underestimate of the molecular weight of the B monomer. Further evidence that the molecular weight of the toxin B chain is at least 6,500 can be obtained from the amino acid analysis of the B chain. Normalizing the values of methionine, histidine, and proline to one residue per B monomer resulted in the amino acid composition seen in Table II. Based on this composition, the molecular weight

of the B subunit would be 6,690. Since the number of cysteines and tryptophans was not assessed, this estimated molecular weight must be considered a lower limit.

Most well-studied toxins conform to a general A-B structural model in which the molecules can be separated into an A domain responsible for the biological activity of the toxin and a B domain mediating the binding of toxin to the target cell surface. Reisbig et al. (16) have demonstrated that the A chain of shigella toxin inhibits protein synthesis in vitro, and we have confirmed these data with toxin purified by the method described in this paper (unpublished data). These studies also provide the first evidence that the B subunit is involved in binding to the cell membrane of sensitive HeLa cell targets. We have investigated the effects of subunit-specific antisera on both cytotoxin neutralization and binding. A and B subunit–specific antibodies neutralized the toxin when incubated together before addition to HeLa cell monolayers. In contrast, when shigella toxin was preincubated with HeLa cells at 4°C to allow binding to the cell surface but not internalization (29, 30), the subsequent addition of antisera resulted in neutralization only when there were A subunit–specific antibodies present. When antibodies directed exclusively to the B subunit were used, including a monoclonal antibody (4D3) and a polyclonal rabbit antiserum, there was no neutralization of prebound toxin. This clearly suggests that anti–B subunit antibodies are directed to a binding domain of the toxin. This hypothesis was directly confirmed by assessing the ability of these sera to inhibit binding of ^{125}I-toxin to HeLa cell monolayers. Anti–B subunit sera inhibited binding by 94% or more, whereas A subunit–specific antibodies reduced binding by ~50%. The reasons for this latter result are not certain, but are likely the consequence of kinetic differences in the binding of free toxin compared with toxin-antibody complexes. The data are therefore most consistent with the involvement of the B subunit in receptor binding. They further suggest that antibodies against the B subunit, whether polyclonal or monoclonal, neutralize shigella toxin by preventing its binding to the HeLa cell surface. Once toxin is bound to the cell surface, only antibody against the A subunit is effective.

These studies have clarified the roles of the A and B subunits of shigella toxin as the biologically active (A) and binding (B) subunits. The availability of the purified subunits and subunit-specific antibodies should now facilitate studies to define the mechanism of action of the A subunit as well as the basis of the binding specificity of the B subunit.

Summary

A simple purification scheme for shigella cytotoxin was devised, resulting in high yields (~50%) and a 1,300-fold increase in specific activity compared with the initial crude bacterial cell lysate. The purified toxin was enterotoxic in ligated rabbit ileal loops and neurotoxic when injected into the peritoneal cavity of mice. Measurement of specific activity of cytotoxin and enterotoxin demonstrated that these two toxicities copurify during the fractionation procedure.

On sodium dodecyl sulfate gel electrophoresis, the toxin migrated as two polypeptide subunits, an A subunit of 32,000 mol wt and a B subunit of 6,500 mol wt. Chemical cross-linking experiments demonstrate that the toxin is a

complex consisting of one A and five B subunits with a molecular weight of 64,000.

Polyclonal rabbit anti-toxin and anti–subunit B antisera were produced as well as subunit-specific mouse monoclonal antibodies. All antibodies preincubated with toxin neutralized cytotoxic effects in HeLa cell monolayers. In contrast, only A subunit–specific antibodies were able to neutralize toxin prebound to the HeLa cell surface. Antibody to the B subunit also inhibited binding of ^{125}I-labeled toxin to these cells by 94% or more. These data demonstrate that the B subunit is involved in shigella toxin binding to the cell surface.

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