

# Pathogenesis of *Shigella* Diarrhea

## SERUM ANTICYTOTOXIN ANTIBODY RESPONSE PRODUCED BY TOXIGENIC AND NONTOXIGENIC *SHIGELLA DYSENTERIAE* 1

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**ABSTRACT** The serum antitoxin response to the cytotoxin contained in preparations of *Shigella dysenteriae* 1 (Shiga's bacillus) exotoxin was studied in natural and experimental infections of man. Natural infection resulted in the rapid appearance of toxin-neutralizing antibody, which disappeared some time between 9 and 18 mo after infection. Experimental infection of human volunteers provided the opportunity to study immunoglobulin class of the antibody in sera obtained serially from 7 to 50 days after infection. Neutralizing antibody was present only in the IgM fraction isolated by sucrose density gradient ultracentrifugation. This was confirmed by the use of solid-phase immunofluorescence. Even though the time-course and immunoglobulin class of the antitoxin antibody response was similar to that previously observed for anti-O polysaccharide antibody, the biologically active cytotoxin was shown to be highly susceptible to destruction by proteolytic enzymes.

Sera from subjects infected with a virulent invasive chlorate-resistant Shiga mutant thought to be "nontoxigenic" also contained antibody which was similarly restricted to the IgM fraction. Biologically active cytotoxin was recovered when this mutant organism was grown in liquid media with controlled iron concentration. The mutant cytotoxin was heat labile, neutralized

by antiwild-type cytotoxin antibody, and was separable by isoelectric focusing into two fractions with pI 7.2 and 6.1 like the wild-type toxin.

These studies show that cytotoxin antigen is produced during in vivo infection with Shiga bacilli, resulting in a serum antitoxin antibody response. Without explanation is the restriction of the antibody to the IgM class and lack of evidence for an IgG antibody to the protein cytotoxin. Finally, mutant strain 725, previously designated "nontoxigenic," was shown to produce biologically active cytotoxin in vitro and, in experimentally infected volunteers, to result in a serum IgM antibody similar to that observed during infection with the wild-type strain.

### INTRODUCTION

The pathogenesis of bacillary dysentery due to Shiga's bacillus, *Shigella dysenteriae* 1, has been the subject of recent controversy (1, 2). At the center of this controversy is the importance and role of two virulence attributes of the organism: (a) the ability to invade epithelial cells (1) and (b) to produce exotoxins with cytotoxic (cell death) and enterotoxic (intestinal fluid secretion) properties (2).

Gemski et al. (1) and Levine et al. (3) have followed an imaginative, direct line of investigation to assess the relative importance of these two properties. Their studies have attempted, through genetic manipulation of the organisms, to isolate each of the virulence attributes in different strains and to determine the capacity of the resultant organism to produce intestinal disease in the most relevant intact natural host, man himself.

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These studies, comparing the wild-type invasive/toxicogenic strain of Shiga's bacillus with a noninvasive/toxicogenic *Shigella-Escherichia coli* mutant hybrid, and a laboratory produced invasive mutant thought to be "nontoxicogenic," have strongly supported the central role of invasion in pathogenesis because the noninvasive but toxicogenic strain was avirulent (3). The invasive/nontoxicogenic strain was also capable of causing disease and it was concluded that enterotoxin itself was of minor, if any, importance (1, 3). However, these well-designed studies suffer from a possible flaw: failure to demonstrate toxin production in vitro may mean only that appropriate culture conditions have not been attained. It may still be true that such strains will produce toxin in the intact host or under different conditions in vitro.

Our studies have sought evidence of in vivo toxin production by these various bacterial strains as well as under in vitro conditions. Because virulent shigellae, by dint of their invasive properties, possess a unique delivery system to the intestinal epithelial cell, minute quantities of toxin might be of significance in vivo but insufficient to detect in vitro. We have reasoned that this delivery system might also bring small quantities of toxin into contact with the immune system of the host with a resultant antibody response. Detection of specific antitoxin antibody would thus be evidence of in vivo production of antigenically related bacterial products in the host. Because of the dual nature of the clinical disease caused by shigella species, diarrhea (a secretory process) and dysentery (characterized by colitis and colonic epithelial cell death), there is a rationale to study both the enterotoxic and cytotoxic properties associated with extracellular proteins elaborated by shigella species. Therefore this study was undertaken to determine the serum anticytotoxin immune response during natural and experimentally induced Shiga bacillus dysentery in man.

## METHODS

Exotoxin produced in 1953 from *S. dysenteriae* 1 (strain 60-R) by the method of van Heyningen and Gladstone (4) and stored at  $-20^{\circ}\text{C}$  since then was the gift of Dr. W. E. van Heyningen. This standard preparation of Shiga neurotoxin, like the recently described enterotoxin (2), contained multiple protein bands by polyacrylamide gel electrophoresis and possessed enterotoxic (fluid secretion), neurotoxic (mouse lethal), and cytotoxic (HeLa cell lethal) activities (5). The specific activities per milligram protein of these three biologic properties were: enterotoxin, 1,149 ileal loop 1-ml/cm doses; 16.9 50% mouse lethal doses; and 23,000 50% HeLa cell lethal doses ( $\text{TC}_{50}$ ).

In terms of mouse lethal activity (neurotoxin), these results indicated significant loss of biologic activity during storage. Further deterioration occurred in these studies when the lyophilized toxin was frozen at  $-70^{\circ}\text{C}$  and thawed repeatedly for various experiments, but not when samples were maintained in the freezer until used.

This toxin was used for all anticytotoxin antibody assays, which were performed by the method of Keusch and Jacewicz (6). Duplicate 1:5 dilutions of serum were preincubated with one  $\text{TC}_{50}$  dose of toxin before inoculation onto a HeLa cell monolayer. Observed cell detachment was expressed as the quantity of biologically active toxin necessary to cause equivalent cell death by reference to a dose-response curve. Cytotoxin antibody activity was expressed as the neutralization index (NI),<sup>1</sup> which is the ratio "actual dose applied (nanogram)/effective dose observed (nanogram)." Previous studies (6) have established that an NI of 1.5 or greater, which corresponds to neutralization of at least 25% of applied toxin, is a specific response to Shiga toxin.

Cytotoxic activity present in partially purified *S. dysenteriae* 1 has recently been shown to be resolvable by isoelectric focusing (IEF) into two fractions isoelectric at pH 7.2 and 6.1, respectively (5, 7). Enterotoxic and neurotoxic activities are present only in the pI 7.2 fraction although it is not yet established that a single protein is responsible for all three properties. With respect to the cytotoxin alone additional experiments were performed to assay antibody in human sera (see below) against both pI 7.2 and 6.1 fractions produced by IEF in a sucrose gradient as described by Keusch and Jacewicz (5), followed by Sephadex G-150 chromatography to remove sucrose and ampholine. For comparison, antibody was assayed simultaneously using unfractionated partially purified starting toxin as well. A standard horse antiserum prepared by Shiga et al. for the League of Nations (8) was run in each assay against starting toxin and the pI 7.2 and 6.1 fractions.

Strain 725, a chlorate-resistant *S. dysenteriae* 1 mutant, isolated by Gemski et al. (1) and designated nontoxicogenic in their studies, was grown in 3% low molecular weight peptone broth adjusted to an iron concentration of 0.1  $\mu\text{g}/\text{ml}$ . Cytotoxin elaboration into the medium supernate was studied as previously described for the wild-type strain (2). Toxin was assayed in HeLa cells as described by Keusch et al. (9).

*Patients.* Acute and convalescent sera were obtained from 23 subjects naturally infected during the 1969 epidemic in Guatemala and generously supplied by Dr. Leonardo J. Mata of Institute of Nutrition of Central America and Panama, Guatemala City. Serial serum specimens were collected from fully informed volunteers experimentally infected with the wild-type and variant Shiga bacillus strains at the Maryland House of Correction, Jessup, Md. (3). These sera were coded by number at the University of Maryland School of Medicine for assay of antibody at The Mount Sinai School of Medicine in New York. The code was not broken until all sera had been assayed.

*Separation of immunoglobulins by sucrose density gradient ultracentrifugation.* Discontinuous sucrose gradients were prepared by overlying equal volumes (1.8 ml) of 10, 15, and 35% sucrose in 0.9% NaCl. Sera were diluted 1:2 with 0.9% NaCl and 0.2 ml was then carefully layered on the surface of the gradient. Separation was obtained by centrifugation at 35,000 rpm for 16 h,  $4^{\circ}\text{C}$  in a SW 50.1 rotor, Beckman L2-65 B ultracentrifuge (Beckman Instruments Inc., Fullerton, Calif.). 20 drop fractions were collected from the top of the gradient with the aid of a Buchler Auto-densi flow (Buchler Instruments, Inc., Fort Lee, N. J.) driven by a peristaltic pump. Immunoglobulin was identi-

<sup>1</sup> Abbreviations used in this paper: IEF, isoelectric focusing; MMM-H, McCoy's modified medium-Hepes buffer; NI, neutralization index.

fied in each fraction of each serum studied by double gel diffusion in agarose against monovalent rabbit antihuman IgG, IgA, and IgM (Beringwerke AG, Marburg-Lahn, West Germany). The two to three fractions containing IgM, the following (intermediate) two fractions, and the four to five fractions with strong reactions to anti-IgG were pooled and termed M, I, and G, respectively. IgA was always present in the G fraction but not in M. No IgM was detected in the G fraction whereas IgG was often present in the M fraction, usually as a faint, diffuse precipitin line which extended to the bottom of the gradient, presumably representing IgG aggregates. The three pools were then dialyzed overnight at 4°C against a large volume of 0.9% NaCl and then overnight in the cold against McCoy's 5a (modified) medium containing 25 mM HEPES buffer (MMM-H). 0.4 ml of the dialyzed immunoglobulin fraction was added to 0.1 ml of MMM-H containing 2.5 TC<sub>50</sub> doses of toxin and 50% fetal calf serum. 0.2 ml of the diluted toxin-immunoglobulin solution thus contained 1 TC<sub>50</sub> of toxin in MMM-H and 10% fetal calf serum. The toxin-serum mixture was incubated at 37°C for 30 min, 4°C for 2 h, and then was inoculated onto monolayers of HeLa cells for determination of the NI.

Lapin antisera prepared by hyperimmunizing rabbits with partially purified Shiga toxin (2) were fractionated on sucrose gradients as described above. Goat antirabbit monospecific IgG was purchased from Miles Laboratories, Inc., Elkhart, Ind. Goat monospecific antirabbit IgM was the gift of Dr. Thomas J. Kindt, The Rockefeller University, New York.

*Isolation of immunoglobulin by affinity chromatography.* Rabbit antihuman IgG and IgM (2.5 ml) were dialyzed against 0.02 M phosphate buffer, pH 6.3, and then fractionated by chromatography on 1.2 × 15-cm columns of DEAE-cellulose equilibrated with the same buffer. The protein-containing fractions, identified by measuring absorbance at 280 nm, were tested by double gel diffusion in agarose against normal human serum to confirm specificity of the rabbit antisera and then were lyophilized.

Duplicate 1-g quantities of cyanogen bromide-activated sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) were weighed into 30 ml of 0.001 N HCl. After 30 min, the mixtures were poured into coarse sintered glass funnels and washed with 500 ml of 0.001 N HCl. The lyophilized antisera were dissolved in 5 ml of coupling buffer (0.1 M NaHCO<sub>3</sub> in 2.92% NaCl) and added to the swollen agarose gel. The mixture was rotated end-over-end for 2 h at room temperature. The gels were then washed with additional coupling buffer and added to 8 ml of 1 M ethanolamine pH 8.0 for 2 additional h at room temperature. After this the gels were returned to the sintered glass funnels, subjected to three washing cycles of 0.1 M acetate buffer, pH 4.0 followed by 0.1 M borate buffer, pH 8.0, and finally 0.15 M Na/K phosphate buffer, pH 7.4 (Na/K PO<sub>4</sub>). The final acetate, borate, and phosphate buffer wash fluids were examined spectrophotometrically at 280 nm to be certain that no unbound protein remained in the gel. Columns were poured in plastic 5-ml pipettes, and washed with Na/K PO<sub>4</sub> buffer followed by 0.1 M glycine buffer, pH 2.8. These washes were also examined for free protein eluting from the columns before being used.

Before the actual experiment the capacity of the columns to absorb the specific immunoglobulin was tested by application of 0.5 ml normal human serum and elution with Na/K PO<sub>4</sub> buffer. 5-ml fractions were collected in Na/K PO<sub>4</sub> buffer. Fractions one and two contained the nonadsorbed protein which was shown by double gel diffusion in

agarose to be depleted of the appropriate immunoglobulin. The specific immunoglobulin bound to the column was then eluted in 1.5-ml fractions with glycine buffer, pH 2.8. The protein-containing fractions were studied by double gel diffusion against specific antisera as above.

Because of the small supply of individual sera, a pool of samples previously shown to contain antibody was prepared from two patients infected with wild-type or invasive nontoxicogenic Shiga strains, respectively. 0.5 ml of serum diluted 1:5 with phosphate buffer containing 0.1% bovine serum albumin was applied to the anti-IgG and anti-IgM columns and eluted with 50 ml of phosphate buffer. After this, the bound immunoglobulin was eluted with glycine buffer/0.1% bovine serum albumin. Fractions were analyzed for specific immunoglobulin content by double gel diffusion in agarose. Four samples for each serum pool were thus obtained; IgG-depleted serum, IgM-depleted serum, isolated IgG, and isolated IgM. These fractions were dialyzed against saline overnight, and then against MMM-H containing antibiotics for tissue culture. Antitoxin antibody was tested as above by addition of 0.4 ml of antibody fraction to 0.1 ml MMM-H containing 2.5 TC<sub>50</sub> doses toxin and 50% fetal calf serum.

*Susceptibility of HeLa cell toxin to proteolytic enzymes.* The heat lability of all three biologic activities and the susceptibility of the mouse lethal action to destruction by papain (2, 6) suggested that Shiga toxin is a protein. This question was investigated further for the HeLa cell toxin by determining the activity of trypsin, chymotrypsin, papain, and protease on biologic activity of the same toxin preparation used for the neutralization experiments. To accomplish this, solid-phase immobilized enzyme columns were purchased from Miles Laboratories, Inc. (enzite columns). Total column activity of the agarose-trypsin, agarose-chymotrypsin, agarose-papain, and agarose-protease matrices was 66 U (pH 8.0), 87 U (pH 8.0), 42 U (pH 8.0), and 97 U (pH 8.5), respectively. Columns were activated according to the recommendations of the manufacturer and washed with 0.05 M Tris buffer, pH 7.5. Washings from each column were shown to be nontoxic for the HeLa cell monolayers. A 2-mg/ml stock solution of toxin was prepared, and 1.5 ml was applied to the column and allowed to enter the gel for varying periods of contact. After the desired time had elapsed, the column was flushed with buffer to elute the protein-containing fractions which were identified and pooled. All 280 nm absorbing material eluted in a volume of 3.6 ml. The design of the experiment was to compare HeLa cell activity of toxin applied to and eluted from the column at 4° vs. 37°C. For this purpose, the column, toxin, and buffer were first equilibrated to the desired temperature. A separate sample of toxin was applied to the column for each of the time periods studied; 5, 10, 20, and 30 min. The eluate was tested for residual toxicity by assay of serial log<sub>10</sub> dilutions in MMM-H as described above.

## RESULTS

*Serum antitoxin activity in naturally acquired Shiga dysentery.* 23 sera were available for titration of serum antitoxin antibody collected at various times during the course and convalescence of patients with Shiga dysentery naturally acquired during the 1969 epidemic in Guatemala (Table I). Neutralizing activity was present in serum as early as the 3rd day of clinical illness, and persisted for at least 9 mo. By 18 mo after illness, how-

TABLE I  
Serum Toxin-Neutralizing and Anti-O Polysaccharide-Hemagglutinating Antibody in Shiga Dysentery

Time of serum	Antibody tested	
	Hemagglutination*	Neutralization†
	No. pos/total	No. pos/total
≤1 wk	0/3	3/3
1-4 wk	6/7	6/7
3-9 mo	3/3	3/3
≥18 mo	1/10	1/10

\* Titer ≥1:40.

† NI ≥1.5.

ever, antibody virtually disappeared. Of 10 sera studied at this time, only 1 was positive with a borderline NI of 1.5. The time-course for the appearance and disappearance of neutralizing antibody paralleled that observed for anti-O polysaccharide-hemagglutinating antibody specific for *S. dysenteriae* 1. However, there was no correlation between the two antibody activities in individual sera.

*Serum antitoxin activity in experimentally induced Shiga dysentery.* Volunteer studies, undertaken as part of a Shigella vaccine development program at the University of Maryland, provided opportunity to prospectively study the development of serum toxin-neutralizing activity. Pre- and serial postchallenge sera were obtained in volunteers orally inoculated with wild-type Shiga bacillus (isolated in Guatemala in 1969) and were studied for neutralizing activity (Fig. 1). Sera from 7 of 8 men before ingestion of the organism, were negative, but all had significant rises in NI by day 7. The titers were subsequently maintained for at least 50 days thereafter. Significant serum toxin-neutralizing activity did not prevent overt disease in the single volunteer with a positive prechallenge serum.

*Response to challenge with noninvasive/toxigenic mutant/hybrid S. dysenteriae 1.* Levine et al. (3) have reported that a noninvasive colonial variant of the wild Shiga strain hybridized with *E. coli* K-12 may be safely fed to man in large numbers, even though the organism can produce toxin in vitro. 12 volunteers fed this organism did not develop serum toxin-neutralizing activity after challenge. Invasiveness, therefore, can be associated with virulence as well as induction of serum toxin-neutralizing activity.

*Response to challenge with invasive nontoxigenic Shiga mutant.* Gemski et al. (1) and Levine et al. (3) were able to isolate a chlorate-resistant Shiga mutant, designated strain 725, which failed to produce detectable toxin in vitro, but retained the virulence hallmark of invasiveness. When 10<sup>8</sup> organisms were fed to volun-

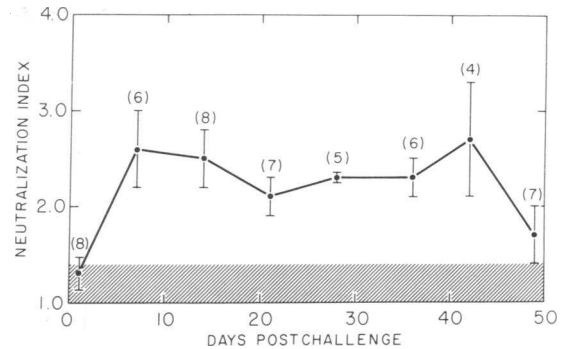


FIGURE 1 Antitoxin antibody titers (NI) in individuals challenged with virulent wild-type *S. dysenteriae* 1. Numbers in parentheses indicate the number of sera at each time point. Shaded area indicates the range of normal values and brackets show  $\pm 2$  SEM.

teers by Levine et al. (3) typical but mild disease developed in half of the volunteers. The serum neutralizing activity in these men is shown in Fig. 2. Compared to the wild strain, 725 infection was associated with a delayed rise in toxin neutralizing activity. 3 wk were required to develop titers comparable to those observed within 1 wk after challenge with the wild-type parent organism. Thereafter the height and duration of the antitoxin antibody response was equivalent for both organisms.

*Antibody response to Shiga toxin and purified fractions obtained by IEF.* Table II reports observed mortality of HeLa cell monolayers exposed to toxin alone or toxin preincubated with various sera. Both antibody negative and positive sera from volunteers challenged with wild-type and the invasive/nontoxigenic strain 725 were studied. Sera previously negative for antibody were again negative when tested against the starting toxin, and pI 7.2 and 6.1 fractions. Previously positive

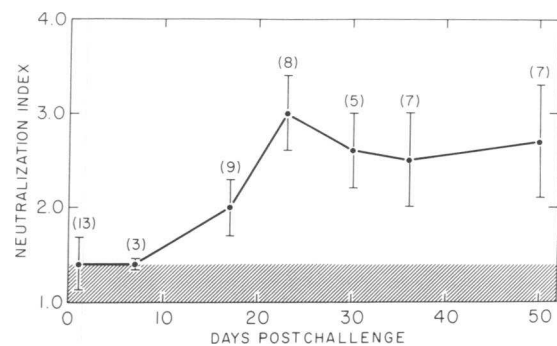


FIGURE 2 Antitoxin antibody titers (NI) in individuals challenged with a virulent laboratory induced mutant nontoxigenic *S. dysenteriae* 1 (strain 725). Numbers in parentheses indicate the number of sera at each time point. Shaded area indicates the range of normal values and brackets show  $\pm 2$  SEM.

TABLE II  
Serum Toxin-Neutralizing Antibody against Partially Purified Shiga Toxin  
and Fractions Obtained by IEF

Serum source	Number of samples	HeLa cell mortality toxin preparation		
		Unfractionated	pI 6.1	pI 7.2
None (Control)	4	51.9±0.87‡	51.4±0.89	51.0±0.22
International standard*	4	31.8±1.23	31.5±1.71	32.7±2.67
Anti-dysentery antiserum				
Wild-type Shiga infection				
NI negative	5	51.6±0.95	50.7±0.52	50.4±0.85
NI positive	5	44.5±2.99	43.5±2.66	43.2±2.01
Strain 725 Shiga infection				
NI negative	5	52.2±0.69	51.5±0.45	51.6±0.71
NI positive	9	44.3±2.64	43.8±2.52	43.9±2.37

\* Final dilution 1:50 compared to 1:5 dilution for all human sera.

‡ Mean±1 SEM.

sera were again positive. There were no significant differences between antibody activity or positive sera against the starting toxin or the two IEF fractions. Horse antibody (International Standard Anti-dysentery Serum) produced by hyperimmunization with toxin contained considerably more toxin-neutralizing activity than the positive human sera.

*Isolation of immunoglobulin fractions by sucrose density gradient centrifugation.* Fig. 3 shows the stability of the neutralizing activity to one freeze-thaw cycle. Although a number of sera with relatively low NI (1.5–2.0) lost activity with thawing, these data indicated that further studies of stored sera with NI  $\geq 2.0$  were feasible.

Two lapin antitoxin antisera were fractionated by sucrose density gradient ultracentrifugation. Complete separation of IgG and IgM was obtained. IgA was not specifically identified but would be expected in the

IgG fraction under these conditions. Toxin neutralization by the whole sera, and isolated G and M fractions is shown in Table III. All activity was recovered in the M fraction.

18 sera from volunteers infected with wild-type Shiga (Fig. 4A) and 45 sera from individuals fed the 725 strain (Fig. 4B) were similarly analyzed. Neutralizing activity was again recovered only in the IgM fraction. The localization was sharp; in no instance did the intermediate fractions or the IgG rich fraction G have any neutralization capacity. There was a highly significant ( $P < 0.001$ ) correlation between the NI of whole serum and the IgM fraction prepared from the same sample.

These data strongly suggested that the serum toxin-neutralizing activity which developed in the course of Shiga bacillus dysentery was, in fact, an antibody. Furthermore, for at least 50 days after challenge all detectable neutralizing antibody activity was recovered in the IgM fraction of serum. Even rabbits hyperimmunized with partially purified toxin preparations developed neutralizing antibody which was found only in the IgM fraction.

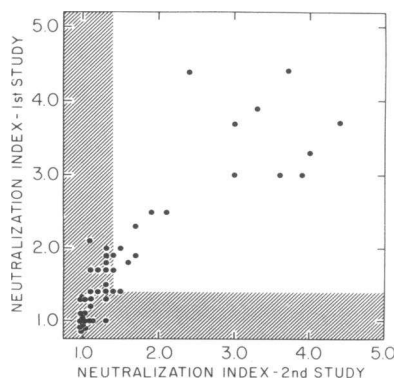


FIGURE 3 Correlation of repeat antitoxin antibody assay of human convalescent sera after freeze-thawing of samples. Shaded areas indicate the range of normal values.

TABLE III  
NI of Whole Sera and Isolated Immunoglobulin  
Fractions Obtained from Experimental  
Rabbit Anti-Shiga-Toxin Antisera

	Rabbit 1	Rabbit 2
Whole serum	2.5	3.9
M fraction	2.3	2.5
G fraction	1.0	1.0

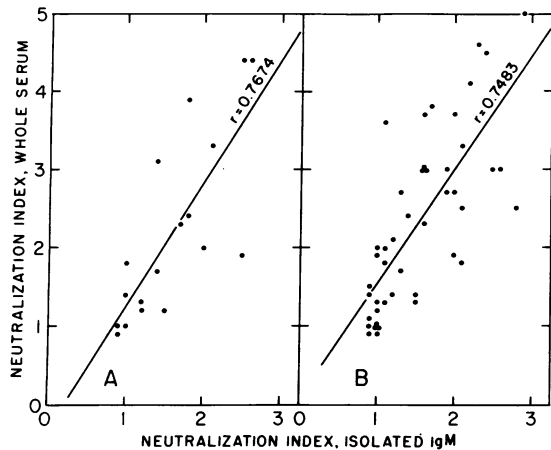


FIGURE 4 Correlation of antitoxin antibody activity in whole human sera and in IgM isolated from the same sample by sucrose density gradient ultracentrifugation. Panel A (left) shows results obtained with sera from subjects infected with wild-type *S. dysenteriae* 1. Panel B (right) shows data from sera taken after infection with a virulent laboratory induced mutant nontoxicogenic *S. dysenteriae* 1 (strain 725).

*Identification of antibody class by solid-phase affinity chromatography.* Sera taken from one volunteer from 4 to 7 wk postchallenge with wild-type Shiga bacilli were pooled (pool 168–171). A second pool (192–195) was made from sera taken at the same times after challenge with the invasive/nontoxicogenic mutant strain 725. These serum pools were passed through the immuno-adsorbant columns containing immobilized rabbit anti-human IgG or IgM as described. Antibody activity of the specific immunoglobulin-depleted fractions and the eluted, isolated IgG and IgM is shown in Table IV. Depletion of IgG had no effect on antitoxin activity whereas removal of IgM completely removed the neutralizing factor. IgM isolated from the 192 to 195 pool had some residual antibody activity, but it was evident that the procedure of attachment to and elution from the column significantly altered biological activity.

TABLE IV  
NI of Whole Sera and Fractions Obtained by Affinity Chromatography on Immunoglobulin Adsorbant Columns

Sample	NI	
	Wild-type Shiga	Nontoxicogenic Shiga 725
Serum pool	2.9	3.7
IgG depleted	2.7	2.6
IgM depleted	1.0	1.0
Isolated IgG	1.0	1.0
Isolated IgM	1.0	1.5

*Cytotoxin production by chlorate-resistant Shiga strain 725.* Because IgM anticytotoxin antibody was detected in volunteers infected with the apparently nontoxicogenic Shiga strain 725, additional attempts were undertaken to detect biologically active toxin using low molecular weight peptone broth with controlled iron content. Under these conditions cytotoxin was recoverable from the medium supernate in four separate experiments. This material was heat labile and neutralized by antibody prepared to the wild-type toxin. Preliminary fractionation on Sephadex G-150 revealed peak activity in the fraction eluting at  $K_{av}$  0.25–0.41 (Table V). Calibration of this column with ovalbumin (mol wt 45,000 daltons) and ribonuclease (mol wt 13,700 daltons) demonstrated that these two proteins eluted at  $K_{av}$  0.34 and 0.69, respectively. Thus a peak of cytotoxin activity elutes from the column in the region expected for wild-type toxin. The considerable activity still present in the later eluting fractions suggests a second peak is present as well with molecular weight somewhat greater than ribonuclease, as previously demonstrated for the wild-type organism (5).

Sephadex G-150 passed crude toxin (encompassing fractions eluting at  $K_{av}$  0.28–0.76) was subjected to IEF in a sucrose gradient column. Fractions were pooled according to pH (based on previous experience with wild-type toxin), dialyzed against 0.02 M NH<sub>4</sub>

TABLE V  
Cytotoxicity Assays of Toxin Preparations from Mutant *S. dysenteriae* 1 Strain 725

Molecular sieve chromatography (Sephadex G-150)	$K_{av}$				
	0.1–0.24	0.28–0.41	0.45–0.59	0.62–0.76	
50% tissue culture lethal dose/mg protein	>500	0.17	0.69	1.85	
IEF (Sucrose gradient column)	Fraction pH				
	7.2	6.9	6.4	6.1	5.7
HeLa mortality, (%)/ $\mu$ g dry wt	42.9	33.2	25.2	44.2	25.9

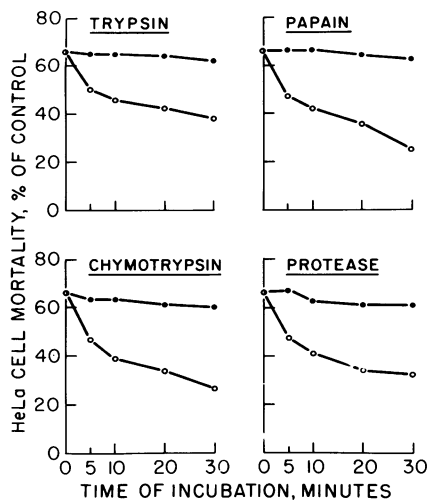


FIGURE 5 Effect of proteolytic enzymes on biologic activity of *S. dysenteriae* 1 enterotoxin. Toxin was interacted with solid-phase immobilized enzymes in agarose columns at 4°C (closed circles) and at 37°C (open circles). A separate sample of the same toxin preparation in 0.05 M Tris buffer, pH 7.5 was applied to each column for each of the time periods indicated and eluted in the same buffer.

HCO<sub>3</sub> buffer, lyophilized, and assayed for cytotoxicity. Two peaks of HeLa cell toxin were found at pH 7.2 and pH 6.1 (Table V), similar to the toxin produced by the wild-type strain. The isolate of strain 725 used to produce this cytotoxin was restudied with respect to its genetic marker, chlorate resistance, and it was confirmed that reversion to or contamination by the wildtype strain had not occurred.

*Sensitivity of HeLa cell toxin to proteolytic enzymes.* Relatively little loss of biologic activity occurred when wild-type Shiga toxin was interacted for up to 30 min at 4°C with proteolytic enzymes immobilized on agarose columns. In contrast, significant loss of biological activity occurred if the reaction was carried out on the same columns at 37°C (Fig. 5). In order of increasing efficacy, trypsin, protease, chymotrypsin, and papain were all capable of inactivating the toxin. By reference to a simultaneous dose-response curve, calculations showed that over 99% of all biologic activity was destroyed by each of the columns when incubated at 37°C for 30 min.

## DISCUSSION

The studies described in this paper demonstrate several points of direct relevance to the possible role of *Shigella* cytotoxin in the pathogenesis of Shiga bacillus dysentery. First of all, serum neutralizing activity against the HeLa cell cytotoxicity of Shiga toxin rapidly develops after the onset of clinical disease. Secondly, this serum factor is an antibody of the IgM type. Thirdly, there is no evidence of a shift in the immunoglobulin class of the antibody from IgM to IgG.

Rather, antibody disappears from serum after a few months. A similar type of serum antibody response to the O polysaccharide antigen of the Shiga bacillus has been described (10). However, the cytotoxin used in these studies is heat labile (9) and destroyed by proteolytic enzymes. In addition, no correlation is apparent between the anti-O and neutralizing antibody activities. Fourthly, and most cogent to the main argument, a laboratory mutant (strain 725) derived from wild-type Shiga bacillus which does not produce readily detectable toxin in vitro (1) also elicits a serum IgM anti-cytotoxin antibody response in volunteers developing clinical illness after oral challenge with the organism. The present studies, which for the first time demonstrate cytotoxin production in vitro by this strain when iron content in the growth medium is controlled and a highly sensitive assay for cytotoxicity is used (9), offer an explanation for the observed antibody response. Preliminary characterization of this toxin does not indicate any difference from cytotoxin produced by the wild-type organism with respect to heat lability, antibody neutralization, or fractionation by IEF. Further studies are necessary to characterize the 725 toxin with respect to other biologic activities associated with wild-type Shiga toxin, that is enterotoxin and neurotoxin.

Wild-type *S. dysenteriae* 1 exotoxin has not been completely characterized. The three biologic activities originally associated with a single protein peak isolated by molecular sieve chromatography, namely enterotoxin, neurotoxin, and cytotoxin have recently been shown to be present in the same fraction at pH 7.2 after IEF in a sucrose gradient column (5) or in polyacrylamide gels (7). These studies have also identified a second cytotoxic fraction at pH 6.1 without enterotoxic or neurotoxic activities. Similarly, a second later eluting cytotoxin without neuro- or enterotoxicity has also been obtained by molecular sieve chromatography (5). Using a calibrated Sephadex G-100 column, the approximate molecular weight of the smaller peak was found to be 20,000 daltons compared to 40,000 daltons for the fraction with all three biologic activities. The precise relationship between these two cytotoxic proteins, and the identity of the three biologic activities in the pI 7.2 fraction as one or more separate molecular species remains to be determined. The present study shows that subjects infected with invasive wild-type Shiga bacilli or the chlorate-resistant 725 mutant develop antibody which neutralizes one TC<sub>50</sub> dose of the isolated pI 7.2 and 6.1 cytotoxins to an equivalent degree. Therefore, either the two cytotoxins are separate proteins produced in vivo as they are in vitro, or the smaller cytotoxin is an antigenically related fragment of the larger molecule.

These studies have relied upon assay for cytotoxicity in the investigation of the antitoxin antibody response of humans with shigellosis due to Shiga's bacillus for two principle reasons. First of all, of the three biological activities associated with partially purified Shiga toxin, cytotoxicity is the most precise, reproducible, and sensitive assay (9, 11). *Vibrio cholerae* and *E. coli* enterotoxins applied to HeLa cell monolayers are not very active in causing cell detachment, and antisera prepared against these toxins do not neutralize the cytotoxicity of Shiga toxin. Although the enterotoxin of *Clostridium perfringens* type A contains a potent cytotoxin activity, this can be differentiated from that of Shiga toxin by antibody studies since there is no cross-neutralization by anti-Shiga and anti-perfringens antisera (12). Similarly, assay for anticytotoxin antibody is specific and reproducible (6, 12) although freeze-thawing of stored sera does further reduce the activity of relatively low titer sera. The second reason is that although we had intended to assay these sera for antineurotoxin and/or antienterotoxin antibodies as well, direct determination of this has not been possible, in part because of limited volumes of patient sera. In addition, we have not been able to consistently find antineurotoxin antibody except in high titered antisera such as the International Antidysentery Serum Standard,<sup>2</sup> possibly because of diminished sensitivity of the intact animal assays in comparison to the tissue culture microassay. We have performed, been limited to the study of anticytotoxin which demonstrates that volunteers infected with either the wild-type or mutant 725 Shiga organisms develop neutralizing antibodies against both IEF cytotoxins to the same degree as they do to the unfractionated Shiga toxin. Taking all of these data into consideration, demonstration of anti-Shiga cytotoxin antibodies in cell culture with human convalescent sera represents a specific antitoxin response to the homologous antigen. It should be stressed, however, that we are not discussing the question of protective antitoxin antibodies, but simply a specific serologic response which indicates exposure to an antigen during *S. dysenteriae* 1 infection in man.

While the rapid development of serum cytotoxin-neutralizing activity within a few days of the onset of clinical symptoms is perhaps explainable by the fact that the early antibody is an IgM immunoglobulin, the observation that the antibody is always IgM, even 50 days after infection, is unusual for known antitoxin antibodies. Ourth and Edsall (13) studied the antibody response of rabbits to tetanus toxoid and both 7S and 19S antibodies were apparently demonstrable. However, antitoxin activity of whole serum was not susceptible to treatment with 2-mercaptoethanol. Because the unit

<sup>2</sup>Keusch, G. T., and D. Seminer. Unpublished observations.

activity of the 7S fraction was 500-fold greater than the 19S fraction, the authors pointed out that less than 1% contamination of the 19S fraction by the 7S antibody could account for the small amount of 19S antitoxin detected. Therefore it is unclear if there was in fact an IgM antitoxin produced at all. Newcomb and Ishizaka (14), studying the antibody response of man to diphtheria toxin found both IgG and IgA antitoxins but no evidence of IgM antitoxin with a sensitive radio-immunodiffusion technique. Ourth, also studying human diphtheria toxin-neutralizing antibody, found activity only in the IgG fraction isolated by DEAE-chromatography (15). In the present study no anti-Shiga cytotoxin-neutralizing antibody could be found in the IgG/IgA sucrose gradient fractions of human convalescent sera. Rather, all demonstrable neutralizing antibody was in the IgM fraction. One possible explanation for these data may be that Shiga cytotoxin is a complex glycoprotein with the protein moiety mediating biologic activity and a carbohydrate antigenic site giving rise to toxin-neutralizing antibodies. However, this cannot be resolved until more purified toxin is available for study. Certainly the unusual nature of the serum antibody response argues against the cytotoxin being a simple protein molecule.

Independent of any question of identity of the antigenic and active site of Shiga cytotoxin, the development of specific neutralizing anticytotoxin antibodies during clinical Shiga bacillus dysentery is evidence that at least an antigenic fragment of toxin which can activate the humoral immune system is produced in vivo. Since IgM-producing plasma cells are present in the lamina propria of the gut it is possible that these cells are responding to antigen being brought to them by the invading virulent bacilli. The delayed rise in antibody in men fed this strain could be construed as evidence of reduced capacity for cytotoxin production by this strain in vivo as well as in vitro compared to the response observed for the wild-type Shiga bacillus. Our preliminary studies with strain 725 indicate that the yield of cytotoxin assayed in the HeLa system is less than that for wild-type toxin produced under the same conditions. Differences in multiplication and/or invasiveness of the mutant strain could also explain the apparent delayed antibody rise in the patients.

Finally, it is clear that it is no longer strictly correct to refer to the mutant 725 strain as an invasive/non-toxicogenic organism. On the basis of the present data, strain 725 is certainly capable of producing at least a cytotoxin which is antigenically related to the cytotoxin elaborated by the wild-type bacillus. Although the question of enterotoxin and neurotoxin production remains unanswered, the argument against a role of toxins in the pathogenesis of Shiga bacillus dysentery



based on the virulence of the 725 strain must be questioned and re-examined. While epithelial cell invasion is of unquestioned importance in the pathogenesis of shigellosis, it is premature to conclude that it is the sole virulence characteristic of the genus.

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