PATHOGENESIS OF SHIGELLA DIARRHEA

VII. Evidence for a Cell Membrane Toxin Receptor Involving $\beta 1 \rightarrow 4$-Linked *N*-Acetyl-D-Glucosamine Oligomers*

BY GERALD T. KEUSCH‡ AND MARY JACEWICZ

(From the Division of Infectious Diseases, Department of Medicine, Mount Sinai School of Medicine, City University of New York, New York 10029)

Shigella dysenteriae 1 (Shiga's bacillus) has two virulence characteristics that may be important in the pathogenesis of shigellosis in man, namely the capacity to invade intestinal epithelial cells and also to produce a protein enterotoxin active on intestinal epithelial cells (1–3). Shiga toxin (ST)[1] is also cytotoxic to HeLa cells in monolayer culture and we have employed this as an assay for investigation of ST activity (4–6). The present study was designed to determine whether or not mammalian cells possess a membrane receptor for Shiga cytotoxin and to characterize the nature of this receptor. The data suggest that mammalian cells do indeed possess a toxin receptor, and that oligomeric $\beta 1 \rightarrow 4$-linked *N*-acetyl-D-glucosamine (D GlcNAc) is in some way involved.

Materials and Methods

Toxin was prepared from *S. dysenteriae* 1, strain 60-R, as previously described (5). This material contained enterotoxic (ileal loop secretion), neurotoxic (mouse lethal) and HeLa cell cytotoxic activities. Cytotoxicity, the most sensitive, quantitative, and reproducible assay, shows that ST may be separated by isoelectric focusing into two cytotoxic fractions, one with isoelectric point (pI) 7.25 (also having the enterotoxic and neurotoxic activities) and the other pI 6.1 (6, 7); both fractions are neutralized to an equivalent degree by experimental antisera versus crude ST and by convalescent human sera from natural and experimental infection (5). The studies reported herein were based entirely upon cytotoxicity assay by the quantitative micromethod of Keusch et al. (4). A single ST preparation which had not been separated by isoelectric focusing was used throughout (5), and all subsequent reference to toxin in this paper is to the cytotoxic activity.

Toxin binding was measured indirectly by means of a consumption assay for toxin. HeLa cell monolayers or isolated rat liver cell membranes were exposed to toxin for varying time periods and conditions, depending on the nature of the experiment. When HeLa cells were studied the supernatant medium was aspirated from monolayers at the end of the experimental period. This

* Supported in part by contract DADA 17-71-C-1042 from the U. S. Army Research and Development Command and grant ROI-AI-13284 from the National Institute of Allergy and Infectious Diseases; presented in part at the National Meeting of The American Federation of Clinical Research, Atlantic City, N. J. 1-2 May 1976.

‡ Recipient of a Research Career Development Award AI-70847 from the National Institute of Allergy and Infectious Diseases.

[1] *Abbreviations used in this paper:* AC, adenylate cyclase; Con A, concanavalin A; D GlcNAc, *N*-acetyl-D-glucosamine; pI, isoelectric point; ΔM, difference in mortality; PHA, phytohemagglutinin; ST, shiga toxin; TC_{50}, 50% lethal dose of toxin; WGA, wheat germ agglutinin.

was immediately inoculated on to fresh HeLa monolayers to determine residual toxicity and assess toxin consumed (bound). Medium without toxin incubated over monolayers, as well as the toxin before exposure to HeLa cells was similarly inoculated on to fresh monolayers. The preincubated monolayers were then washed three times and fed with fresh toxin-free medium to assay toxicity of the cell bound toxin. Cytotoxicity was determined in all monolayers after 20 h at 37°C (4). When consumption of toxin by liver cell membranes was studied the membrane toxin or membrane medium mixture was centrifuged after incubation at 40,000 *g* for 40 min at 4°C to sediment the cell membranes. Residual toxicity was again assayed in the supernate by using fresh HeLa cell monolayers. Toxin not exposed to membranes but otherwise treated in identical manner was also assayed. Incubation and centrifugation did not alter toxic activity.

Removal of cytotoxicity from the medium by HeLa monolayers was found only in association with evidence of toxicity to the cell monolayer (*vide infra*) and was considered to represent binding of toxin to cells. Consumption of toxin from the medium in the presence of liver cell membranes was similarly considered to indicate toxin binding. However, these studies do not permit direct quantitative assessment of actual binding of toxin molecules, which in turn presents a problem for expression of the relative extent of binding under the different experimental conditions used in this study. Therefore, we have simply recorded the difference in mortality (ΔM) due to toxin incubated with cells or membranes compared to toxin incubated in the absence of cells or membranes. Under the conditions employed with a 50% lethal dose of toxin (TC_{50}) the usual ΔM observed with either cells or membranes was about 12%; however, by reference to a dose-response curve this represents binding of over 90% of the toxin present.

Toxicity to HeLa cells in monolayer was also taken to be the result of toxin binding. In experiments designed to characterize the membrane receptor, HeLa cells were pretreated with various enzymes and lectins, or toxin was incubated with potentially competitive inhibitors; a decrease in toxicity after treatment was considered as evidence that the treatment was destroying or competitively inhibiting the toxin receptor. These data are expressed as percent inhibition of binding calculated as:

$$\left(1 - \frac{\text{toxicity (percent M) to treated cells}}{\text{toxicity (percent M) to untreated cells}}\right) \times 100.$$

When rat liver cell membranes were studied in similar experiments, percent inhibition of binding was calculated in a similar fashion by using the ΔM:

$$\left(1 - \frac{\text{toxin uptake } (\Delta M) \text{ by treated membranes}}{\text{toxin uptake } (\Delta M) \text{ by untreated membranes}}\right) \times 100.$$

Rat liver cell membranes were prepared from freshly excised livers of adult Sprague-Dawley rats by the method of Neville (8). Animals were killed by a blow to the head and their livers quickly removed and placed in ice cold 0.25 M sucrose. The tissue was trimmed of fat and connective tissue and finely minced before homogenization in a vol of 50 ml of cold 0.25 M sucrose, employing a Dounce homogenizer (Kontes Co., Vineland, N. J.) with 15 strokes of a loose pestle and 5 strokes of a tight pestle. The homogenate was filtered through two layers of gauze and centrifuged at 600 *g* for 10 min. The supernate was then sedimented at 12,000 *g* for 30 min at 4°C. The resultant supernate was quickly adjusted to 0.1 M NaCl and 0.2 M $MgSO_4$ and centrifuged at 40,000 *g* for 40 min at 4°C. The supernate was decanted and the membranes were resuspended and washed twice in 0.05 M Tris-HCl buffer, pH 7.4, by centrifugation at 40,000 *g*. The final washed membrane preparation was resuspended in 0.05 M Tris and adjusted to a protein concentration of 2 mg/ml by using the method of Lowry et al. (9) with bovine serum albumin as standard. Electron microscopy of the membrane preparations, kindly performed by Dr. Michael Gerber, showed membrane vesicles of varying size, with virtually no intact mitochondria or mitochondrial fragments present.

Crab shell chitin (Sigma Chemical Co., St. Louis, Mo.) was hydrolyzed in acid according to the method of Rupley (10). Chitin oligosaccharides were separated on a charcoal-celite column with a linear 0–60% ethanol gradient (10).

Lysozyme activity was assayed by using lyophilized *Micrococcus luteus* substrate (Sigma Chemical Co.). *M. luteus* was suspended in 0.05 M Tris-HCl buffer, pH 7.4, at a concentration of 0.25 mg/ml and adjusted to yield a suspension with A_{460} of 0.7. 2 ml of this suspension was placed in

a standard 1-cm cuvette and 6 U of lysozyme was added in a vol of 25 μl. The contents were mixed by inversion and the subsequent change in A_{460} was monitored with a recording spectrophotometer for a period of 10 min.

The following chemicals and enzymes were commercially obtained: neuraminidase (*Vibrio cholerae*) from Behring Diagnostics, American Hoechst Corp., Somerville, N. J.; phytohaemagglutinin from Burroughs Wellcome & Co., Greenville, N. C.; trypsin, phospholipase A, galactose oxidase, β-galactosidase, lysozyme, hyaluronidase IV, hyaluronidase V, β-glucuronidase, methyl β-D-galactopyranoside, methyl α-D-galactopyranoside, isopropyl β-D-thiogalactopyranoside, stachyose, D-galactose-β-D-thiogalactopyranoside, raffinose, fetuin, thyroglobulin, ovomucoid, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, *M. luteus*, and concanavalin A from Sigma Chemical Co.; pronase, phospholipase C, *p*-aminophenyl β-D thiogalactopyranoside, galactose, galactosamine, glucosamine, mannose, mannosamine, and wheat germ agglutinin from Calbiochem, San Diego, Calif.; highly purified wheat germ agglutinin from Pharmacia Fine Chemicals Inc., Piscataway, N. J.; α-glucosidase, β-glucosidase, α-mannosidase, and β-glucuronidase from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; and bovine β-lipoprotein (IV-1) and bovine β-lipoprotein (III-0) from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio.

The following reagents were received as gifts: mixed bovine brain gangliosides (Dr. W. E. van Heyningen) and purified crab shell chitin oligosaccharides from dimer (chitobiose) through hexamer (chitohexose) (Dr. J. W. Rupley).

Results

Demonstration of a Toxin Receptor. Fig. 1 shows results of binding experiments by using rat liver cell membranes and toxin diluted to 5 TC_{50} doses/ml. Toxin uptake was directly related to membrane protein added (upper left panel) and on the basis of these studies further experiments were conducted by using a final concentration of membrane protein of 1 mg/ml and 5 TC_{50} doses of toxin/ml. Under these conditions uptake of toxin was directly related to time and temperature of incubation, but inversely related to the ionic strength of the buffer used. Preincubation of membranes with toxin, followed by extensive washing, blocked uptake of toxin during a second exposure (Fig. 2). Toxin inactivated by heating at 90°C for 30 min was far less effective in blocking toxin uptake than the unheated material.

By using three different cell lines, HeLa, WI-38, and mouse Y-1 adrenal cells, toxin binding was found to correlate with observed biological activity (Fig. 3). Only the HeLa cell removed toxin from the medium (right panel) concomitant with a cytotoxic effect on the monolayer (left panel). The persistence of toxicity in the medium overlying the two insensitive cell types was important in validating this indirect consumption assay and as evidence for the presence of a cell membrane receptor for Shiga toxin on susceptible cells.

Characterization of the Receptor. Three basic approaches were used to characterize the membrane receptor. These included (*a*) enzymatic destruction of the receptor, (*b*) competitive inhibition of toxin binding with a variety of sugars, oligosaccharides, and glycoproteins, and (*c*) specific receptor blockade by using lectins with known binding specificities.

ENZYMATIC DESTRUCTION OF THE RECEPTOR. Table I shows the effect of pretreatment of liver cell membranes at 37°C for 30 min with eight enzymes. Receptor activity was reduced by proteolytic enzymes, phospholipases, and lysozyme. The effect of trypsin was inhibited completely by ovomucoid, a potent inhibitor of tryptic activity. Because of its enzymic specificity, the effect of

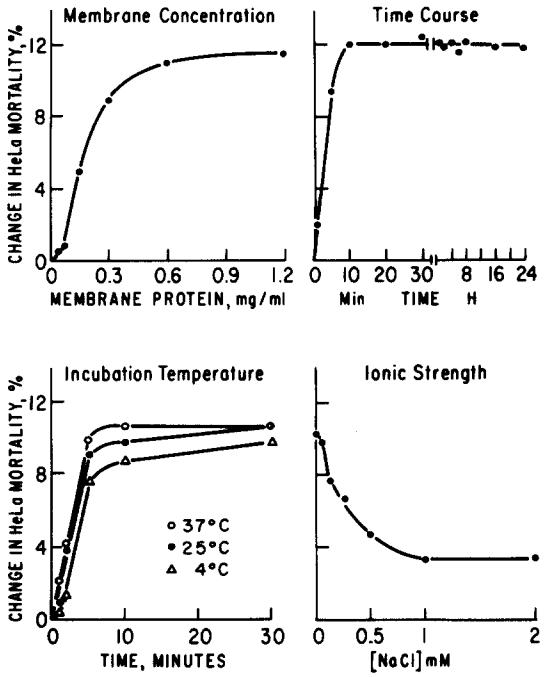

FIG. 1. Binding of Shiga cytotoxin by rat liver cell membranes as a function of the quantity of membranes added (upper left), time of incubation, (upper right) incubation temperature (lower left), and salt concentration (lower right). These experiments were done with a final concentration of membrane protein of 1 mg/ml, except when the quantity of membrane protein added was being varied, and 5 TC_{50} doses of toxin/ml.

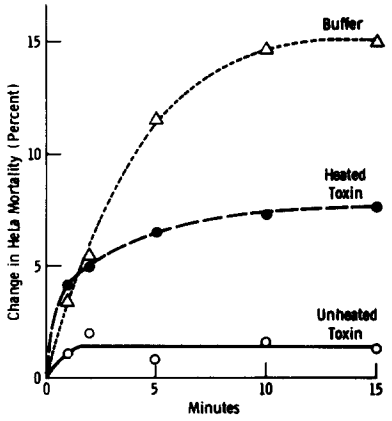

FIG. 2. Binding of Shiga cytotoxin by rat liver cell membranes preincubated with unheated toxin, heated (90°C, 30 min) toxin, or buffer. Membranes were washed three times after preincubation and then studied to determine their ability to bind fresh unheated toxin.

lysozyme was particularly interesting. Since lysozyme activity can be inhibited by salt, the effect of increasing NaCl concentration on both enzyme activity, with *M. luteus* substrate, and on the toxin receptor was studied. Rat liver membranes were preincubated with lysozyme in the presence of varying quanti-

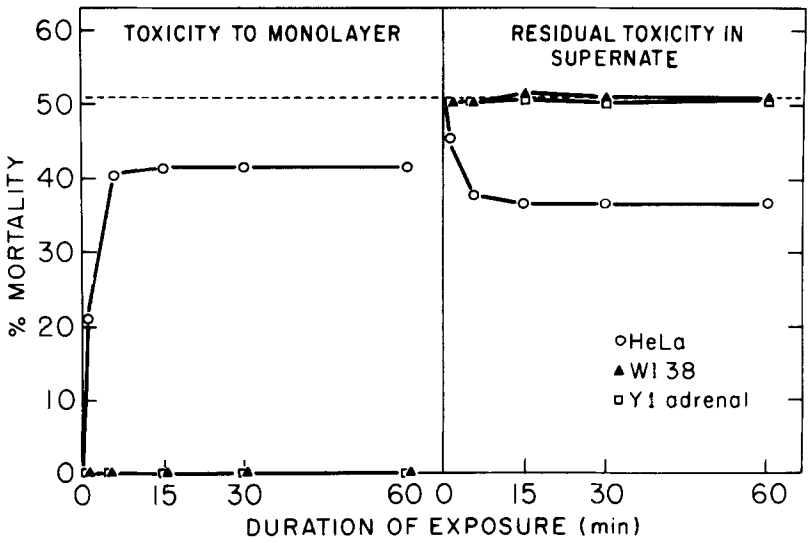

FIG. 3. Binding of Shiga cytotoxin by HeLa, WI-38, or Y-1 adrenal cells in monolayer culture. Cytotoxicity to the monolayer after varying periods of exposure to toxin is shown in the left panel; the horizontal dashed line indicates percent mortality of HeLa monolayers exposed for 20 h to Shiga cytotoxin. Residual cytotoxicity (unbound toxin) in the supernatant medium removed from the monolayers after varying time periods is shown in the right panel, and presented as percent mortality of HeLa monolayers exposed for 20 h to the toxin containing supernates.

TABLE I

Effect of Enzymatic Treatment of Rat Liver Cell Membranes on Binding of Shiga Cytotoxin

Enzyme	Activity/tube	Inhibition of binding
	U	%
Trypsin	16,000	62
	32,000	100
Pronase	18	81
Phospholipase A	0.22	92
Phospholipase C	0.6	58
Galactose oxidase	20.0	4
β-galactosidase	1.2	0
Neuraminidase	66.0	0
Lysozyme	5,000.0	100

ties of NaCl, followed by thorough washing before addition of toxin (Fig. 4). There was a parallel inhibition of lysozyme action on *M. luteus* substrate and on the toxin receptor of the liver membranes by increasing salt concentration.

Table II shows results of pretreating intact viable HeLa cell monolayers with different enzymes on their ability to bind toxin as determined by direct cytotoxicity. Each enzyme employed was first titrated to establish the highest concentration which itself did not cause either cell death or cell detachment from the glass during a 30-min incubation at 37°C. Of the enzymes studied, only lysozyme had a demonstrable inhibitory effect on toxin binding. Addition of natural

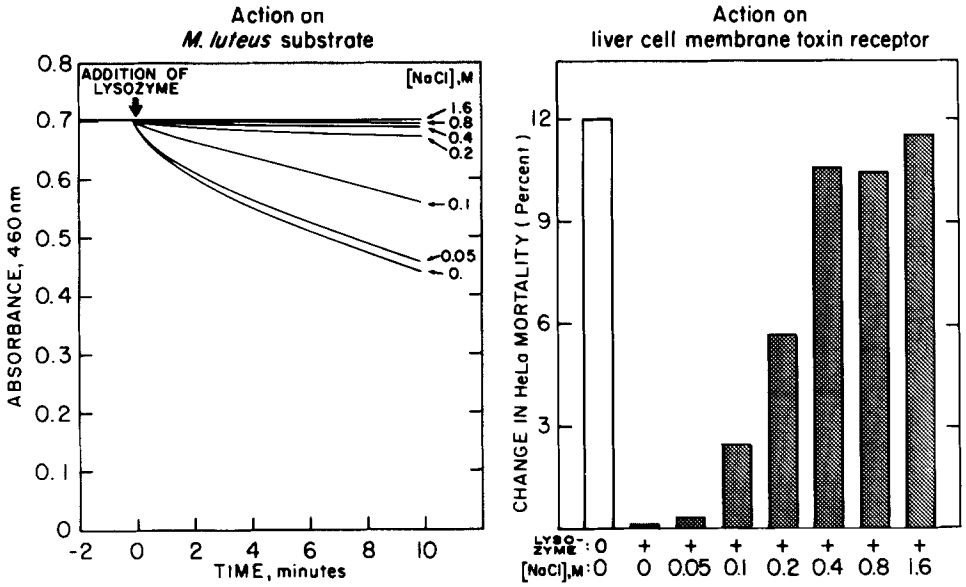

FIG. 4. Effect of NaCl concentration on lysis of *M. luteus* by lysozyme (left panel) and on binding of Shiga cytotoxin to lysozyme-treated rat liver cell membranes (right panel). Toxin binding to untreated membranes is shown by the clear bar, and to lysozyme-treated membranes by the hatched bars. The molarity of NaCl in the buffer is indicated along the abscissa.

TABLE II
Effect of Enzymatic Pretreatment of HeLa Cells on Binding of Shiga Cytotoxin

Enzyme	Activity/monolayer	Inhibition of binding
	U	%
Hyaluronidase IV	30	5.5
Hyaluronidase V	30	3.2
α-glucosidase	0.1	0
β-glucosidase	0.08	0.8
α-mannosidase	0.02	1.3
Galactose oxidase	0.2	1.1
β-glucuronidase	0.4	0
β-galactosidase	0.06	0.9
Neuraminidase	6.6	0
Lysozyme	5,000	18.7

substrate, *M. luteus*, to the enzyme and HeLa monolayers inhibited the effect of the enzyme on the HeLa cell membrane receptor (Table III).

These results suggested that the cell membrane receptor for ST was a glycoprotein in some way involving a lysozyme sensitive substrate. This was further investigated by inhibition experiments.

COMPETITIVE INHIBITIONS. Table IV shows the effect on ST binding to HeLa cells of 13 glycoproteins, substituted sugars, and ganglioside, all previously

TABLE III
Inhibition of Lysozyme Effect on HeLa Cell Binding of Shiga Cytotoxin by M. luteus

M. luteus added	Inhibition of binding	
	No Lysozyme	Lysozyme, 5,000 U
mg/ml	%	
0	0	18.7
1	2.8	14.1
10	1.0	5.0
100	2.9	4.5

TABLE IV
Effect of Substituted Sugars, Oligosaccharides, and Glycoproteins on Binding of Shiga Cytotoxin to HeLa Cells

Inhibitor	Concentration	Inhibition of binding
	mM	%
Carbohydrates		
p-aminophenyl-β-D-thiogalactopyranoside	200	3.2
Methyl β-D-galactopyranoside	100	1.0
Methyl α-D-galactopyranoside	100	1.5
Isopropyl β-D-thiogalactopyranoside	100	3.2
D-galactosyl β-D-thiogalactopyranoside	100	0
Stachyose (α-D-Gal-α-D-Gal-α-D-Glu-β-D-Fru)	30	2.7
Raffinose (α-D-Gal-α-D-Glu-β-D-Fru)	50	3.2
	mg/ml	
Glycoproteins and Ganglioside		
Fetuin	0.5	0
Thyroglobulin	15.0	0
Bovine β-lipoprotein (IV-1)	0.1	0
Bovine β-lipoprotein (III-0)	1.0	0.2
Ovomucoid	30.0	0.2
Ganglioside (mixed bovine brain)	1.0	0

demonstrated to inhibit the binding of cholera toxin to its receptor on rat liver cell membranes (11). None of these compounds, nor 10 simple or amino sugars (Table V A), or cholera toxin (Fig. 5), competitively inhibited ST binding to the HeLa cell. In contrast, chitin oligosaccharide lysozyme substrates (10) were effective competitive inhibitors (Table V B). Optimal inhibition was found with the trimer *N,N',N"* triacetyl chitotriose; the tetramer was somewhat less efficient, whereas D-GlcNAc and chitohexaose were virtually without effect.

RECEPTOR BLOCKADE. To examine the specificity of these findings we determined the effect of three lectins with known structural binding specificities as receptor blockers, including phytohemagglutinin (PHA), concanavalin A (Con-A), and wheat germ agglutinin (WGA). These lectins have been previously shown to have binding affinities for α-linked D-*N*-acetyl-galactosamine (PHA), carbohydrates containing α-D-mannopyranosyl, α-D-glucopyranosyl, or β-D-fructofuranosyl residues at their nonreducing ends (Con A), or *N,N',N"*-triace-

TABLE V

Inhibition of Binding of Shiga Cytotoxin to HeLa Cells: Effect of Simple and Aminosugars and Chitin Oligosaccharides

	Inhibitor	Concentration	Inhibition of binding
		mM	%
A	Methyl α-D-mannoside	100	0
	Galactose	50	0
	Galactosamine	50	0
	N-acetyl-D-galactosamine	100	2.7
	Methyl β-D-galactoside	100	0
	Glucosamine	50	2.0
	N-acetyl-D-glucosamine	100	7.5
	Methyl α-D-glucoside	50	1.0
	Methyl β-D-glucoside	50	2.5
	Mannose	50	0
B	*N,N'*-diacetyl chitobiose	1	8.3
	N,N',N''-triacetyl chitotriose	1	45.5
	N,N',N'',N'''-tetraacetyl chitotetraose	1	40.0
	N,N',N'',N''',N''''-pentaacetyl chitopentaose	1	17.2
	N,N',N'',N''',N'''',N'''''-hexaacetyl chitohexaose	1	3.6

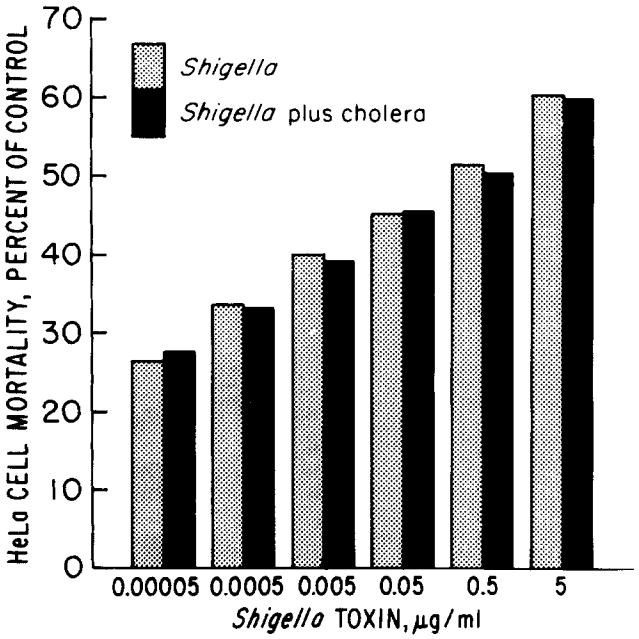

FIG. 5. Dose-response of Shiga cytotoxin in HeLa cell monolayers in the presence (dark bars) or absence (hatched bars) of 5 μg/ml of cholera enterotoxin.

tyl chitotriose (WGA) (12–14). The effect of pretreatment of liver cell membranes with these three lectins is shown in Table VI. Only WGA efficiently blocked consumption of ST by the membranes. Table VII shows the results of similar experiments by using intact HeLa cell monolayers. The maximum concentra-

TABLE VI
Lectin Inhibition of Binding of Shiga Cytotoxin to Rat Liver Cell Membranes

Lectin concentration	Inhibition of toxin binding, % Lectin studied		
	PHA	Con A	WGA
μg/ml			
0.01	0	3.3	49.0
0.1	1.6	5.7	84.1
1.0	7.3	5.7	92.2

TABLE VII
Lectin Inhibition of Binding of Shiga Cytotoxin to HeLa Cell Monolayers

Lectin concentration	Inhibition of toxin binding, % Lectin studied		
	PHA	Con A	WGA
μg/ml			
0.01	0	0	6.3
0.05	NT*	0	15.8
0.1	NT	0.7	22.2
0.5	NT	0	27.5
1.0	NT	0	34.9
5.0	NT	NT	52.6
10.0	NT	NT	69.5

* NT, Not tested because of cell agglutination by this concentration of lectin.

tion of lectin tested was limited in these experiments by agglutination of the HeLa cells, particularly noted in the presence of PHA. Again, only WGA affected toxin uptake.

Discussion

These studies indicate that there is a membrane receptor for Shiga cytotoxin on HeLa and rat liver cell membranes. Three distinct lines of evidence all point to the presence of an oligomeric $\beta 1\rightarrow$ 4-linked D GlcNAc determinant in the binding site: (*a*) it is destroyed by lysozyme, (*b*) it is competitively inhibited by the lysozyme substrates *N,N',N"*-triacetyl chitotriose and *N,N',N",N'"*-tetracetyl chitotetraose, and (*c*) it is blocked by wheat germ lectin, which has binding specificity for *N,N',N"*-triacetyl chitotriose. With the exception of proteolytic enzymes, which might be expected to disrupt any glycoprotein membrane receptor, and phospholipase A and C, which grossly altered the membrane preparations used, all other specific enzymes, haptens and lectins tested in this study were without effect on the binding of Shiga cytotoxin.

These characteristics of the Shiga toxin receptor are clearly distinct from those described for the cholera toxin receptor, a sialidase resistant monosialosyl ganglioside, G_{M1} (15–18). Furthermore, neither cholera toxin nor ganglioside were able to competitively inhibit the binding of Shiga toxin to either HeLa cells

or rat liver cell membranes. It has been shown that the binding of cholera toxin to cell surface membrane receptors activates adenylate cyclase (AC), with a resultant increase in intracellular cAMP levels (19). Recent evidence indicates that Shiga toxin is also able to activate AC in proximal rabbit small intestine (20). If Shiga toxin binds to jejunal cells through a chitin oligosaccharide receptor on their surface, it would suggest either that there are at least two enterotoxin paths to activation of AC in small bowel, or a common mechanism after initial binding. Holmgren and Lönnroth (21) and Bennett et al. (22) have recently proposed models for cholera toxin activation of AC in which initial binding of toxin to a G_{M1}-containing receptor is followed by interaction of the active part of the toxin molecule with a second receptor which actually results in stimulation of AC. Whatever the mechanism, activation of AC is believed to lead to secretion of isotonic fluid and therefore the effects of cholera and Shiga toxins on the intestine would both be limited by AC activity. Thus there would be no additive effects of maximum secretory doses of the two toxins, previously observed to be the case by Steinberg et al. (23).

On the basis of the known secretory responsiveness of small intestine to topical application of ST (1, 20, 23) and the reported resistance of rat colon (24), it is reasonable to speculate that presence or absence of a cell surface toxin receptor is involved in this differential susceptibility. The toxin is also cytotoxic to some cells in culture, to rabbit ileal epithelial cells, and causes an inflammatory enteritis in the latter tissue similar to the colitis caused by invading intact bacteria in the large bowel (2). Therefore toxin could play a role in the dysentery (colitis) phase of clinical shigellosis as well. If these speculations are indeed true it might suggest that the direct role of bacterial invasion in pathogenesis is, in essence, to allow development of a microcolony within the intestinal epithelium resulting in local (? intracellular) production of toxin. This hypothesis might also explain why *Shigella-Escherichia coli* hybrids, which invade but do not multiply in the intestinal mucosa, are avirulent (25) since bacterial multiplication would be necessary for toxin production. It is necessary to also postulate a site-specific mechanism mediating bacterial penetration of the bowel since shigella are known to invade the colon but not the jejunum (26). Thus pathogenesis and clinical manifestations of shigellosis may depend on site specific localization of surface receptors on small and large intestinal epithelial cells for toxin and whole bacteria, and on intrinsic capacity of the infecting organism to produce toxin and to penetrate the intestinal mucosa.

The chitin oligosaccharide membrane receptor on HeLa cells for Shiga cytotoxin described in the present study suggests that affinity chromatography on a chitin column may be useful for toxin purification (27), a necessary step in the development of more quantitative methods for the study of the binding of toxin to its receptor. Affinity chromatography might also shed light on the relationships of cytotoxic, enterotoxic, and neurotoxic activities in partially purified Shiga toxin. We have recently found that both cytotoxicity and neurotoxicity are retained by chitin oligosaccharides attached to Sepharose 4-B, and that both activities are released by M NaCl (27; G. T. Keusch and M. Jacewicz, unpublished observations). These preliminary studies imply a similar receptor for at least these two biologic activities which are found together with enterotoxin in the separated pI 7.25 toxin.

Summary

The binding of *Shigella dysenteriae* 1 cytotoxin to HeLa cells in culture and to isolated rat liver cell membranes was studied by means of an indirect consumption assay of toxicity from the medium, or by determination of cytotoxicity to the HeLa cell monolayer. Both liver cell membranes and HeLa cells removed toxicity from the medium during incubation, in contrast to WI-38 and Y-1 mouse adrenal tumor cells, both of which neither bound nor were affected by the toxin. Uptake of toxin was directly related to concentration of membranes added, time, and temperature, and indirectly related to the ionic strength of the buffer used.

The chemical nature of the membrane receptor was characterized by using three principal approaches: (*a*) enzymatic sensitivity; (*b*) competitive inhibition and (*c*) receptor blockade studies. The receptor was destroyed by proteolytic enzymes, phospholipases (which markedly altered the gross appearance of the membrane preparation) and by lysozyme, but not by a variety of other enzymes. Of 28 carbohydrate and glycoprotein haptens studied, including cholera toxin and ganglioside, only the chitin oligosaccharide lysozyme substrates, per *N*-acetylated chitotriose, chitotetraose, and chitopentaose were effective competitive inhibitors. Greatest inhibition was found with the trimer, *N,N',N''* triacetyl chitotriose. Of three lectins studied as possible receptor blockers, including phytohemagglutinin, concanavalin A, and wheat germ agglutinin, only the latter, which is known to possess specific binding affinity for *N,N',N''* triacetyl chitotriose, was able to block toxin uptake.

Evidence from all three approaches indicate, therefore, existence of a glycoprotein toxin receptor on mammalian cells, with involvement of oligomeric $\beta 1 \rightarrow 4$-linked *N*-acetyl glucosamine in the receptor. This receptor is clearly distinct from the G_{M1} ganglioside thought to be involved in the binding of cholera toxin to the cell membrane of a variety of cell types susceptible to its action.

We thank Dr. Elvin A. Kabat for his careful and critical reading of this manuscript and Dr. Michael Gerber for electron microscopy of liver cell membrane preparations.

Received for publication 15 April 1977.

References

1. Keusch, G. T., G. F. Grady, L. J. Mata, and J. McIver. 1972. The pathogenesis of Shigella diarrhea. I. Enterotoxin production by *Shigella dysenteriae* 1. *J. Clin. Invest.* 51:1212.
2. Keusch, G. T., G. F. Grady, A. Takeuchi, and H. Sprinz. 1972. The pathogenesis of Shigella diarrhea. II. Enterotoxin-induced acute enteritis in the rabbit ileum. *J. Infect. Dis.* 126:92.
3. Levine, M. M., H. L. DuPont, S. B. Formal, R. B. Hornick, A. Takeuchi, E. J. Gangarosa, M. J. Snyder, and J. P. Libonati. 1973. Pathogenesis of *Shigella dysenteriae* 1 (Shiga) dysentery. *J. Infect. Dis.* 127:261.
4. Keusch, G. T., M. Jacewicz, and S. Z. Hirschman. 1972. Quantitative microassay in cell culture for enterotoxin of *Shigella dysenteriae* 1. *J. Infect. Dis.* 125:539.
5. Keusch, G. T., M. Jacewicz, M. M. Levine, R. B. Hornick, and S. Kochwa. 1976. Pathogenesis of Shigella diarrhea. Serum anticytotoxin antibody response produced by toxigenic and non-toxigenic *Shigella dysenteriae* 1. *J. Clin. Invest.* 57:194.
6. Keusch, G. T., and M. Jacewicz. 1975. The pathogenesis of Shigella diarrhea V. Relationship of Shiga enterotoxin, neurotoxin, and cytotoxin. *J. Infect. Dis.* 131:S33.

7. McIver, J., G. F., Grady, and G. T. Keusch. 1975. Production and characterization of exotoxins of *Shigella dysenteriae* Type 1. *J. Infect. Dis.* **131**:559.
8. Neville, D. M., Jr. 1968. Isolation of an organ specific protein antigen from cell-surface membrane of rat liver. *Biochim. Biophys. Acta.* **154**:540.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
10. Rupley, J. A. 1964. The hydrolysis of chitin by concentrated hydrochloric acid, and the preparation of low-molecular weight substrates for lysozyme. *Biochim. Biophys. Acta.* **83**:245.
11. Cuatrecasas, P. 1973. Interaction of *Vibrio cholerae* enterotoxin with cell membranes. *Biochemistry.* **12**:3547.
12. Borberg, H., I. Yesner, B. Gesner, and R. Silber. 1968. The effect of N-acetyl D-galactosamine and other sugars on the mitogenic activity and attachment of PHA to tonsil cells. *Blood.* **31**:747.
13. Goldstein, I. J., C. M. Reichert, and A. Misaki. 1974. Interaction of concanavalin A with model substrates. *Ann. N. Y. Acad. Sci.* **234**:283.
14. Goldstein, I. J., S. Hammarstrom, and G. Sundblad. 1975. Precipitation and carbohydrate-binding specificity studies on wheat germ agglutinin. *Biochim. Biophys. Acta.* **405**:53.
15. Holmgren, J., I. Lönnroth, J. E. Mansson, and L. Svennerholm. 1975. Interaction of cholera toxin and membrane G_{M1} ganglioside of small intestine. *Proc. Natl. Acad. Sci. U. S. A.* **72**:2520.
16. King, C. A., and W. E. van Heyningen. 1973. Deactivation of cholera toxin by a sialidase resistant monosialosylganglioside. *J. Infect. Dis.* **127**:639.
17. Cuatrecasas, P. 1973. Gangliosides and membrane receptors for cholera toxin. *Biochemistry.* **12**:3558.
18. van Heyningen, S. 1974. Cholera toxin: interaction of subunits with ganglioside G_{M1}. *Science (Wash. D. C.).* **183**:656.
19. Sharp, G. W. G. 1973. Action of cholera toxin on fluid and electrolyte movement in the small intestine. *Annu. Rev. Med.* **24**:19.
20. Charney, A. N., R. E. Gots, S. B. Formal, and R. A. Giannella. 1976. Activation of intestinal mucosal adenylate cyclase by *Shigella dysenteriae* 1 enterotoxin. *Gastroenterology.* **70**:1085.
21. Holmgren, J., and I. Lönnroth. 1976. Cholera toxin and the adenylate cyclase-activating signal. *J. Infect. Dis.* **133**:S64.
22. Bennett, V., E. O'Keefe, and P. Cuatrecasas. 1975. Mechanism of action of cholera toxin and the mobile receptor theory of hormone receptor-adenylate cyclase interaction. *Proc. Natl. Acad. Sci. U. S. A.* **72**:33.
23. Steinberg, S. E., J. G. Banwell, J. H. Yardley, G. T. Keusch, and T. R. Hendrix. 1975. Comparison of secretory and histological effects of shigella and cholera enterotoxins in rabbit jejunum. *Gastroenterology.* **68**:309.
24. Donowitz, M., and H. J. Binder. 1976. Effect of enterotoxins of *Vibrio cholerae*, *Escherichia coli*, and *Shigella dysenteriae* Type 1 on fluid and electrolyte transport in colon. *J. Infect. Dis.* **134**:135.
25. LaBrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal. 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. *J. Bacteriol.* **88**:1503.
26. Rout, W. R., S. B. Formal, R. A. Giannella, and G. J. Dammin. 1975. Pathophysiology of shigella diarrhea in the rhesus monkey: intestinal transport, morphological, and bacteriological studies. *Gastroenterology.* **68**:270.
27. Keusch, G. T., I. Parikh, and M. Jacewicz. 1977. Affinity chromatography purification of Shigella toxin on a sepharose-chitin column. *Clin. Res.* **25**:490A.