Identification of the Carbohydrate Receptor for Shiga Toxin Produced by Shigella dysenteriae Type 1*

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The binding of Shiga toxin isolated from the bacterium Shigella dysenteriae type 1 to a series of glycolipids and to cells or cell homogenates has been studied. Bound toxin was detected using either 125I-labeled toxin or specific monoclonal antibody and 125I-labeled anti-antibody. Overlay of toxin on thin-layer chromatograms with separated glycolipids and binding to glycolipids coated in microtiter wells established that the toxin specifically bound to Galα1-4Galβ (galabiose) placed terminally or internally in the oligosaccharide chain. No glycolipid shown to lack this sequence binds the toxin. Most of the glycolipids with internally placed galabiose were not active, indicating a steric hindrance for toxin access to the binding epitope.

Binding of toxin to HeLa cells in monolayers could be inhibited by preincubation of the toxin with galabiose covalently linked to bovine serum albumin (BSA), but not with free oligosaccharides containing galabiose or with lactose coupled to BSA. This demonstrated that the inhibition is specifically dependent on galabiose and requires multivalency of the disaccharide to be efficient. The inhibitory effect was successively enhanced by increasing the substitution on BSA (7, 18, and 25 mol of galabiose/mol of BSA). The BSA-coupled galabiose could also prevent the cytotoxic effect on HeLa cells (detachment of killed cells).

There are cell lines with a dense number of receptor sites, but which are resistant to toxin action (uptake and inhibition of protein synthesis) which may suggest two types of receptor substances which are functionally different and unevenly expressed. In analogy with the mechanism earlier formulated for cholera toxin, we propose glycolipid-bound, bilayer-close galabiose as the functional receptor for membrane penetration of the toxin, while galabiose bound in glycoproteins affords binding sites but is not able to mediate penetration.

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Shigella (Shiga) toxin is a protein exotoxin produced by Shigella dysenteriae type 1 and other members of the genus Shigella and may be an important virulence factor in bacillary dysentery of man (1, 2). Recent observations indicate that various pathogenic strains of Escherichia coli (3), Vibrio cholerae, and Vibrio parahemolyticus (4) produce a similar toxin referred to as Shiga-like toxin or Vero toxin, suggesting that Shiga toxin may represent a virulence factor present in a spectrum of human diseases.

The cytotoxic effect of Shiga toxin is inhibition of protein synthesis (5, 6). The molecule is composed of one A subunit of $M_r = 30,500$ and several B subunits of $M_r = 5,000$ (7). The A subunit enzymatically inactivates the 60 S ribosomal subunit (8), leading to inhibition of aminoacyl-tRNA binding in the peptide elongation reaction (9). The B subunits are assumed to carry binding sites for the obligatory receptor-mediated uptake of toxin molecules (10). Although it has been shown that less than 1 pg of toxin per ml of culture medium is able to kill sensitive cells overnight, many cell lines are completely resistant to the toxin (11). However, several resistant cells bind a large number of toxin molecules (about 106/cell) with apparently high affinity ($K_d = 10^{-10} M^{-1}$), indicating that the difference between sensitive and resistant cells may involve more than binding (11).

The chemical nature of the receptor has been studied by Keusch and Jacewicz (12), who concluded that oligomers of β1-4-linked GlcNAc residues in the protein-bound form may be the receptor for the toxin. Their conclusion was based on partial sensitivity of the putative receptor to lysozyme, limited inhibition of toxin binding to cells with oligosaccharide, and partial receptor blockade with wheat germ agglutinin.

In the present work, we report on the binding of purified Shiga toxin to glycosphingolipids. Extending a preliminary study (13), we have established a specific binding to structurally well-characterized glycolipids with the disaccharide Galα1-4Galβ as a common denominator. Moreover, inhibition of toxin binding and cytotoxicity to HeLa cell monolayers using synthetic receptor analogues was demonstrated.

MATERIALS AND METHODS

RESULTS

Binding of Shiga Toxin to Glycolipids—Highly purified 125I-labeled Shiga toxin was earlier tested for binding to glycolipids separated on a thin-layer chromatogram or coated in microtiter wells (13). The results indicated that the toxin had an...
apparently high-avidity binding to glycolipids having in common Galα1-4Galβ residues in a terminal or internal position of saccaride chains. We have now extended this to a larger series of glycolipids (Table I) and also used monoclonal antibody to detect bound toxin (Figs. 1 and 2), with essentially the same results.

In Fig. 1, the overlay result is shown for some selected glycolipid fractions. To be considered negative for toxin binding (as summarized in Table I), a glycolipid of at least 1 μg should produce no darkening on autoradiography using the same or similar conditions as for Fig. 1. As shown, there are several major spots as detected with anisaldehyde (Fig. 1A) which do not show up on the autoradiogram (Fig. 1B), documenting selectivity of toxin binding. All glycolipids that bound the toxin contained the disaccharide Galα1-4Galβ. Glycolipids lacking this structure did not bind toxin. Substance No. 3 (globoside) bound only weakly (weak autoradiographic darkening compared to anisaldehyde intensity) and is shown in Table I with a plus sign in parentheses. However, several glycolipids carrying the Galα1-4Gal disaccharide in an internal position did not bind the toxin. Forsman hapten (No. 4), which has a GaINAcβ1-3 linked to the nonreducing end of globoside (No. 3; P antigen), did not bind the toxin. This was also the case when Galβ1-3 (No. 5), R-Galβ1-3 (Nos. 6-8), or GaINAcβ1-3 (No. 9) was linked to globoside. Substance No. 11 where Galα1-3 has been extended on globotriaosylceramide (No. 2; P antigen) was active. However, linkage of one or more additional Galα1-3 (Nos. 12 and 13) on the nonreducing end of globotriaosylceramide interfered with binding. Estimation of the extent of binding to glycolipids coated in microtiter wells (Fig. 2) suggests that substances No. 2 (P antigen) and 10 (P1 antigen) bind the strongest.

Table II shows the results of studies of the binding of 125I-labeled Shiga toxin to human erythrocytes that had been coated with purified glycolipids. Erythrocytes coated with globotriaosylceramide (No. 2) or globotetraosylceramide (No. 3) bound 15-27 times more than uncoated erythrocytes, or erythrocytes coated with isoglobotriaosylceramide (No. 14).

Binding of Toxin to Cell Monolayers—In order to study the effect of receptor analogues on cell surface binding, the binding kinetics of 125I-labeled Shiga toxin at 0 °C to HeLa cell monolayers was established. Fig. 3 shows the binding of labeled toxin (1 μg/ml) as a function of time. After 60 min, about 50% of the toxin was bound, and, after 4 h, maximal binding had occurred. In the presence of 100-fold excess unlabeled toxin, the amount of bound toxin was reduced to about 10% of that with labeled toxin alone. Thus, unlabeled toxin competed effectively with labeled toxin, indicating a low degree of nonspecific adsorption. Fig. 4 shows the binding at 0 °C with increasing concentrations of 125I-toxin. Incubations were conducted for 5 h at toxin concentrations from 0.01-25 μg/ml. The largest increase in binding was seen in the range from 0.1-1.0 μg/ml; at concentrations above 5.0 μg/ml, only a small gradual increase was seen. The amount of toxin bound at 5 μg/ml indicates that approximately 1 x 10^7 toxin molecules were bound per HeLa cell. The data show that Shiga toxin binding to these cells was rapid, specific, and saturable. The association of toxin to HeLa cell monolayers was also examined at 25 °C and 37 °C. Toxin uptake at 25 °C as a function of time was about 170% over that observed at 0 °C, whereas at 37 °C, uptake was about 125% over that at 0 °C.

TABLE I

<table>
<thead>
<tr>
<th>No.</th>
<th>Structurea</th>
<th>Bindingb</th>
<th>Source and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Galα1-4GalβCer</td>
<td>+</td>
<td>Human meconium (32)</td>
</tr>
<tr>
<td>2</td>
<td>Galα1-4Galβ1-4GlcβCer</td>
<td>+</td>
<td>Human erythrocyte (20)</td>
</tr>
<tr>
<td>3</td>
<td>Galα1-NAcβ1-3Galα1-4Galβ1-4GlcβCer</td>
<td>(+)</td>
<td>Rat small intestine (33)</td>
</tr>
<tr>
<td>4</td>
<td>Galα1-NAcβ1-3Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Human erythrocyte (20)</td>
</tr>
<tr>
<td>5</td>
<td>Galα1-3Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Dog small intestine (34)</td>
</tr>
<tr>
<td>6</td>
<td>Fuco1-2Galβ1-3Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Human kidney (35, 37)</td>
</tr>
<tr>
<td>7</td>
<td>Galα1-NAcβ1-3(Fuco1-2)Galβ1-3Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Human meconium (36)</td>
</tr>
<tr>
<td>8</td>
<td>NeuAc2-3(NeuAc2-6)Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Human kidney (37)</td>
</tr>
<tr>
<td>9</td>
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<td>-</td>
<td>Human erythrocyte (38)</td>
</tr>
<tr>
<td>10</td>
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<td>-</td>
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<tr>
<td>11</td>
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<td>-</td>
<td>Human erythrocyte (40)</td>
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<tr>
<td>12</td>
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<td>-</td>
<td>Rat small intestine (41)</td>
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<tr>
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<td>-</td>
<td>Rat small intestine (41)</td>
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<tr>
<td>14</td>
<td>Galα1-3Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Dog small intestine (34)</td>
</tr>
<tr>
<td>15</td>
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<td>-</td>
<td>Rat small intestine (33)</td>
</tr>
<tr>
<td>16</td>
<td>Galα1-3Galα1-4Galβ1-4GlcNAcβ1-3Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Ratβ gl erythrocyte (42)</td>
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<tr>
<td>17</td>
<td>Galα1-3(Fuco1-2)Galβ1-4GlcNAcβ1-3Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Human erythrocyte (21)</td>
</tr>
<tr>
<td>18</td>
<td>Galα1-4(Fuco1-2)Galβ1-4GlcNAcβ1-3Galα1-4Galβ1-4GlcβCer</td>
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<td>Dog small intestine (43)</td>
</tr>
<tr>
<td>19</td>
<td>GlcNAcβ1-3Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Malignant melanoma (3)</td>
</tr>
<tr>
<td>20</td>
<td>GaINAcβ1-3Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Human meconium (44)</td>
</tr>
<tr>
<td>21</td>
<td>Galα1-3Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Mouse small intestine (45)</td>
</tr>
<tr>
<td>22</td>
<td>Galα1-3Galα1-4Galβ1-4GlcβCer</td>
<td>-</td>
<td>Mouse small intestine (45)</td>
</tr>
</tbody>
</table>

*The Galα1-4Gal disaccharide has been underlined to indicate its presence in a terminal or nonterminal position.

For binding results, + indicates a good binding (black spot on autoradiogram larger than a corresponding anisaldehyde-detected spot, e.g. No. 10 of Fig. 1), (+) indicates a weak binding (spot 3 of Fig. 1), and — indicates nonbinding. To be convincing as a negative binder, the substance should produce no darkening in the autoradiogram at at least 1 μg level of material.


Carbohydrate Receptor for Shiga Toxin

Inhibition of Binding of Toxin to Cultured Cells in Monolayer—Blocking of binding at 0°C or uptake at 25°C of labeled toxin to HeLa cell monolayers was studied using varied saccharides and glycoconjugates (Fig. 5). In these experiments, the toxin concentration was 0.25 μg/ml, i.e., where binding/uptake was linear (Fig. 4). After premixing toxin and dilutions of the various inhibitors, the mixtures were incubated with the cell monolayers at 25°C or 0°C. No inhibition of binding to cells was observed when galabiose (Galα1-4Gal) or globotetraose (Galα1-3Galα1-4Galα1-3Galα1-4Glcα1-4Gle) was preincubated with toxin at concentrations ≤4 mg/ml (Fig. 5). However, using galabiose covalently linked to BSA1 (Galα1-4Galβ3-BSA; 25 mol of galabiose/mol of BSA), a significant inhibition of toxin binding could be demonstrated. At 0°C, preincubation of toxin with 1 μg/ml Galα1-4Galβ3-BSA resulted in a 50% blocking of toxin binding to HeLa cells. At 25°C, 50% inhibition required a higher concentration of Galα1-4Galβ3-BSA (250 μg/ml). In contrast, lactose covalently linked to BSA (Galβ3-4Glcβ3-BSA) did not inhibit binding of toxin to HeLa cells at either temperature (≤4 mg/ml).

Inhibition of toxin binding by Galα1-4Galβ3-BSA was also observed in Vero cell monolayers. Inhibition (60%) of toxin binding to Vero cells was observed with Galα1-4Galβ3-BSA at 0 and 25°C concentrations of 1 μg/ml and 125 μg/ml, respectively. Galabiose and Galα1-4Glcβ3-BSA at concentrations of ≤4 mg/ml had no effect on binding of toxin to the Vero cell monolayers at either temperature.

Chitobiose, chitotriose, and a chitobiose-BSA conjugate were tested for inhibition of toxin uptake by HeLa and Vero cells since data from Keusch and Jacewicz (12) indicated that

\[ \text{oligomer} \begin{array}{l}
\text{α1-4-linked GlcNAc residues may be the receptor for Shiga toxin. No inhibition of } \text{125I-toxin association was}
\end{array} \text{observed in our binding assays at 0°C and 25°C with either chitobiose or chitotriose up to concentrations of 2 mg/ml, or with chitobiose-BSA up to 4 mg/ml.}

Since Galα1-4Galβ3-BSA, in contrast to free disaccharide, inhibited the binding of the toxin to HeLa cell monolayers, it was of interest to determine the effect of varying numbers of galabiosyl residues per BSA molecule on toxin binding. Results indicated that the higher the number of galabiosyl residues linked to BSA, the greater was its inhibitory effect on toxin binding to HeLa cell monolayers (Fig. 6). Glycoconjugates containing 25 mol of galabiose/mol of BSA gave the best inhibition (50% inhibition at 1 μg/ml), whereas conjugates with only 7 mol of galabiose/mol of BSA gave the weakest inhibition. The latter molecule failed to cause 50% inhibition of toxin binding to HeLa monolayers even at 100 μg/ml.

Cytotoxicity of Shiga Toxin to HeLa Cells and Inhibition by Glycoconjugate—Since Galα1-4Galβ3-BSA blocked toxin binding to HeLa cells, it was of interest to determine if the Galα1-4Galβ3-BSA conjugate also could prevent internalization of toxin and thereby block cytotoxicity. Toxin (10 ng/ml) was preincubated with serial dilutions of Galα1-4Galβ3-BSA (25 mol/mol, ≤2 mg/ml) and added to HeLa cells at 0°C. After 60 min, rabbit Shiga toxin antiserum was added to neutralize unbound toxin, and the cells were then allowed to incubate at 37°C overnight. In the presence of excess Galα1-4Galβ3-BSA (≤255-fold), cells were completely protected from Shiga toxin cytotoxicity (Fig. 7A). The protective effect was lost as Galα1-4Galβ3-BSA was diluted. When cells were incubated with toxin alone for 60 min and then followed by antiserum, only slight protection was observed. In agreement with results by Ekild and Olsnes (10), we observed that cells cannot be rescued by the addition of antiserum after toxin binding. At 0°C, approximately 50% maximal binding of Shiga toxin occurred in 60 min at 0.1 μg/ml. Finally, when toxin, Galα1-4Galβ3-BSA, and antiserum were premixed before applying to cells, cytotoxicity was neutralized (Fig. 7B).

Inhibition of Protein Synthesis in a Cell-free System—The A subunit of Shiga toxin inhibits cell-free protein synthesis by inactivation of the 60 S ribosomal subunit (8). We examined whether Galα1-4Galβ3-BSA glycoconjugate affected the inhibition of cell-free synthesis by Shiga toxin in rabbit reticulocyte lysate reaction mixtures (7). Activated toxin was incubated with varying concentrations of Galα1-4Galβ3-BSA at 2–7.6 mg/ml. After 30 min, aliquots were assayed for their ability to inhibit cell-free protein synthesis. As shown in Table III, Galα1-4Galβ3-BSA (≤2 mg/ml) had no protective effect on inhibition of protein synthesis by Shiga toxin.

Discussion

Our initial hypothesis (13) that Galα1-4Galβ3 may carry the specific binding epitope of the receptor for the Shiga toxin is substantiated by the results of the present studies. Binding of 125I-labeled highly purified toxin was studied using glycolipid mixtures or isolated glycolipids developed on a thin-layer chromatogram (Fig. 1) or coated in microtiter wells (Fig. 2). The common recognition marker of glycolipids that bind toxin was Galα1-4Galβ3 contained in the terminal or internal position of the oligosaccharide chains (Table I). Interestingly, this disaccharide is also recognized by E. coli causing urinary tract infections of man (46, 47). The binding of the bacteria to natural glycolipids including those of target cells for adhesion has been studied in detail (48). A characteristic feature of adhesion is that all glycolipids containing Galα1-4Galβ3 in an internal position bound the bacteria with similar avidity regardless of the nature of the saccharide extension. These

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1 The abbreviations used are: BSA, bovine serum albumin; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; RBC, red blood cells; CETF, 2-(carboxymethyl)-thio)ethyl.
results indicate that the receptor binding site of the Shiga
toxin (Table I) and that of E. coli are closely related but not
identical. The binding saccharide is part of the P blood group
system. The relation between urinary tract infection and P
blood group status has been reported (49); no such relation
has been revealed in the case of shigellosis. A probable expla-
nation for the hemolytic uremic syndrome occasionally seen
following infection with S. dysenteriae type 1 bacteria (50) is
that Shiga toxin which has gained entrance to the bloodstream
bonds to receptors in the kidney.

Binding of labeled toxin could be demonstrated also to
chicken erythrocytes that had been coated with glycolipids
with the appropriate Galα1→4Galβ-BSA structure (Table II). These
results are in agreement with the solid phase assays (Figs. 1
and 2).

Binding of labeled Shiga toxin was also seen with HeLa
cell monolayers (Fig. 3). Using a toxin concentration of 1 μg/
ml, the association observed was most efficient at 25 °C,
followed by 37 °C, and then 0 °C. The increase in association
was most rapid during the first 60 min, thereafter, it leveled
off and approached saturation at 4–5 h. The binding of labeled
was specific. Addition of a 100-fold excess of unlabeled
toxin reduced binding of labeled toxin by 90% (Fig. 3). These
results show that labeled and unlabeled toxin competed for
the same binding sites.

Most of the association of toxin at 0 °C and 25 °C took
place between 0.01 and 5 pg/ml. A 10-fold increase in labeled
toxin concentration increased the association only 15–20%.
The number of binding sites observed in these experiments
varied between 5 × 10^6 and 1 × 10^7, results in general agree-
ment with those reported by Eiklid and Olsnes (10) and by us
(51) using cell suspensions.

The binding of toxin to HeLa cells could be inhibited by
preincubation of the toxin with Galα1→4Galβ-BSA glycocon-
jugate which caused 50% inhibition of toxin binding, whereas
the concentration at 25 °C had to be at least 250-fold higher
(Fig. 5). The inhibition of toxin binding was specific since
neither unsubstituted BSA nor lactose linked to BSA could
prevent the binding of toxin to the cells. The Galα1→4Gal
disaccharide had to be covalently linked to a carrier to cause
inhibition. Neither the disaccharide nor globotetraose caused
inhibition in concentrations 100-fold higher than the Galα1→
4Galβ-BSA concentrations which caused 50% inhibition of
toxin binding. The effect of multivalency was evident when
three Galα1→4Galβ-BSA glycoconjugates with different molar
ratios of saccharide to BSA were tested as inhibitors (Fig. 6).
The conjugate with 25 mol of saccharide/mol of BSA was
twice as effective as an inhibitor as the conjugate with a ratio
of 18 mol of the disaccharide/mol. However, the inhibiting
activity was drastically reduced when the saccharide/BSA
ratio was only 7. We interpret this to mean that at a Galα1→
4Gal density as low as 7, the distance between 2 or more
galabiosyl residues, is too large to allow efficient binding by
two or more of the B subunits of the same toxin molecule.

Interestingly, our own results (to be reported in detail else-
where) are in line with this, demonstrating with the overlay
assay on glycolipids that resistant cells are practically lacking
glycolipid 1 (Table I), a prominent glycolipid of sensitive cells.

Earlier studies using HeLa cells in suspension, i.e. after
tryptsinization, showed that higher concentrations of the
Galα1→4Galβ-BSA conjugate were required for inhibition of
toxin binding compared to cells in monolayer (51). We inter-
pret this to indicate that trypsinized cells may have more
accessible receptor sites, possibly in short-chain glycolipids,
untreated cells in monolayer.

The Galα1→4Galβ-BSA conjugate had no effect on the cell-
free activity, i.e. blocking of in vitro protein synthesis of Shiga
toxin. The inhibition of protein synthesis is caused by subunit
A (M, = 30,500 subunit) (8, 9). Our results suggest that the
glycoconjugate did not bind to this material. These results
lead us to conclude that toxin binding is mediated through
the B subunits found in the holotoxin and are in accord with
the multivalency required for inhibition of binding to cells
and blocking of cytotoxicity by the glycoconjugate. The struc-
ture of Shiga toxin is analogous to that of cholera toxin and
neither A nor B subunit contains the catalytic site (53). The
receptor for cholera toxin is the G_M ganglioside found in
mammalian cell membranes. It has been shown that the B
subunit binds the receptor and the A subunit carries the
enzymatic activity (53). Binding can be inhibited by the intact
ganglioside and by the isolated G_M, oligosaccharide at phys-
iological temperatures (52, 54). Apparently, binding of Shiga
toxin to its receptor(s) may take place in a similar manner;
however, multivalency is an important factor for efficient
binding.

Keusch and Jacowicz (12) earlier reported that chitin oli-
gososaccharides were able to inhibit toxin binding to HeLa cells
(45% inhibition using 0.63 mg/ml chitotriose). Using the
present assay conditions, we were not able, however, to find
any inhibition with up to 2 mg/ml chitobiose of chitotrioise or with up to 4 mg/ml of a chitobiose-BSA conjugate. The observation by Keusch and Jacewicz (12) that the lectin wheat germ agglutinin could block binding of the toxin either to HeLa cells or rat liver cell membranes may be explained by binding of the lectin to receptor-active trihexosylceramide (substance 2, Table I), which has been shown to bind the lectin in addition to terminal GlcNAcβ (55). Alternatively, lectin binding to GlcNAcβ may cause a steric hindrance for toxin binding to its receptors. From the present data, it appears that Galα1-4Galβ is the receptor for Shiga toxin, although similar structures like Galα1-4Gala or GalP-4Galp have not been tested as they are not yet available in suitable form.

REFERENCES

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Carbohydrate Receptor for Shiga Toxin

Determination of the carbohydrate receptor for Shiga toxins produced by Salmonella typhimurium type 0129

Materials and Methods

Toxin

Toxin from Shigella dysenteriae type 1 strain 81-205 was purified from cell lysates as described by Brown et al. (25). It was stored at -20°C. Briefly, the procedure involved isoelectric focusing and preparative isoelectric focusing. The procedure allows recovery of material with a specific cytotoxic activity of 0.1-0.20 pCi/mcg protein, assayed in a quantitative microtiter HeLa cell cytotoxicity assay.

Labeling of toxin

Toxin 110 ng/mil was labeled with either 100 pg/mil or 200 pg/mil 125-I using the Bolton-Hunter reagent (22). The solution was dialyzed against PBS, adjusted to pH 6.5, and layered onto a 1 ml column of anion-exchange resin. After washing with 10 ml of PBS, the column was incubated for 12 h. Finally, the wells were washed four times with PBS, dried and exposed tomlinography. The chemical structures (including those previously published by others) were established by mass spectrometry (23, 24) and degradation studies (25-27).

Glycolipid fractions

Glycolipids from diverse sources were separated into total acid and non-reducing glycolipid fractions and were partially purified by thin-layer chromatography. A total glycolipid fraction was separated from crystallized material by repeated chloroform-water extraction. Cell-line-derived glycolipids, diacyl, and non-dialysis-cellulose and acid-labile carbohydrates were isolated by cation exchange chromatography, gel filtration, and preparative isoelectric focusing. The procedure allows recovery of material with a specific cytotoxic activity of 0.1-0.20 pCi/mcg protein, assayed in a quantitative microtiter HeLa cell cytotoxicity assay.

Binding of toxin to glycolipids in microtiter wells

Solid-phase radioactivity was done as described (27) with slight modifications. Fifty ng of glycolipids in methanol was added to each well and the solvent was allowed to evaporate overnight. After coating the wells, the plates were stored at 4°C for 5 days (in solution, unbound toxin 11.4 pg/mil was added to the wells and incubated for 4 h. The wells were washed 5 times with PBS, followed by incubation at room temperature for 1 h with 50 µl of monoclonal antibody (described above). After washing 5 times with PBS, 125-I-labeled Fab (50 µl) was added and the plates incubated for 12 h. Finally, the wells were washed 5 times with PBS and the radioactivity measured by cutting the wells and counting in a gamma counter.

Coating of purified glycolipids onto chicken erythrocytes

Suspensions (3% in PBS) of chicken erythrocytes were incubated with various purified glycolipids (1 mg/ml) at 37°C for 2 h. After washing, erythrocytes were resuspended in PBS and then incubated with 125-I toxin (3 ng/mil) for 1 h at 25°C. Erythrocytes were treated with repeated centrifugation and resuspension in PBS. Radiolabeled bound to cells was counted in a gamma counter.

Cell cultures

HeLa cells (line CCTC) were obtained from the Walter Reed Army Institute of Research (11) and the HeLa cell line (green monkey kidney) from the National Tumor Institute. Monolayers of HeLa cells were grown and used in drug sensitivity tests. Cells were grown in Eagle's minimal essential medium (MEM) supplemented with the final calf serum (5%), 10% MEM-BSA, 1% glutamine, and 10% cortisone of streptomycin and penicillin (Gibco). Cell cultures were inoculated at 4 x 10^4 cell/ml for 3 days (HeLa) or 7 days (Vero). Monolayers were fixed by overnight incubation in microtiter wells with MEM medium as described by Dentley and Greaves (35).

Binding of 125-I-labeled Shiga toxins to cells in monolayer

Culture medium was replaced with fresh medium containing 25 µg/ml neomycin (100 µg/ml) and the monolayers were then preincubated at 37°C for 30 min. After washing, medium was then added to a final concentration of 125-I toxin (3 ng/mil) for 1 h at 25°C. Erythrocytes were treated by repeated centrifugation and resuspension in PBS. Radiolabeled bound to cells was counted in a gamma counter.

Measurement of cytotoxic binding

Serum dilutions of dextran sulfate-BSA receptor analogues were mixed with Shiga toxin (100 pg/mil) and incubated in PBS containing 80 µg/mil at 37°C for 30 min. Culture medium was replaced with fresh medium containing 25 µg/ml neomycin and the monolayers were then incubated at 37°C for 2 h. After washing, the wells were fixed and stained with crystal violet. The amount of staining was determined by measuring the absorbance of the stained dye at 544 nm for each well (11).

Cell-free protein synthesis

Monolayers were treated with rabbit reticulocyte reaction mixtures using globin mRNA (21). In brief, toxin was activated with ribonuclease (10 ng/ml) in 10% dimethyl sulfoxide and dissolved with GTP (100 µg/ml). 3H-lysine into trichloroacetic acid-soluble, precipitable material was assayed.

Ph: 2. Binding of Shiga toxin to glycolipid coated in microtiter wells. Wells (triplicated) were coated with glycolipids and further treated as described. Inhibition studies were carried out with monoclonal antibody and 125-I labeled anti-toxin. The numbers on the curves refer to the glycolipids listed in Table 1.

FIG. 3. Binding kinetics of 125-I-labeled Shiga toxin to HeLa cell monolayers. Cells were treated as described in Methods. The bar indicates the time periods described.

FIG. 4. Binding of different concentrations of Shiga toxin to HeLa cell monolayers. Cells were treated as described in Methods. The bar indicates the time periods described.

FIG. 5. Inhibition of binding of 125-I-labeled Shiga toxin to HeLa cell monolayers. Cells were treated as described in Methods. The bar indicates the time periods described.
Carbohydrate Receptor for Shiga Toxin

FIG. 4. Effect of varying the number of galactosyl residues per NAA molecule on the inhibition of binding of Shiga toxin to Hela cell monolayers. 75 μg/ml 125I-labeled toxin; 30 min at 0°C; 0.1 ml NAA diluted 1:125 in PBS-buffered medium was added, and the plates were incubated at 37°C overnight. Cytotoxicity was measured as described in Materials and Methods. (A) Toxin + glycoconjugate, antiserum added after 60 min; (B) Toxin + glycoconjugate, no antiserum. (C) Premixed toxin + glycoconjugate + antiserum (A); toxin + glycoconjugate, no antiserum (B). The inset shows the actual stained cell monolayers after overnight incubation. Glycoconjugate concentrations were increased by twofold in each well at a starting concentration of 0.9 μg/ml. The black areas show the regions of cell death.

FIG. 5. Blocking of cytotoxic activity of Shiga toxin by Gal-1-4GalNAc NAA. Toxin (0.1 ml at 0.4 μg/ml, final concentration) was mixed with varying concentrations of Gal-1-4GalNAc NAA (0.1 ml at 2 μg/ml) for 15 min at 0°C. Rabbit antiserum (0.1 ml diluted 1:125 in PBS-buffered medium) was added, and the plates were incubated at 37°C overnight. Cytotoxicity was measured as described in Materials and Methods. (A) Toxin + glycoconjugate, antiserum added after 60 min; (B) Toxin + glycoconjugate, no antiserum. (C) Premixed toxin + glycoconjugate + antiserum; toxin + glycoconjugate, no antiserum. The inset shows the actual stained cell monolayers after overnight incubation. Glycoconjugate concentrations were increased by twofold in each well at a starting concentration of 0.9 μg/ml. The black areas show the regions of cell death.

TABLE II
Binding of 125I-labeled Shiga toxin to chicken erythrocytes coated with purified glycolipid
Suspensions (1% in PBS) of chicken erythrocytes were incubated with various purified glycolipids (1 μg/ml), as described under Materials and Methods. After incubation with 125I-labeled toxin (13 ng/ml), radioactivity bound to erythrocytes was measured (cpm).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity bound to cells (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxin only</td>
<td>1700</td>
</tr>
<tr>
<td>Toxin + acetylated HEC</td>
<td>4300</td>
</tr>
<tr>
<td>Toxin + globotriacontiosecozyme - coated cells</td>
<td>12400</td>
</tr>
<tr>
<td>Toxin + globotriacontiosecozyme - coated cells</td>
<td>104800</td>
</tr>
<tr>
<td>Toxin + isoglobotriacontiosecozyme - coated cells</td>
<td>792</td>
</tr>
</tbody>
</table>

TABLE III
Effect of Gal-1-4 Gal-NAc on inhibition of protein synthesis by Shiga toxin
The assay was performed in rabbit reticulocyte reaction mixtures using globoside/NAc (4%). Incorporation of [3H]-leucine into trichloroacetic acid precipitable material was assayed. Incorporation with no added toxin; 103, 700 ± 1960.

<table>
<thead>
<tr>
<th>Gal-1-4 Gal-NAc</th>
<th>[3H]-leucine incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal-1-4 Gal-NAc</td>
<td>[-]</td>
</tr>
<tr>
<td>2500</td>
<td>55,600</td>
</tr>
<tr>
<td>500</td>
<td>41,600</td>
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<tr>
<td>125</td>
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<tr>
<td>7.8</td>
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<tr>
<td>1.9</td>
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<tr>
<td>0.75</td>
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<tr>
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<tr>
<td>0.036%</td>
<td>97,000</td>
</tr>
</tbody>
</table>

Incorporation with no added toxin; 103, 700 ± 1960.