Oral Therapeutic Agents with Highly Clustered Globotriose for Treatment of Shiga Toxigenic Escherichia coli Infections

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Shiga toxin (Stx) is a major virulence factor in infection with Stx-producing Escherichia coli (STEC). We developed a series of linear polymers of acrylamide, each with a different density of trisaccharide of globo-triaosylceramide (Gb3), which is a receptor for Stx, and identified Gb3 polymers with highly clustered trisaccharides as Stx adsorbents functioning in the gut. The Gb3 polymers specifically bound to both Stx1 and Stx2 with high affinity and markedly inhibited the cytotoxic activities of these toxins. Oral administration of the Gb3 polymers protected mice after administration of a fatal dose of E. coli O157:H7, even when the polymers were administered after the infection had been established. In these mice, the serum level of Stx was markedly reduced and fatal brain damage was substantially suppressed, which suggests that the Gb3 polymers entrap Stx in the gut and prevent its entrance into the circulation. These results indicate that the Gb3 polymers can be used as oral therapeautic agents that function in the gut against STEC infections.

Shiga toxin (Stx)–producing Escherichia coli (STEC), including E. coli serotype O157:H7, causes gastrointestinal diseases in humans that are often followed by potentially fatal systemic complications, such as acute encephalopathy and hemolytic-uremic syndrome (HUS) [1–4]. During infection, STEC colonizes the gut and releases Stx into the gut lumen. The toxin is then absorbed into the circulation and causes vascular damage in specific target tissues, such as the brain and kidney, resulting in systemic complications. Therefore, the development of an effective Stx adsorbent that functions in the gut or an Stx neutralizer that functions in the circulation would be a promising approach to finding a viable therapy.

Stx is classified into 2 closely related subgroups, Stx1 and Stx2. Epidemiologic and experimental studies have suggested that Stx2 has greater clinical significance than does Stx1. Stx2–producing STEC strains are associated with the development of HUS in humans more frequently than are Stx1–producing strains [5], and Stx2–producing strains were found to cause more-severe neurologic symptoms in an experimental study of STEC–infected piglets [6]. Both Stx1 and Stx2 consist of a catalytic A subunit that has RNA N-glycosidase activity and inhibits eukaryotic protein synthesis and a pentameric B subunit that recognizes and binds to the functional cell-surface receptor globotriaosylceramide [Gb3; Galα(1–4)-Galβ(1–4)-Glcβ1-ceramide] [4, 7, 8]. Because multiple interactions of the B subunit with the trisaccharide moiety of Gb3, are known to be essential to high-affinity binding of Stx to its receptor, several Stx neutralizers containing the trisaccharide
in multiple configurations have been developed [9–12].

Recently, we developed a series of carbosilane dendrimers carrying various numbers of the trisaccharides (referred to as “SUPER TWIG”) and identified a SUPER TWIG with 6 trisaccharides [SUPER TWIG (1/6)] as an Stx neutralizer functioning in the circulation [13]. Intravenous administration of SUPER TWIG (1/6) protected STEC-challenged mice, even when SUPER TWIG (1/6) was administered after the infection had been established, which indicates that SUPER TWIG (1/6) is a promising therapeutic agent for use against STEC infection in humans [13]. On the other hand, development of an Stx adsorbent that functions in the gut is important, because oral administration of this type of agent can be widely applicable not only to treatment of individuals with STEC infection, but also to treatment of those at risk of such infections. Recently, oral administration of a genetically manipulated bacterium expressing these trisaccharides on its surface was reported to protect mice after challenge with a fatal dose of STEC [12, 14]. However, no synthetic compound has previously been developed that effectively adsorbs Stx, especially Stx2, present in the gut.

In this study, we used a series of linear polymers of acrylamide with different numbers of the trisaccharide of Gb3 to develop Stx adsorbents that would function in the gut. We found that Gb3 polymers with highly clustered trisaccharides specifically bound to both Stx1 and Stx2 with high affinity and markedly inhibited the cytotoxic activities of these toxins. The Kd values of the most active Gb3 polymer to the B subunits of Stx1 and Stx2 were even lower than those of SUPER TWIG (1/6), which indicates that this Gb3 polymer binds to the B subunits more strongly than does SUPER TWIG (1/6). Finally, oral administration of the Gb3 polymers protected mice after challenge with a fatal dose of E. coli O157:H7, which suggests that the Gb3 polymer could be used as an oral therapeutic agent to treat STEC infections in humans.

**MATERIALS AND METHODS**

**Materials.** Polymers with carbohydrates used in this study were synthesized as described elsewhere (K.M., A.M., T.W., and D.T., unpublished data), and were characterized by 1H nuclear magnetic resonance spectroscopy to confirm their structures. In brief, globotriaosyl derivatives with a polymerizable aglycon were prepared from D-galactose and D-lactose by a slight modification of the method of Matsuoka et al. [15]. Elongation of the aglycon as a spacer arm was performed by a radical addition of aminoethanethiol to the C=C double bond, followed by acryloylation to produce the acrylamide-type carbohydrate monomer. These water-soluble monomers were polymerized by a standard radical polymerization protocol [16] to produce white, powdery glycopolymers of high molecular weight after lyophilization. The molar ratio of oligosaccharide to acrylamide of each polymer was determined by 1H nuclear magnetic resonance spectroscopy. The average molecular weights of the polymers were estimated by size-exclusion chromatography in water using a Shodex Asahipak GS-510 7E column. Calibration curves were obtained using pullulan standards (5.8, 12.2, 23.7, 48, 100, and 186 kDa; Shodex Standard P-82). Free trisaccharide was kindly provided by Kyowa Hakko Kogyo (Tokyo). Recombinant Stx1 and Stx2 were prepared according to methods described elsewhere [17]. Recombinant histidine-tagged Stx1 B subunit (1B-His) and Stx2 B subunit (2B-His), in which 6 histidine residues were added at the carboxy termini of the B subunits, were prepared as follows: From the pUC118 vector and the pCH283 vector, which contained the complete coding sequences of Stx1 and Stx2, respectively (constructs were kindly provided by S. Yamasaki and T. Hamabata, International Medical Center of Japan, Tokyo) [18], an NcoI-BamHI fragment was prepared by polymerase chain reaction with the primers 5′-AGAGCGATCGGACGCGTATTGTGTAACCT-3′ and 5′-AGAGGATCCGACGAAAATAACTTCGCT-3′ for Stx1 and 5′-AGAGGATCGGATTTGTGCAAAACCTTGCTG-3′ for Stx2. The fragments obtained were ligated into the Ncol-BamHI site of the pET-28a vector (Novagen). Competent E. coli BL21DE(3) cells (Novagen) were then transformed with these vectors. The transformed BL21DE(3) cells were cultured in 1 L ofuria-Bertani broth (DiFCO) supplemented with 30 μg/mL kanamycin (Nacalai Tesque) at 37°C to midexponential phase. The cultures were subsequently treated with 1.0 mmol/L isopropyl β-d-thiogalactopyranoside (Wako Pure Industries) for 4 h at 37°C. Collected cell pellets were lysed in 10 mL of PBS containing 600 U/mL polymyxin B (Sigma). After centrifugation, the resulting supernatants were incubated with 100 μL of Ni2+-charged resin (Novagen) for 2 h at 4°C. After extensive washing of the beads, soluble 1B-His and 2B-His were eluted from the beads by incubation with elution buffer (1 mol/L imidazole, 500 mmol/L NaCl, and 80 mmol/L Tris-HCl; pH 7.9) for 5 min at 25°C. Phospholipid vesicles containing either Gb3 or globotetraosylceramide (Gb4) were prepared using phosphatidylcholine and either glycolipid (molar ratio, 24:1), as described elsewhere (X. T. Zeng, K. Nishikawa, and Y. Natori, unpublished data). 125I-labeled Stx1 (125I-Stx1) and 125I-Stx2 were prepared by the iodine monochloride method, as described elsewhere [19].

**Cells.** Vero cells were maintained in DMEM supplemented with 10% fetal calf serum. Cells were seeded in 24- and 96-well plastic microplates for binding and cytotoxicity assays, respectively.

**Kinetic analysis of Gb3 polymer binding to immobilized 1B-His and 2B-His.** Gb3, polymer binding to immobilized 1B-His and 2B-His was quantified using a BLAcore instrument [20]. Ni2+ was fixed on a nitrotriacetic acid sensor chip (BLAcore), and recombinant 1B-His or 2B-His (10 μg/mL) was injected...
into the system, where it was immobilized on the chip. Various concentrations of compounds were injected (time 0) over the immobilized 1B-His or 2B-His at a flow rate of 20 μL/min for at least 3 min to reach plateau at 25°C. The resonance unit (RU) is an arbitrary unit used by the BIAcore system. The RU value obtained without recombiant protein was subtracted from the data obtained from immobilized 1B-His or 2B-His to correct for the background. The binding kinetics were analyzed by Scatchard plot, using BIAevaluation software, version 3.0 (BIAcore).

**125I-Stx binding assay.** For the binding assay, Vero cells were treated with 1 μg/mL 125I-Stx1 or 125I-Stx2 (7 × 10⁴ or 3.8 × 10⁵ cpm/μg of protein, respectively) in the absence or presence of the desired amount of a given compound or with unlabeled Stx1 or Stx2 (50 μg/mL) for 30 min at 4°C. After extensive washing, the cells were dissolved in lysis solution (0.1 mol/L NaOH and 0.5% SDS). Recovered radioactivity was measured by a γ-counter (Packard). Specific binding of these radiolabeled Stxs was confirmed by the complete inhibition of the unlabeled Stxs (data not shown).

**Cytotoxicity assay.** For the cytotoxicity assay, subconfluent Vero cells in a 96-well plate were treated with Stx1 or Stx2 (10 pg/mL) in the absence or presence of the desired amount of a given compound for 72 h. The relative number of living cells was determined by using a WST-1 Cell Counting Kit (Wako Pure Industries).

**Mouse infection protocol.** Specific pathogen–free, 3-week-old female C57BL/6 mice that had been weaned were purchased from Charles River Laboratories. The animals were fed a low-protein diet (5% protein) for 2 weeks to achieve protein calorie malnutrition [21]. At 5 weeks of age, mice were infected intragastrically with cfu of *E. coli* which produces both Stx1 and Stx2, as described elsewhere [21]. The animals were fed the low-protein diet even after the start of the infection. Seven or 8 infected mice received Gb3 polymers (data not shown).

RESULTS

**Direct, high-affinity binding of Gb, polymers to the Stx B subunit.** We developed a series of linear polymers of acrylamide, each with a different density of the trisaccharide of Gb, [Galα(1-4)-Galβ(1-4)-Glcβ1-] or lactose (Lac) [Galβ(1-4)-Glcβ1-], through a spacer that binds the sugar group to the core structure (figure 1). The molar ratio of oligosaccharide to acrylamide was varied as shown in table 1. Polymers were indicated as X:Y, in which X and Y represent the number of carbohydrate-assembled and carbohydrate-free acrylamide units, respectively. Because the molar content of oligosaccharide per weight differed among these polymers (table 1), the concentration of each polymer was given as micromolar concentrations of trisaccharide or Lac in the following experiments, which enables direct comparison of their activities on a per-oligosaccharide basis.

With 1B-His and 2B-His immobilized on a BIAcore sensor chip, *K*ₐ for each polymer for the B subunit pentamer of Stx1 or Stx2 was determined by Scatchard plot analysis. Gb, polymer 1:0, which had the most densely clustered trisaccharides, directly bound to both 1B-His and 2B-His with very high affinity (figure 2). The *K*ₐ values determined by Scatchard plot analysis were 0.34 and 0.68 μmol/L, respectively (table 2), both of which are one-half of those for SUPER TWIG (1:6) (0.72 and 1.3 μmol/L), when comparison is made on a per-trisaccharide basis.
Figure 1. Structures of globotriaosylceramide (Gb3) and lactose polymers. Linear polymers of acrylamide with trisaccharide of Gb3 [Galα(1-4)-Galβ(1-4)-Glcβ1-] or lactose [Galβ(1-4)-Glcβ1-] are shown.

(K. Nishikawa, K. Matsuoaka, K. Hino, K. Igai, D. Terunuma, and Y. Natori, unpublished data), which indicates that the Gb3 polymer binds to the B subunits more strongly than does SUPER TWIG (1). Under the same conditions, phospholipid vesicles containing Gb3, but not Gb4, at a molar ratio of 25:1 specifically bound these recombinant B subunits, confirming their specific recognition of the trisaccharide of Gb3 (data not shown). In contrast, Lac polymer 1:0, which has a structure that is almost the same as that of Gb3 polymer 1:0, except for the terminal sugars, bound to neither 1B-His nor 2B-His (figure 2), which suggests that the terminal galactose of the trisaccharide is strictly required for high-affinity binding to the B subunits. Interestingly, the $K_d$ values of Gb3 polymers 2:17, 1:11, and 1:12 for the Stx2 B subunit were 2, 6, and 10 times higher, respectively, than the value of Gb3 polymer 1:0, whereas the $K_d$ values of all of these Gb3 polymers for the Stx1 B subunit were in a similar range (figure 2 and table 2). These results indicate that more highly clustered trisaccharides in the Gb3 polymers are required for high-affinity binding to the Stx2 B subunit, clearly demonstrating a different sugar-clustering effect in the recognition of trisaccharide between Stx1 and Stx2.

Inhibition of the biological activities of Stx by Gb3 polymers. All of the Gb3 polymers markedly inhibited the binding of $^{125}$I-Stx1 and $^{125}$I-Stx2 to Vero cells, one of the cell types most sensitive to Stx (figure 3A). The IC$_{50}$ values of Gb3 polymer 1:0 for $^{125}$I-Stx1 and $^{125}$I-Stx2 binding were 0.33 and 0.34 μmol/L (table 3), which are similar to and 10 times lower than those of SUPER TWIG (1)6 (0.33 and 3.5 μmol/L, respectively), when comparison is made on a per-trisaccharide basis. These results indicate that the inhibitory effect of Gb3 polymer 1:0 is superior to that of SUPER TWIG (1)6. In contrast, no inhibitory effect was observed with Lac polymer 1:0 or free trisaccharide, even at a concentration of 100 μmol/L (figure 3A).

The Gb3 polymers effectively inhibited the cytotoxic activity of Stx1 and, to a lesser extent, Stx2 (figure 3B). The IC$_{50}$ value of Gb3 polymer 2:17 for Stx2 was 18.8 μmol/L, which is 23 times higher than that of Gb3 polymer 1:0 (0.82 μmol/L), whereas the value for Stx1 was 0.16 μmol/L, or 3 times higher than that of Gb3 polymer 1:0 (0.049 μmol/L) (table 3). These results indicate that the dependency of the inhibitory effect on the trisaccharide density of each polymer was more clearly observed for Stx2, which further supports the hypothesis that the sugar-clustering effect in the recognition of trisaccharide for Stx1 is different from that for Stx2. No inhibitory effect was observed with Lac polymer 1:0 or free trisaccharide, even at a

Table 1. Molar content of the trisaccharide of globotriaosylceramide (Gb3) or lactose (Lac) in linear polymers of acrylamides.

<table>
<thead>
<tr>
<th>Polymer, X:Y</th>
<th>Density, mol × 10^{-3}/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gb3</td>
<td></td>
</tr>
<tr>
<td>1:0</td>
<td>1.4</td>
</tr>
<tr>
<td>2:17</td>
<td>0.75</td>
</tr>
<tr>
<td>1:11</td>
<td>0.66</td>
</tr>
<tr>
<td>1:12</td>
<td>0.63</td>
</tr>
<tr>
<td>Lac</td>
<td></td>
</tr>
<tr>
<td>1:0</td>
<td>1.7</td>
</tr>
</tbody>
</table>

a The molar ratio of oligosaccharide to acrylamides was determined by $^1$H nuclear magnetic resonance spectroscopy and described as X:Y, in which X and Y represent the nos. of carbohydrate-assembled and carbohydrate-free acrylamide units, respectively.
Figure 2. Kinetic analysis of globotriaosylceramide (Gb₃) polymer binding to immobilized Shiga toxin (Stx) B subunits, using a BIAcore system. Recombinant histidine-tagged Stx1 and Stx2 B subunits were immobilized on a nitrilotriacetic acid sensor chip (BIAcore), and the indicated amount of each compound (in µg/mL)—Gb₃ polymer 1:0, Gb₃ polymer 1:12, or lactose (Lac) polymer 1:0—was injected at time 0 over the immobilized B subunits at a flow rate of 20 µL/min for 3 min to reach plateau.

Table 2. Results of kinetic analysis of the binding of globotriaosylceramide (Gb₃) polymers to His-tagged Shiga toxin (Stx) B subunits, using a BIAcore system.

<table>
<thead>
<tr>
<th>Gb₃ polymer</th>
<th>Stx1 B subunit</th>
<th>Stx2 B subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₑ mean ± SE</td>
<td>RUₘₐₓ mean ± SE</td>
</tr>
<tr>
<td>1:0</td>
<td>0.34 ± 0.05</td>
<td>468 ± 28</td>
</tr>
<tr>
<td>2:17</td>
<td>0.44 ± 0.11</td>
<td>614 ± 60</td>
</tr>
<tr>
<td>1:11</td>
<td>0.43 ± 0.12</td>
<td>604 ± 80</td>
</tr>
<tr>
<td>1:12</td>
<td>0.60 ± 0.06</td>
<td>560 ± 6</td>
</tr>
</tbody>
</table>

NOTE. RUₘₐₓ, maximal resonance unit.

concentration of 100 µmol/L (figure 3B). Each polymer itself did not affect the cell viability (data not shown). These results demonstrate that Gb₃ polymers with highly clustered trisaccharides effectively inhibited the biological activities of not only Stx1 but also Stx2 against the target cells, which is consistent with direct and high-affinity binding of the polymers to the Stx B subunits, as described above.

Effect of Gb₃ polymers in vivo. Next, we investigated the inhibitory effects of Gb₃ polymers on the lethality of Stx-producing E. coli O157:H7 infections in mice. We used mice with protein calorie malnutrition, which are very susceptible to infection with E. coli O157:H7 [21]. In this model, the establishment of infection can be diagnosed by the detection of Stx both in stool on day 2 and in serum on day 3 after intra-gastric injection of E. coli O157:H7 [21]. We administered Gb₃ polymers intragastrically twice a day for 3 consecutive days (days 3–5). All the control animals developed neurologic symptoms after postinfection day 5 and succumbed to the infection by day 12 (figure 4). In contrast, all 5 of the mice treated with Gb₃ polymer 1:0 and 3 of the 4 mice treated with Gb₃ polymer 1:12 survived (P < .001 and P < .01, respectively) for >30 days without any neurologic symptoms (figure 4). Treatment with other Gb₃ polymers (2:17 and 1:11) also reduced lethality (2 of 2 mice survived in each group; figure 4). These results clearly indicate that the Gb₃ polymers can protect mice after challenge with a fatal dose of E. coli O157:H7, even when the polymers are administered after the infection has been established.

Stx2 content, which is more closely related to the lethality of E. coli O157:H7 infections than Stx1 content in this mouse model, was measured in serum and stool samples. The Stx2 content in serum from mice treated with Gb₃ polymers 1:0 and 1:12 decreased to an undetectable level by day 4, when the serum level of Stx2 had reached its maximum without treatment (table 4) [21]. Interestingly, the Stx2 content in stool was also substantially reduced by treatment with Gb₃ polymers 1:0 and 1:12, to one-half of and less than the control value, respectively (table 4). In an in vitro test, a high concentration of Gb₃ polymer 1:0 (≥0.5 mg/mL) did not affect the results of ELISA for detection of Stx2, even in the presence of serum or stool (data not shown), which confirms that there is a sub-
Figure 3. Inhibitory effects of globotriaosylceramide (Gb₃) polymers on the biological activities of Shiga toxin (Stx) in Vero cells. A, Results of ¹²⁵I-labeled Stx1 (¹²⁵I-Stx1) and ¹²⁵I-Stx2 binding assay. Data are presented as the percentage of activity in the absence of polymers (mean ± SE; n = 3 or 4). B, Results of cytotoxicity assay using Vero cells. Data are presented as the percentage of cell viability in the absence of Stxs (mean ± SE; n = 3). Filled diamonds, Gb₃ polymer 1:0; filled circles, Gb₃ polymer 2:17; filled triangles, Gb₃ polymer 1:11; filled rectangles, Gb₃ polymer 1:12; open rectangles, lactose polymer 1:0; open triangles, free trisaccharide.

DISCUSSION

In this study, we used a series of linear polymers of acrylamide, each with a different density of the trisaccharide of Gb₃, to develop an Stx adsorbent that functions in the gut. We found that the Gb₃ polymers with highly clustered trisaccharides specifically bound to both Stx1 and Stx2 with high affinity and markedly inhibited the biological activities, such as binding activity and cytotoxic activity toward the target cells, of these toxins.

Table 3. IC₅₀ of globotriaosylceramide (Gb₃) polymers for the biological activities of Shiga toxin (Stx) toward Vero cells.

<table>
<thead>
<tr>
<th>Gb₃ polymer</th>
<th>IC₅₀ (μmol/L) ± SE</th>
<th>In binding assay</th>
<th>In cytotoxicity assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stx1 (n = 4)</td>
<td>Stx2 (n = 3)</td>
<td>Stx1 (n = 3)</td>
</tr>
<tr>
<td>1:0</td>
<td>0.33 ± 0.04</td>
<td>0.34 ± 0.05</td>
<td>0.05 ± 0.004</td>
</tr>
<tr>
<td>2:17</td>
<td>0.33 ± 0.04</td>
<td>0.38 ± 0.07</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>1:11</td>
<td>0.25 ± 0.03</td>
<td>0.60 ± 0.13</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>1:12</td>
<td>0.33 ± 0.04</td>
<td>0.55 ± 0.13</td>
<td>0.30 ± 0.06</td>
</tr>
</tbody>
</table>

stantial mass reduction of Stx2 by Gb₃ polymer 1:0 treatment in vivo.

Because E. coli O157:H7 infection causes severe brain damage in a mouse model in which protein calorie malnutrition was used, pathological changes in cerebral blood vessels, such as cell infiltration and hemorrhage, were investigated in Gb₃ polymer 1:0–treated and untreated mice. In untreated control mice, cell infiltration (figure 5A) and hemorrhage (figure 5B) were observed in the cerebral cortex on day 5 after infection. Demyelinated nerve fibers were not noticed at the brain stems of the control animals, despite marked cell infiltration (figure 5C), which is consistent with findings we have published elsewhere [21]. In contrast, no histological changes were observed in the brains of Gb₃ polymer 1:0–treated mice, even at day 30 after infection (data not shown). In the hippocampus of untreated mice, immunoreactions for Stx2 were detected (figure 5D); those reactions were absent in the brain of Gb₃ polymer 1:0–treated mice (figure 5E). These results suggest that Gb₃ polymer 1:0 suppressed the lethality of E. coli O157:H7 infection by reducing the serum level of Stx2 and subsequent Stx2-associated fatal brain damage.
length of Gb₃ can have an important effect on the extent to which Stx1 and Stx2 bind to Gb₃ [23]. When these data are considered with the results of our present study, it is highly possible that not only the high density of trisaccharides, but also the long alkyl spacer present in the Gb₃ polymers is required for high-affinity binding to Stx2. In a recent report, in which self-assembled monolayers of Gb₃ mimics that contain the tri-saccharide with alkyl chains of different lengths were used, it was demonstrated that Stx2, but not Stx1, preferred a longer alkyl chain for high-affinity binding [24], which further supports our contention.

We found that oral administration of the Gb₃ polymers protected mice against a fatal dose of *E. coli* O157:H7 and that Stx2 content in serum samples from such mice was substantially reduced, compared with levels in serum from untreated mice. Although these Gb₃ polymers are heterogeneous in their molecular size, the average molecular sizes of Gb₃ polymers 1:0 and 1:12 were determined to be 36 and 73 kDa, respectively, by gel permeation chromatography (data not shown), both of which can be calculated to contain ~50 trisaccharides/molecule of these compounds. Judging by all of these findings, it is highly possible that Gb₃ polymers bind to Stx2 in multiple ways to form large complexes in the gut, thereby inhibiting the entrance of Stx2 into the circulation and resulting in a reduction in the serum level of Stx2. Interestingly, we found that the Stx2 content in stool samples was also reduced by treatment with Gb₃ polymers. Although the precise mechanism of this reduction remains to be elucidated, this phenomenon may reflect another aspect of the mechanism by which the Gb₃ polymers effectively function as oral therapeutic agents in the gut.

In a previous report, it was shown that oral administration of another chemically synthesized Stx adsorbent (Synsorb-Pk; Synsorb Biotech), which consists of globotrisaccharide covalently linked to silica particles [9, 25], did not protect mice against oral challenge with STEC, although it delayed time to

### Table 4. Quantification of Shiga toxin (Stx) 2 on day 4 after infection with *Escherichia coli* O157:H7 in stool and serum samples from mice treated with globotriaosylceramide (Gb₃) polymers or saline.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean concentration of Stx2 ± SE*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In stool, pg/mg (n = 3)</td>
</tr>
<tr>
<td>Gb₃ polymer</td>
<td></td>
</tr>
<tr>
<td>1:0</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>1:12</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>Saline</td>
<td>71 ± 6</td>
</tr>
</tbody>
</table>

**NOTE.** — lower than the limit of detection.

* The limits of detection were 12 pg/mg of stool and 18 pg/mL of serum, respectively.

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### Figure 4. Inhibitory effect of globotriaosylceramide (Gb₃) polymers on the lethality of infection with *Escherichia coli* O157:H7 in mice. Mice with protein calorie malnutrition were infected intragastrically with *E. coli* O157:H7 strain N-9 (2 × 10⁶ cfu) on day 0. Gb₃ polymer (25 µg/g of body weight) or saline alone was administered intragastrically to the mice (control group, n = 12; Gb₃ polymer 1:0, n = 5; Gb₃ polymer 2:17, n = 2; Gb₃ polymer 1:11, n = 2; Gb₃ polymer 1:12, n = 4) twice a day on days 3–5 after infection. Data are the survival time of each mouse. The data were analyzed by Kaplan-Meier survival analysis or, when no mice had died by the end of the observation, by Fisher’s exact test.

Toxins. The Kᵢ values of the Gb₃ polymer 1:0, the most active Gb₃ polymer, for the B subunits of Stx1 and Stx2 were even lower than those of SUPER TWIG (1:6), which indicates that the Gb₃ polymer binds to both Stxs more strongly than does SUPER TWIG (1:6). Interestingly, we found that the sugar-clustering effect in the recognition of trisaccharide for Stx1 was different from that for Stx2 and that more highly clustered trisaccharides in the Gb₃ polymers are required for high-affinity binding to Stx2. This observation provides an important insight into the development of Stx adsorbents, especially against Stx2, which has greater clinical importance than Stx1.

Recently, Dohi et al. [22] reported a linear polymer of acrylamide that contained the trisaccharide attached to a spacer of a phenyl group. This compound substantially inhibited the cytotoxic activity of Stx1 toward human renal adenocarcinoma ACHN cells, another cell type that is very sensitive to Stx, but did not show any inhibitory effect on Stx2 at concentrations ≤100 µmol/L on a per-trisaccharide basis. The major difference between this compound and the Gb₃ polymers developed in the present study is the length of the spacer arm through which the trisaccharide group binds to each core structure. On the other hand, it is generally accepted that the fatty acid chain...
Figure 5. Histological examination and immunostaining of Shiga toxin (Stx) 2 in the brains of mice treated with globotriaosylceramide (Gb3) polymer 1:0 (E) or not treated (A–D) and infected with Escherichia coli O157:H7. Sections of the cerebral cortex were used for histological examination. The sections were stained with hematoxylin-eosin (A and B; original magnification, ×450) or Luxol fast blue (C; original magnification, ×150). Stx2 present in sections of the hippocampus was detected using specific antibody against Stx2 (D and E; original magnification, ×450). The sections were stained afterward with hematoxylin.

death by 1 day [26]. The Stx-binding capacity of Synsorb-Pk is at least 100,000 times lower than that of the Gb3 polymers estimated from our present results (table 3 and figure 3A); this is probably the result of the low density of trisaccharide displayed on the surface of Synsorb-Pk, which is ~2000 times lower than that of the Gb3 polymers [9]. Therefore, the marked inhibitory effect of the Gb3 polymers on the lethality of STEC infections may be mainly attributed to the superiority of the capacity of Gb3 polymers to bind toxin.

All of the results of our present study indicate that Gb3 polymers can be used as an oral therapeutic agent to treat STEC infections in humans. This type of agent is expected to have significant therapeutic advantages, because it can be widely applicable not only to individuals with STEC infection, but also to those at risk for such infections.

Acknowledgments

We thank Shinji Yamasaki and Takashi Hamabata for providing us with the pUC118 vector and the pCH283 vector that contained the complete coding sequences for Shiga toxins 1 and 2, respectively.

References

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