Restricted Expression of Shiga Toxin Binding Sites on Mucosal Epithelium of Mouse Distal Colon

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Shiga toxins (Stx) are some of the major virulence factors of enterohemorrhagic Escherichia coli strains such as serotype O157:H7. To analyze how Stx might initially gain access to the bloodstream from sites of infection, frozen sections of mouse colon were immunohistochemically examined for binding sites for recombinant binding subunits (Stx1B). Binding sites were selectively expressed on the epithelium in the distal half of the mouse colon, whereas the proximal half did not exhibit any binding sites. In agreement with this observation, we also demonstrated the distal-part-restricted distribution of glycolipids that bind to Stx1B in the mouse colon. For comparison, the binding sites of several control lectins were also examined. Selective binding to the distal part of the colon was not seen with any other control lectins, including Griffonia simplicifolia lectin-I isolectin B4 (GS-I-B4), which shares α-galactose specificity with Stx1B. Partial overlapping of the specificities of Stx1B and GS-I-B4 was seen by assay with globotriose-conjugated multivalent ligands. The results indicate that Stx1B is stricter in the recognition of carbohydrate determinants than GS-I-B4 when examined with biological ligands.

Infection by enterohemorrhagic Escherichia coli (EHEC) strains, such as serotype O157:H7, can result in life-threatening complications, including hemolytic-uremic syndrome (HUS) and central nervous system involvement (19). Shiga toxins (Stx), also called “verotoxins,” are exotoxins and virulence factors of EHEC. The Stx consist of a toxin component (A subunit) that inhibits protein synthesis and a cell-binding component (B subunit) (1, 23). B subunits form a pentamer and recognize specific carbohydrate determinants, such as those displayed on the glycolipid globotriaosylceramide (Gb3; Galα1-3Galβ1-4Glcβ1-1Ceramide) (10, 12).

Glycolipid ligands specific for Stx on endothelial cells have gained much attention, especially in the context of damage to the brain microvasculature. The expression of Gb3 has been reported to increase on human cerebral endothelial cells upon in vitro treatment with proinflammatory cytokines, such as tumor necrosis factor (2). In mice, upon experimental infection by E. coli O157:H7, an increase in the serum Stx level was followed by apoptosis of brain cells. The presence of Gb3 as a functional ligand has been demonstrated in mouse brain (9).

Despite these studies of ligands on endothelial cells in target organs, intestinal glycoconjugates have not been clearly demonstrated to be involved in the entry of Stx at the sites of infection. Furthermore, the binding sites for Stx on the intestinal epithelium at or near the sites of EHEC infection have not been well characterized. How Stx initially gains access to the bloodstream upon EHEC infection is still not fully understood, although one possible mechanism has been proposed: i.e., neutrophil transmigration at a site of infection may play a significant role in the entry of Stx (5). In this study, we analyzed the binding sites for Stx in mouse colon by using a sensitive immunohistochemical probe as well as a biochemical approach.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female CD-1 (ICR) mice were purchased from SLC Japan (Shizuoka, Japan) and used at 6 to 8 weeks of age. Animal care and experiments were performed in accordance with the ethical guidelines for the animal facilities of the University of Shizuoka.

Reagents. Recombinant binding subunits of Stx1 (Stx1B) were expressed in E. coli JM105 (pVT1-B5) cells, and proteins were purified to homogeneity by anion-exchange chromatography and chromatofocusing as described previously (16). The purified Stx1B subunits were labeled with digoxigenin (DIG-Stx1B) as described previously (16). A multivalent carbohydrate ligand (FITC-Gb3-PLL) consisting of a poly-L-lysine (PLL) backbone conjugated with globotriose and tagged with fluorescein isothiocyanate (FITC) was prepared as described previously (7). Horseradish peroxidase (HRP)-labeled sheep anti-digoxigenin (DIG Fab fragments (HRP-anti-DIG) were purchased from Roche Diagnostics (Tokyo, Japan). Biotinylated Aleuria aurantia lectin (AAL), biotinylated Lycopersicon esculentum lectin (LEL), biotinylated Ulex europaeus agglutinin I (UEA-I), biotinylated Arachis hypogaea lectin (PNA), biotinylated Griffonia simplicifolia lectin-I isolectin B4 (GS-I-B4), HRP-avidin D, and a 3-aminooxyethylcarbazole (AEC) substrate kit were purchased from Vector (Burlingame, Calif.), and HRP-rabbit anti-FITC antibody was purchased from Dakopatts (Glostrup, Denmark). Bovine serum albumin (BSA) fraction V and PLL were purchased from Sigma (St. Louis, Mo.), and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Wako Pure Chemicals (Osaka, Japan). Standard glycolipids Galβ3, and galactosylceramide (GalCer; Galβ1-1Cer) were prepared from porcine erythrocytes and bovine brain, respectively, as described previously (15).

Immunohistochemistry. Mouse kidneys or colon fragments were embedded in OCT compound (Miles, Elkhart, Ind.) and then frozen in a liquid nitrogen bath. Immunostaining was performed as described previously (6, 20). In brief, cryostat sections (10 μm thick) were picked up on PLL-coated slides and air dried. The sections were fixed in acetone for 30 s at 20°C. Nonspecific binding sites were blocked with 3% BSA in calcium- and magnesium-free Dulbecco’s phosphate-buffered saline.
were combined and evaporated. Each sample was dissolved in 4 ml of chloroform, was cut into small pieces. Kidneys were crushed with a mortar and pestle. Proximal (less than 3 cm below the cecum) and distal (more than 5 cm below the cecum) parts of the colon from 10 mice were separately pooled. Each part of the colon was cut into small pieces. Kidneys were crushed with a mortar and pestle. The tissue samples were lyophilized and weighed: proximal colon, 330 mg; distal colon, 383 mg; kidneys, 688 mg (dry weight). Each dry sample was serially extracted with chloroform-methanol (2:1, 1:1, and then 1:2), and the extracts were combined and evaporated. Each sample was dissolved in 4 ml of chloroform-methanol (2:1), and aliquots of 10 μl (colon) or 5 μl (kidney) were separated on a thin-layer chromatography (TLC) plate (Polygram Sil G, Macherey-Nagel, Germany) in chloroform-methanol-water (65:35:8 [vol/vol/vol]) as described previously (16). As references, GalCer (2.5 μg) and Gb3 (5 μg) were also applied. The plates were subjected to immunostaining with DIG-Stx1B or orcinol-sulfuric acid as described previously (16).

ELISA. A solid-phase binding assay with FITC-Gb3-PLL was performed as described previously (7). In brief, purified Stx1B or one of the biotin-conjugated lectins was allowed to adsorb to the wells of a multilayer immunologic assay (ELISA) plate (catalog no. 9018; Costar, Corning, N.Y.) in PBS for 16 h at 4°C. Nonspecific binding sites were blocked with 1% BSA-PBS for 2 h at 20°C. To detect the binding of carbohydrate ligands, FITC-Gb3-PLL (100 μl per well) was added with PBS containing 0.1% Tween 20 (Sigma) and 0.1% BSA (PBS-Tween) to the wells. After incubation for 1 h at 20°C, the bound ligands were washed three times with PBS-Tween. As a control, the wells were incubated with 100 μl of either DIG-Stx1B (4 μg/ml) or unlabeled Stx1B (data not shown). These results establish the specificity of the binding.

Comparison of the binding pattern with those of other lectins. We simultaneously compared the binding patterns of various lectins to clarify whether or not the binding pattern of Stx1B is just a reflection of general differences in the glycoconjugates depending on the position in the colon. Representative results are shown in Fig. 1, and the data are summarized in Table 2. We examined 10 mice and found no variation from mouse to mouse in the lectin binding patterns in the colon tissue. No other lectins caused such a great difference in the staining patterns between the proximal and distal parts of the colon as that produced by Stx1B, although some differences were observed. GS-I-B4, which recognizes α-galactose residues (4), produced strong staining of the apical surface of the proximal colon, while such a position-specific pattern was not seen for other components of the colon. NPA, which recognizes the asialo-Gaβ1,3GalNAc moiety (21), produced intense apical staining of the distal part of the colon, while it stained the crypts of Lieberkühn rather intensely in the proximal colon. LEL, which recognizes the poly-N-acetyllactosamine structure (8), did not stain the epithelium intensely. Two fucose-specific lectins, UEA-I (13, 14) and AAL (26), produced intense staining of the epithelium, especially the crypts of Lieberkühn. However, no difference in binding was observed between the proximal and distal parts of the colon. The lamina propria of the colon was not stained by Stx1B, UEA-I, or AAL. In contrast, GS-I-B4, PNA, and LEL stained the lamina propria rather strongly, and the staining patterns were not different between the proximal and distal parts of the colon. Therefore, the binding pattern of Stx1B, which was highly selective for the epithelium of the distal part of the mouse colon, was unique among those of all lectins tested.

Comparison of carbohydrate binding activity. We examined whether the carbohydrate specificity could be partially common to Stx1B and other lectins. We investigated this by means of a solid-phase binding assay using multivalent ligands tailored for Stx1B. As we have already reported (7), the ligands

FIG. 1. Comparison of the patterns of binding of Stx and various lectins to mouse colon. Acetone-fixed frozen sections of the proximal (<4 cm below the cecum) (A, C, D, G, H, K, and L) or distal (>5 cm below the cecum) (B, E, F, I, J, M, and N) part of the colon were examined separately. The sections were stained with DIG-Stx1B plus HRP-anti-DIG (A, B, C, and E), biotin-GS-I-B4 (D and F), biotin-PNA (G and I), biotin-LEL (H and J), biotin-UEA-I (K and M), or biotin-AAL (L and N). The binding of biotin-conjugated lectins was revealed with HRP-avidin D. The distal part of the colon expressed binding sites for Stx1B (B and E). In contrast, the proximal part of the colon was not stained by Stx1B (A and C). Other lectins did not show a similar site-selective binding pattern. Arrowheads indicate the luminal surface. Arrows indicate the lamina propria (A, C, H, I, and J) or crypts of Lieberkühn (B, E, G, K, L, M, and N). Bars represent 25 μm (A and B [original magnification, ×360]) or 100 μm (C to N [original magnification, ×90]).

RESULTS

Site-selective expression of binding sites for Stx in mouse colon. Frozen sections of mouse colon were examined for binding sites for recombinant B subunits (DIG-Stx1B) by an immunohistochemical approach. The binding patterns were dramatically different between the proximal and distal parts of the colon. No staining was observed in the proximal part of the colon, indicating the absence of carbohydrate ligands (Fig. 1A and C). In contrast, the epithelium of the distal part of the colon possessed a number of binding sites for DIG-Stx1B, especially in the crypts of Lieberkühn and on the surface of the lumen (Fig. 1B and E). To determine where the transition takes place, frozen sections of mouse colon were examined at 1-cm intervals. Table 1 summarizes the data obtained for four individual mice. The binding of DIG-Stx1B to samples of the colon at positions close to the cecum (less than 4 cm below) was marginal. In contrast, sections from positions far from the cecum (more than 5 cm below) exhibited intense staining of the mucosal epithelium and luminal surface. Such an uneven distribution in the staining pattern was statistically significant (P < 0.005 by the Friedman test).

To check specificity of the binding, DIG-Stx1B was applied to the tissue sections in the presence of 50 times excessive amounts of unlabeled Stx1B. Staining of the epithelium of the distal colon was completely inhibited by unlabeled Stx1B (data not shown). Binding of DIG-Stx1B to the renal tubules and collecting ducts from mouse kidneys (6) was also eliminated by unlabeled Stx1B (data not shown). These results establish the specificity of the binding.

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bound to the immobilized Stx1B (Fig. 2A). An α-galactosidespecific lectin (GS-I-B4) produced significant binding of the ligands, whereas β-galactoside-reactive lectins (PNA and LEL) did not. A weak interaction with fucose-specific UEA-I was observed, but the level of background binding without the ligands was higher than that produced by other lectins. Similarly, the level of binding of second reagents was too high to evaluate ligand binding to immobilized AAL (data not shown). To confirm the adsorption of lectins, the biotin tags of immobilized lectins were detected (Fig. 2B). The amounts of immobilized lectins, as judged by the binding of HRP-avidin D, were saturated when more than 0.05 μg of lectins was added per well. The results confirmed that sufficient amounts of immobilized lectins were available in the ligand binding experiments when the lectins were immobilized at 0.5 μg per well.

**Restrict expression of glycolipid ligands in the distal colon.** To determine whether the selective binding of Stx1B could be due to the differential distribution of glycolipid Gb3 in the colon, tissue fragments from the proximal (less than 3 cm below) and distal (more than 5 cm below) parts of the colon were separately extracted with chloroform-methanol. After separation on a TLC plate, binding of DIG-Stx1B was examined (Fig. 3). A strong signal reflecting the binding of DIG-Stx1B to the band that exhibited similar mobility to the standard Gb3 was observed for the sample from the distal part of the colon (lane 9). On the other hand, only a faint band was seen at the corresponding position for the sample from the proximal part of the colon (lane 8). Extracts of kidneys gave a band exhibiting DIG-Stx1B reactivity at the corresponding position (lane 10), but the staining intensity of the band was much weaker than that in the case of the distal colon. Nonspecific adsorption of Stx1B to faster-migrating materials found in both the distal and proximal colon extracts (lanes 8 and 9) was observed. These materials could be neutral lipids or cholesterol under the present TLC conditions. As we have already reported (16), DIG-Stx1B bound to purified GalCer (lane 6).

**DISCUSSION**

Binding sites for Stx1B in the mouse colon were examined by an immunohistochemical approach. The binding sites were restricted to the epithelium of the distal part of the colon. The proximal part of the colon did not show any binding. Because a dramatic transition in the staining pattern was seen in the middle of the colon, the expression of carbohydrate ligands seems to be a reflection of the developmental origin and blood supply: in humans, the proximal part is of midgut origin and is supplied by the superior mesenteric artery, and the distal part is of hindgut origin and is supplied by the inferior mesenteric artery (17).

Although EHEC strains cause hemorrhagic colitis, it is not well known how Stx initially gain access to the bloodstream at an early stage of infection. It has not been determined whether the cytotoxicity of Stx against intestinal epithelial cells could...
lead to entry of the Stx into the bloodstream due to a breach in the epithelial barrier. On the other hand, in an experiment involving a polarized T84 human cell line, the translocation of Stx (apical to basal) was enhanced when neutrophil transmigration (basal to apical) was induced by chemotactic factors (5). In addition, Stx have been reported to induce the production of interleukin-8 (IL-8) by an Hct-8 colonic carcinoma cell line (24). These studies suggested that neutrophil chemotactic factors (including IL-8) locally available at the sites of infection might induce neutrophil transmigration. The transient reduction in the barrier function of the epithelium caused by the neutrophil transmigration was suggested to be responsible for the passive transfer of Stx into the tissue parenchyma. However, these observations were based on experiments involving cell lines, and the concentration of Stx used to demonstrate translocation was high. Hence, whether passive translocation is the only mechanism responsible for the entry of Stx into the bloodstream remains uncertain.

In clinical situations, studies of the colonic pathology of children who had developed HUS demonstrated that the left and transverse portions of the colon (distal parts of the colon) were the most severely and earliest affected regions of the colon (18). Although one should be cautious about extending speculation about the distribution of Gb3 in the mouse, it will be interesting to see how Gb3 might be distributed in the human colon in relation to human diseases.

There are several publications describing a mouse model in which streptomycin-treated mice were orally challenged with EHEC strains (11, 25). The EHEC strains heavily colonized the entire gastrointestinal tract without any pathological changes in colon tissues. Although extensive renal tubular necrosis was observed in moribund mice, no lesions were reported in the colon epithelium. However, more recent studies revealed intestinal hemorrhage and the depletion of goblet cells from the colon tissues in a model of EHEC infection of germfree mice (22).

The differential expression of the binding sites for Stx1B is not trivial, because several control lectins did not produce differential staining of the proximal and distal colon. Nevertheless, this partial overlap in specificity, GS-I-B4 did not produce a
staining pattern restricted to the distal colon. This is probably due to the abundant and rather wide distribution of glycoconjugates with terminal α-galactose, which is not necessarily a part of the Galα1-4Gal determinant, in the mouse colon. Two lectins with β-galactoside specificity (PNA and LEL) did not bind to FITC-Gb₃-P.LL. They bound with a different pattern to mouse colon. In the case of PNA, the staining of the apical surface was restricted to the distal part of the colon, whereas the cells in the crypts of Lieberkühn were only stained in the proximal part of the colon. AAL, a lectin related to UEA-I in specificity, also bound to the mouse colon epithelium regardless of the position in the colon. The results indicate that the highly selective expression of the ligands for Stx in the distal part of the colon is unique among those of the lectins examined in the present study.

We next investigated the biochemical basis of the differential expression of the ligands in the epithelium of mouse colon. Extractions from the distal part of the colon contained glycolipids that bound to DIG-Stx1B. These are presumably Gb₃ because they showed mobility similar to that of the standard glycolipid on a TLC plate. On the other hand, the proximal part of the colon was essentially devoid of the corresponding glycolipid ligands. This pattern of the distribution of glycolipid ligands in the mouse colon agrees with that observed with the immunohistochemical approach.

In conclusion, the Stx binding sites in the mouse colon were found to be restricted to the epithelium of the distal part of the colon. The results may suggest that epithelial glycolipid ligands selectively expressed in the distal part of the colon may contribute to the entry of toxins upon EHEC infection. However, one could not exclude the possibility that the avid binding of Stx to the glycolipid ligands may prevent Stx from penetration into underlying tissues or the bloodstream. It would be interesting to see whether Stx could exert selective toxicity against epithelial cells from the distal colon.

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