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Binding of the Fibrillar CS3 Adhesin of Enterotoxigenic *Escherichia coli* to Rabbit Intestinal Glycoproteins Is Competitively Prevented by GalNAcβ1-4Gal-Containing Glycoconjugates

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We have attempted to characterize the binding specificity of the coli surface 3 (CS3) subcomponent of colonization factor antigen II of enterotoxigenic *Escherichia coli*, by means of an immunoblot method in which the binding of fimbriated bacteria to sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated rabbit intestinal cell membranes was evaluated. Isolated CS3 fibrillae as well as bacteria expressing CS3 on their surface bound to several intestinal cell membrane structures, i.e., structures present in the electrophoretic front and in the 30- to 35-kDa range and, most prominently, 120- to 140-kDa structures. Delipidization and protein digestion of the rabbit brush borders revealed that CS3 bound to structures of a proteinaceous nature. Sodium *meta*-periodate oxidation of the intestinal cell membranes abolished all their CS3 binding activity, indicating that CS3 bound to carbohydrate moieties of glycoproteins. The binding of CS3 to the separated intestinal proteins could also be inhibited by preincubation with the lectin derived from *Maackia amurensis*, indicating that CS3 bound to galactoproteins in the rabbit intestine. Inhibition experiments using equimolar amounts of various gangliosides demonstrated that GM1, asialo-GM1, and GM2 inhibited the binding of CS3 equally well, whereas GM3 was not as effective. These results suggested that the critical CS3 binding epitope consisted of the carbohydrate sequence GalNAcβ1-4Gal. This was supported by electron microscopic experiments showing that this disaccharide, O linked to bovine serum albumin via a spacer, localized around CS3-positive bacteria but not at all around corresponding CS3-negative mutants. Furthermore, CS3-expressing bacteria recognized this neoglycoprotein when it was immobilized on nitrocellulose. The GalNAcβ1-4Gal disaccharide has also been implicated as a binding structure for other pathogenic bacteria such as enteropathogenic *E. coli* and *Pseudomonas aeruginosa*.

Enterotoxigenic *Escherichia coli* (ETEC) is one of the major causes of morbidity and mortality among children in developing countries (1). These enteric pathogens give rise to disease by colonizing the small intestinal mucosa and thereafter elaborating one or two enterotoxins, heat-stable toxin (ST) and heat-labile toxin (LT), which induce massive fluid and electrolyte secretion into the gut lumen. Colonization encompasses the adhesion of bacteria to the intestinal epithelium and possibly also to the mucus layer by means of fimbrial or fibrillar antigens named colonization factor antigens (CFAs). Hitherto, a large number of CFAs have been described, the most well-established ones being CFA/I (6), CFA/II (16), and CFA/IV (30). The last two are composed of antigenically distinct structures called coli surface antigens, of which CS1, CS2, and CS3 belong to CFA/II and CS4, CS5, and CS6 belong to CFA/IV. In addition, a whole series of new adhesive structures of a fimbrial nature have been found on clinical isolates which were negative for CFA/I, CFA/II, and CFA/IV (19).

Very little is known about the receptors or binding structures for the different CFAs. CFA/I can bind to free sialic acid (5), sialic acid-containing glycoproteins (7, 25, 33), and GM2 (3, 17). It has been shown that CS3-expressing bacteria bind to differentiated HT-29 cells and that this binding could be inhibited by polylactosaminoglycan-containing glycopeptides isolated from human meconium (22). Furthermore, it has been demonstrated that all of the CFA/II subcomponents can bind to asialo-GM1 in thin-layer chromatograms (24).

In this study, we have attempted to characterize the binding specificity of CS3-mediated adhesion of ETEC to intestinal glycoproteins isolated from the mucosa of the rabbit small intestine. The rationale for this approach is the known ability of CS3-expressing ETEC bacteria to attach to both human and rabbit intestinal mucosae (13, 33). The binding of purified CS3 fibrillae, CS3-expressing bacteria, and selected lectins of known carbohydrate specificity was assayed. Inhibition experiments were also performed using well-characterized antibodies and various glycoconjugates of defined structures. The reason for studying these fibrillae is that CS3 is ubiquitously expressed on CFA/II-carrying bacteria with the exception of a few rare CS2-only strains. Furthermore, CS3-only strains and strains expressing CS1-CS3 or CS2-CS3 adhere equally well to intestinal cells (13, 22), implying that CS3 plays the major role in the ability of CFA/II strains to bind to enterocytes.

MATERIALS AND METHODS

Intestinal brush border membranes. A modification of the procedure described by Miller and Crane (20) was used for the preparation of brush border membranes from the small intestines of adult and 6- to 7-day-old New Zealand White rabbits. Briefly, segments of small intestine were excised and thoroughly washed with ice-cold phosphate-buffered saline (PBS), pH 7.2. With a glass slide, the mucosa was scraped off in cold 5 mM EDTA, pH 8.0, and homogenized in a blender (Waring Products, New Hartford, Conn.) at maximum speed for 1 min. The homogenate was then filtered through a nylon mesh and centrifuged at 200

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× *g* for 10 min to remove any whole cells or cell aggregates. Subsequently, the supernatant containing the intestinal brush borders was sedimented by centrifugation at 10,000 × *g* for 10 min at 4°C and washed twice in 5 mM EDTA. The final pellet was resuspended in PBS and stored at −18°C until used.

Bacteria. The following enterotoxigenic *E. coli* strains were used: E1392-75 (O6:K15:H16 $CS1^+$ $CS3^+$ ST^+ LT^+), 58R61 (O6:H16 $CS2^+$ $CS3^+$ ST^+ LT^-), E19446 (O139:H28 $CS1^-$ $CS3^+$ ST^+ LT^+), 60R315 (O139:H28 $CS1^-$ $CS3^-$ ST^- LT^-), and 60R936 (O139:H28 $CS1^+$ $CS3^-$ ST^- LT^-). Strains were kindly provided by B. Rowe, Colindale Public Health Laboratory, London, United Kingdom.

Purified CFAs. Purified CFA/II (CS1-CS3) was prepared from strain E1392-75 by homogenization with a Waring blender followed by salt and isoelectric precipitation and column chromatography (12).

Delipidization of rabbit intestinal brush borders. Lipids were removed from rabbit intestinal brush borders by two extractions with chloroform-methanol-water (4:8:3, vol/vol/vol) (29). The delipidized cell membrane fraction was collected as a sediment after centrifugation at 10,000 × *g* for 10 min and resuspended in PBS. The capacity of CS3 to bind to the delipidized fraction was assayed by the immunoblot method described below.

Proteinase digestion of rabbit intestinal brush borders. A preparation of rabbit intestinal brush borders containing approximately 30 μg of protein was incubated with 0.1 mg of proteinase K (Sigma Chemical Co., St. Louis, Mo.) and 1% (wt/vol) sodium dodecyl sulfate (SDS) for 2 h at 65°C. The rabbit brush borders were then washed in PBS and thereafter assayed for the presence of CS3-binding activity in the immunoblot assay described below.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in 11% polyacrylamide slab gels essentially as described by Laemmli (15) with the modification that bisacrylamide was replaced by *N,N*-dialyltartardiamide of corresponding molarity as a cross-linker. Pyronin Y (Sigma) was used as a marker of the electrophoretic front. All other reagents, including molecular-weight reference protein kits, were purchased from Bio-Rad Laboratories (Richmond, Calif.).

Immunoblot assay. The immunoblot assays were performed essentially as described by Towbin et al. (31). Nitrocellulose strips with transblotted intestinal brush border preparations or the neoglycoprotein GalNAcβ1-4Gal–*O*-2-(2-carbomethoxyethylthio)ethyl (CETE)–bovine serum albumin (BSA) (Bio-Carb Chemicals, Lund, Sweden, and Oxford Glycosystems, Oxford, United Kingdom) were blocked with 1% BSA for 20 min, washed for 10 min with two changes of PBS, and thereafter incubated with either purified CFA/II or CFA/II-expressing bacteria. Purified CFA/II was diluted to 3 μg/ml in an 0.1% BSA–PBS–0.05% Tween (BSA-PBS-T) solution. Whole bacteria were used in concentrations of 10^8 to 10^9 bacteria per ml in the same solution, with the addition of 0.5% (wt/vol) D-mannose. Purified fimbriae or whole bacteria were incubated at 37°C for 2 h. Following three 5-min washes with PBS–0.05% Tween (PBS-T), the nitrocellulose strips were incubated for 2 h with a mouse monoclonal antibody (MAb) against CS3, i.e., MAb 10:2 (18), diluted 1/500 in BSA-PBS-T. Next, the strips were again washed thrice in PBS-T before the final incubation with horseradish peroxidase-labelled rabbit anti-mouse antibody (Dakopatts, Copenhagen, Denmark) at a dilution of 1/200 in BSA-PBS-T. Thorough washing with PBS-T followed by PBS preceded incubation with the enzyme substrate, which included H_2O_2 and 4-chloro-1-naphthol (Bio-Rad). All incubations were performed at room temperature with an orbital shaker except for the incubation step including whole bacteria, which took place at 37°C without shaking.

Sodium *meta*-periodate oxidation of rabbit intestinal brush borders. Sodium *meta*-periodate (Sigma) was used at a 100 mM concentration in a 0.1 M sodium acetate buffer, pH 5.2. The rabbit intestinal brush borders were oxidized by this solution either before or after SDS-PAGE and electrotransfer to nitrocellulose. In either case the brush borders were incubated with the sodium *meta*-periodate solution in the dark at room temperature overnight. Following incubation, the brush borders were washed thrice in PBS, either by sedimenting the brush borders at 10,000 × *g* or by incubating the nitrocellulose strips with the transferred brush border membranes in PBS.

Inhibitors of CS3 binding. The following substances were tested for their ability to inhibit the binding of CS3 to the electrophoretically separated intestinal cell membranes: a mucin-like glycopeptide fraction and its defucosylated and desialylated derivative, isolated from a pool of meconium of Swiss newborn children (22); bovine glycophorine, human erythrocyte ghosts, and desialylated bovine caseinoglycomacropeptide (23); neoglycopeptides consisting of the disaccharides GalNacβ1-4Gal and Galβ1-4Glc, both of which were β-anomerically O linked to a spacer covalently bound to BSA (Bio-Carb Chemicals); monosaccharides and gangliosides (Sigma); nonlabelled lectins (Boehringer Mannheim Biochemica, Mannheim, Germany); and Galβ1-3GalNAc disaccharide (generously provided by J.-E. Månsson, Göteborg, Sweden). All inhibitors were diluted in BSA-PBS-T, and when needed, the pH of the solution was adjusted to around 7 with NaOH. Asialo-GM1 was first diluted in pure methanol before further dilution with BSA-PBS-T. A polyclonal rabbit antiserum directed against asialo-GM1 was also tested for CS3 inhibitory activity (Wako Chemicals GmbH, Neuss, Germany).

In addition, a glycoprotein detection kit based on digoxigenin-labelled lectins was employed in the determinations of the glycoprotein nature of the CS3-binding rabbit intestinal proteins. Briefly, the intestinal rabbit proteins which had been electrophoretically resolved and transblotted onto nitrocellulose sheets were blocked with a blocking solution, and then labelled lectins, antidigoxigenin antibody conjugated to alkaline phosphatase, and the appropriate chromogen and enzyme substrate were sequentially added, all according to instructions given by the manufacturer (Boehringer Mannheim). Nonlabelled lectins were used in inhibition experiments of CS3 binding at a concentration of 1 mg/ml.

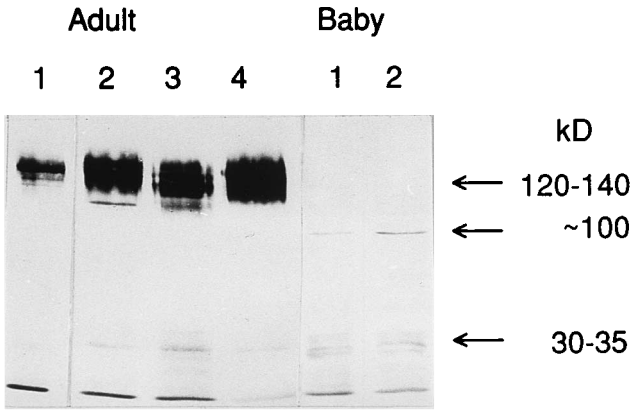


FIG. 1. Binding of purified CFA/II fimbriae to electrophoretically separated and transblotted intestinal proteins from adult and infant rabbits. Bound CS3 was detected with MAb 10:2 (anti-CS3).

Hemagglutination assays. The capacity of CS3-only bacteria to hemagglutinate human A erythrocytes with and without incorporated GM1 was tested in microtiter plates as previously described (10).

Electron microscopic studies. The capacity of two gold-labelled neoglycoproteins, consisting of either of two disaccharides, i.e., Galβ1-3GalNAc or GalNAcβ1-4Gal, both in β-anomeric configuration and O linked to BSA (Ey Laboratories Inc., San Mateo, Calif.), to bind to a CS3-expressing ETEC strain (E19446) and its CS3-negative mutant (60R315) was studied by electron microscopy. Bacteria were grown on CFA agar overnight, washed once in PBS, and directly suspended in distilled water. Ten microliters of the bacterial solution was added to Formvar-carbon-coated copper grids (Ted Pella Inc., Redding, Calif.) and left to settle on the grids for 2 min. Thereafter, the grids were immediately placed on drops of BSA-PBS-T containing the gold-labelled neoglycoproteins diluted 1/20. After a 45-min incubation period, the grids were initially washed in 2 drops of BSA-PBS-T and thereafter in 8 drops of distilled water. CS3 fibrillae were visualized by placing grids coated with bacteria (see above) on drops containing MAb 10:2 diluted 1/200 in BSA-PBS-T. Subsequently, grids were moved to drops containing gold-labelled anti-mouse antibodies (Ey Laboratories) diluted 1/25 in BSA-PBS-T for 15 min. All specimens were stained in 1% ammonium-molybdate for 1.5 min and examined with a JEM-1200X transmission electron microscope (JEOL Ltd., Tokyo, Japan).

RESULTS

Binding of CS3 to rabbit intestinal brush borders. When testing the binding of purified CS3 fibrillae to intestinal brush borders derived from four different adult rabbits, only slight differences in the binding profiles of CS3 to cell extracts from different animals were observed (Fig. 1). CS3 bound to the electrophoretic front as well as to bands in the 30- to 35-kDa range and very strongly to 120- to 140-kDa binding structures. Binding of CS3 to electrophoretically separated intestinal membranes derived from infant rabbits was also studied. Infant rabbit enterocytes lacked the 120- to 140-kDa bands encountered in the adult rabbit intestine but had instead a 100-kDa protein which bound CS3 (Fig. 1). In subsequent experiments only brush borders from adult rabbit intestines were used, mainly because of the strong binding of CS3 to the 120- to 140-kDa bands.

After repeated chloroform-methanol extractions of the rabbit intestinal brush borders, CS3 still bound strongly to the SDS-PAGE-separated brush border components, indicating that these binding structures were of a proteinaceous nature. This was confirmed by proteinase K digestion of rabbit brush borders, which totally abolished all CS3 binding activity. By

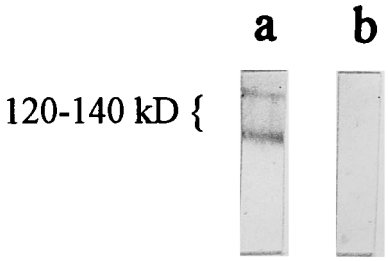


FIG. 2. Sodium *meta*-periodate oxidation of transblotted rabbit intestinal glycoproteins. Nonoxidized (lane a) and oxidized (lane b) glycoproteins were incubated with the CS3-only strain E19446. A smaller quantity of rabbit cell membrane extract was used in this set of experiments to achieve complete oxidation of the carbohydrate moieties of the glycoproteins, which is why the bands stained more weakly in lane a than in the other figures.

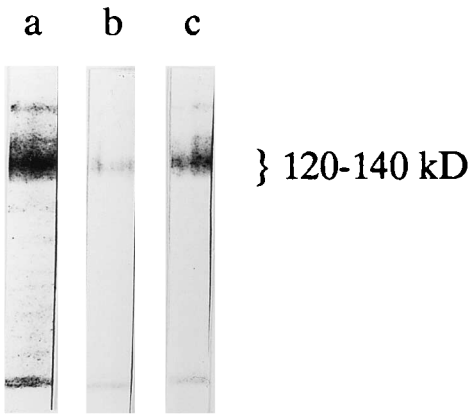


FIG. 3. Inhibitory capacity of meconium-derived glycopeptides on the binding of CS3-expressing bacteria. The CS3-only strain E19446 was used at a concentration of 10^9 cells per ml and was mixed with buffer (lane a), 2.5 mg of mucin-type meconium-derived glycopeptides per ml (lane b), and the same preparation as in lane b but devoid of sialic acid and fucose substitutions (lane c).

treating the brush borders with sodium *meta*-periodate, we could oxidize away virtually all the binding of CS3, indicating that these proteins were glycoproteins and that CS3 bound to their carbohydrate portions (Fig. 2).

Next, we compared the profiles of binding of purified CS3 and whole bacteria expressing CS3 to proteins derived from adult rabbit intestinal cells and found that they were virtually identical. Furthermore, bacteria coexpressing the CS1 subcomponent with CS3 bound in the same fashion to the 120- to 140-kDa proteins as did CS3-only bacteria (not shown). However, bacteria only expressing CS1 did not recognize the 120- to 140-kDa glycoproteins. To ascertain that the various ETEC strains were not expressing the mannose-sensitive type 1 fimbriae, incubations with the intestinal proteins were always performed in the presence of 0.5% D-mannose. Furthermore, it was found that the strains tested did not agglutinate guinea pig erythrocytes when grown on CFA agar and that the presence of mannose had no effect on the binding of the test strains to the rabbit brush border proteins (not shown).

Furthermore, when CS3-expressing bacteria were coincubated with high concentrations of a MAb directed against CS3, bacterial binding to the intestinal proteins was abolished (not shown).

Inhibition of the binding of CS3 to rabbit intestinal brush border glycoproteins. In an attempt to further characterize these CS3-binding glycoproteins, we tested the capacity of a series of glycopeptides isolated from meconium of newborn Swiss children to inhibit the binding of bacteria expressing CS3 to the SDS-PAGE-separated rabbit intestinal proteins. We found that a mucin-like fraction of meconium containing only O-linked glycopeptides inhibited CS3 binding completely at a concentration of 2.5 mg/ml (Fig. 3). The same fraction, devoid of sialic acid and fucose residues, also inhibited CS3 binding, although to a lesser extent. However, neither the monosaccharide fucose nor sialic acid was on its own able to inhibit the binding of CS3 (Table 1).

We also tested the CS3 inhibitory capacity of glycopeptides containing exclusively N-linked and O-linked oligosaccharide chains. It was apparent that glycopeptides isolated from human erythrocyte ghosts of the B blood group (contains solely N-linked glycopeptides) exhibited no inhibitory capacity whatsoever and those derived from bovine glycophorine (contains solely O-linked oligosaccharides) were weak inhibitors (Table 1).

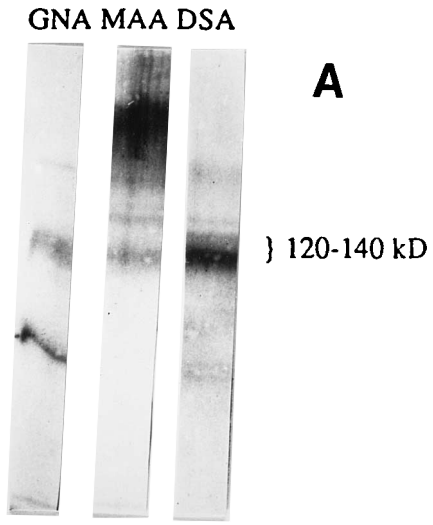
Next, we tested the ability of a series of lectins to bind to the 120- to 140-kDa proteins. These studies showed that the lectins isolated from *Datura stramonium* (DSA), *Maackia amurensis* (MAA), and *Galanthus nivalis* (GNA) all bound to these proteins (Fig. 4A). However, only pretreatment of the blotted intestinal proteins with the MAA lectin resulted in complete inhibition of the binding of CS3-expressing bacteria to the 120- to 140-kDa glycoproteins (Fig. 4B). In addition, MAA inhibited binding of CS3 to low-molecular-mass bands. MAA has specificity for the disaccharide sialic acidα2-3Gal, yet neither sialic acid nor galactose was on its own able to inhibit bacterial binding (Table 1). We also tested the inhibitory capacity of the lectin from *Evonymus europaea* and found that this lectin inhibited binding of bacteria only to the molecules of the electrophoretic front.

In order to determine the sugar sequence that CS3 recognized in the rabbit intestinal glycoproteins, we took advantage of the fact that glycolipids are better characterized than and not as complex as glycoproteins. Thus, glycolipids only have one carbohydrate chain, unlike glycoproteins, which frequently have very complex carbohydrate moieties with branching oligosaccharide chains. For these reasons, we compared the abilities of various gangliosides to inhibit bacterial binding to the rabbit intestinal glycoproteins. We found that GM1 and asialo-GM1 were about equally good inhibitors when tested at equimolar concentrations and virtually abolished the binding of CS3 to the rabbit intestinal proteins (Fig. 5). Furthermore,

TABLE 1. Capacity of monosaccharides and glycopeptides to inhibit the binding of CS3-expressing bacteria to rabbit intestinal glycoproteins

Putative inhibitor	Concn (mg/ml)	Molarity (mM)	Inhibition
L-Fucose	10	61	No
D-Galactose	10	56	No
N-Acetylgalactosamine	10	45	No
Glucose	9	50	No
Sialic acid	10	32	No
MMG[a]	2.5	ND[b]	Yes (4+[c])
Asialo- or afuco-MMG	2.5	ND	Yes (2+)
Bovine glycophorine	10	ND	Yes (1+)
Human erythrocyte ghosts[d]	10	ND	No

[a] MMG, mucin-like meconium glycopeptides.
[b] ND, not determined.
[c] On a scale of 1+ to 4+.
[d] Human erythrocytes expressing B-blood-group antigens.



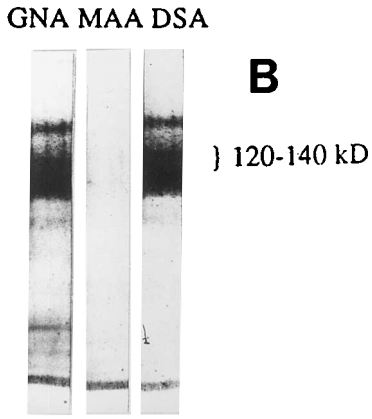


FIG. 4. Capacity of lectins to bind to blotted rabbit intestinal glycoproteins and to inhibit the binding of CS3-expressing bacteria. (A) Digoxigenin-labelled lectins derived from *G. nivalis* (GNA; specific for terminal mannose), *M. amurensis* (MAA; specific for sialic acidα2-3Gal), and *D. stramonium* (DSA; specific for Galβ1-4GlcNAc or GlcNAc alone) incubated with the transblotted rabbit intestinal proteins. (B) SDS-PAGE-separated rabbit brush borders preincubated with nonlabelled lectins before the addition of CS3-only bacteria (strain E19446) at a concentration of 10^9/ml.

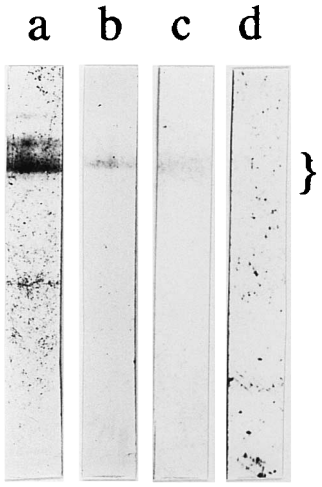


FIG. 5. The capacity of gangliosides to inhibit the binding of CS3-expressing bacteria to rabbit intestinal proteins. Bacteria (strain E19446) were incubated with buffer (lane a), 1 mM asialo-GM1 (lane b), and 1 mM GM1 (lane c). In lane d, the blotted intestinal proteins were preincubated with polyclonal rabbit antiserum against asialo-GM1 before the addition of bacteria. The 120- to 140-kDa bands are marked on the right.

when the nitrocellulose strips with the electrotransferred intestinal proteins were preincubated with polyclonal rabbit antiserum directed against asialo-GM1, the binding of bacteria expressing CS3 was completely abolished (Fig. 5). However, neither the B subunit of cholera toxin nor that of *E. coli* LT, both of which bind to GM1 with high affinity, bound to the 120- to 140-kDa proteins; consequently they were not able to inhibit the binding of CS3 to these proteins. Furthermore, none of the monosaccharides of the oligosaccharide chain of GM1, that is, galactose, *N*-acetylgalactosamine, neuraminic acid, and glucose, were able to inhibit bacterial binding (Table 1).

With the aim of determining if CS3 bound to any of the disaccharide combinations found in the oligosaccharide chain of GM1, we tested the ability of the disaccharides GalNacβ1-4Gal and Galβ1-4Glc, each covalently coupled to BSA via a spacer, to inhibit CS3 binding. Neither of these neoglycoproteins inhibited bacterial binding even at concentrations as high as 2.5 mg/ml. When testing the inhibitory capacity of free disaccharide Galβ1-3GalNAc (0.25 mM) as well as desialylated bovine caseinoglycomacropeptide (10 mg/ml), known to contain multiple Galβ1-3GalNAc residues, we found that neither had any inhibitory effect. Instead, we evaluated the inhibitory capacity of these disaccharides in the conformations existent in naturally occurring glycolipids. These analyses revealed that GM1 and GM2 were strong and about equipotent inhibitors, whereas GM3 was less effective (Fig. 6). Furthermore, incorporation of GM1 into human A erythrocytes resulted in higher hemagglutinating titers of CS3-only bacteria than when nontreated erythrocytes were used.

These findings implied that GalNAcβ1-4Gal was the critical sugar sequence of the CS3 binding structure. Further evidence for this assumption came from experiments in which the GalNAcβ1-4Gal disaccharide covalently bound to gold-labelled BSA via a spacer was used for examination and labelling of bacterial cell surfaces by electron microscopy. Indeed, this gold-labelled neoglycoprotein localized in great numbers around CS3-expressing bacteria and hardly at all around the corresponding CS3-deficient mutant (Fig. 7A and B). A similar distribution of gold particles around CS3-positive bacteria was seen with use of anti-CS3 MAb followed by gold-labelled anti-

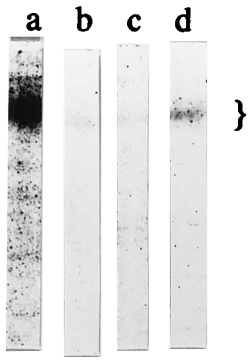


FIG. 6. Comparison of capacities of different gangliosides to inhibit the binding of CS3-expressing bacteria (strain 19446) to rabbit intestinal brush borders. Bacteria were incubated with buffer (lane a) and 0.8 mM GM1 (lane b), GM2 (lane c), and GM3 (lane d). The 120- to 140-kDa bands are marked on the right.

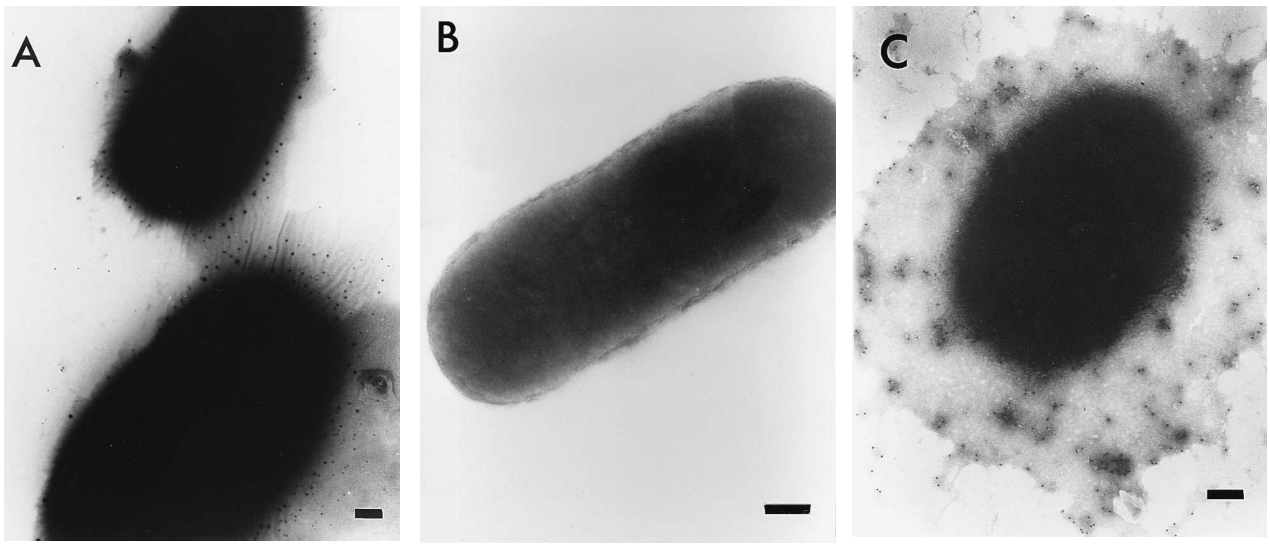


FIG. 7. Electron micrographs of CS3-positive and -negative bacteria. (A) CS3-positive bacteria (strain E19446) incubated with a neoglycoprotein consisting of GalNAcβ1-4Gal disaccharide covalently bound to gold-labelled BSA; (B) CS3-negative bacterium (strain 60R315) incubated with the same neoglycoprotein as described above; (C) CS3-positive bacterium (strain E19446) incubated with the anti-CS3 MAb 10:2 followed by gold-labelled anti-mouse antibodies. All bars = 200 nm.

mouse antibodies (Fig. 7C). Control experiments employing Galβ1-3GalNac covalently linked to gold-labelled BSA revealed that this neoglycoprotein localized around CS3-positive as well as CS3-negative bacteria, probably reflecting nonspecific binding.

Finally, the neoglycoprotein consisting of GalNAcβ1-4Gal coupled to BSA via a spacer functioned as binding site for CS3-expressing bacteria when immobilized on nitrocellulose (Fig. 8). However, the neoglycoprotein had to be electrophoretically separated by SDS-PAGE and then transferred to nitrocellulose in order for CS3 to recognize it, since CS3 could not bind to it when the neoglycoprotein was dot blotted directly onto nitrocellulose.

DISCUSSION

To conclude, we have been able to show that CS3-expressing ETEC binds to a GalNAcβ1-4Gal-containing neoglycoprotein and that GalNAcβ1-4Galβ-containing glycoconjugates can act as inhibitors of CS3 binding to immobilized rabbit intestinal glycoproteins. It has previously been described that CS3 is able to bind efficiently to enterocytes of both human (13) and rabbit (33) origins, in addition to colonizing human (16) as well rabbit (28) intestine. Besides, CS3 is both immunogenic and a protective antigen (16, 28). In a previous study we have shown that this fibrillar antigen bound very strongly to 120- to 140-kDa proteins of the rabbit intestine (33). Simultaneously, it was shown that CS3-expressing bacteria bound to enterocyte-like differentiated HT-29 cells and that this binding could be inhibited both by a lectin from *Evonymus europeae* with specificity for fucose and B-blood-group related antigens and by mucin-type glycopeptides derived from human meconium (22). The inhibitory capacity of this lectin was attributed to steric hindrance rather than to the recognition of the precise carbohydrate sequence in the HT-29 cell membrane to which CS3 bound. It was surmised that the inhibitory activity of the mucin-type glycopeptide fraction was due to its content of polylactosaminoglycans (22).

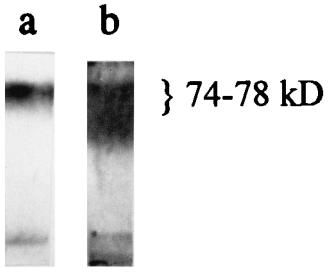


FIG. 8. Binding of CS3-positive bacteria to the transblotted neoglycoprotein consisting of GalNAcβ1-4Gal disaccharide coupled to BSA. Twenty micrograms of protein was electrophoretically separated by SDS-PAGE and then electrotransferred to nitrocellulose. Lane a, glycoprotein incubated with CS3-positive bacteria; lane b, glycoprotein detected by Amido-Schwartz protein stain.

In the present study our aim was to further characterize the binding structure(s) for CS3 in the rabbit intestine. We found that purified CS3 fibrillae and bacteria expressing CS3, either alone or in conjunction with CS1 fimbriae, bound virtually identically to the electrophoretically separated intestinal brush borders. The binding of CS3-carrying bacteria was totally abolished by coincubating the bacteria with a MAb directed against CS3, indicating that the observed bacterial binding indeed could be attributed to CS3.

Age-associated differences of the molecular sizes of binding structures for CS3 were demonstrated in the rabbit intestine. CS3 bound more strongly to the brush border membranes of the adult rabbit intestine than to those of the infant one, probably reflecting greater numbers of binding epitopes in the adult gut. It is well known that there are age-dependent differences in glycosylation of both proteins and lipids in the mammalian intestine (21). For instance, the intestinal epithelial cells of infant rabbits lack glycocalyx. In porcine ETEC infections, there is a decrease with age of ETEC infections caused by bacteria carrying K99 and F41 fimbriae due to alterations in both numbers of and distribution of fimbrial receptors in the intestine. It is known that human ETEC is able to colonize both infant and adult rabbit intestines (6, 28). However, since animals were killed after 18 h in all studies of

intestinal colonization by ETEC in infant rabbits (6), it is unclear whether ETEC might be able to persist in the infant rabbit intestine for equally long periods, i.e., 5 to 8 days, as in the adult rabbit intestine (28). In humans, however, it does not seem that the decreased illness-to-infection rate observed with age in ETEC disease could be attributed to differences in the glycosylation of binding structures for CFAs, since both children from areas where ETEC is endemic and adults from areas where ETEC is not endemic succumb in high frequency to ETEC disease.

The 120- to 140-kDa structures identified in the intestines of adult rabbits exhibiting CS3-binding capacity were determined to be proteins since these structures remained in completely delipidized rabbit intestinal brush borders. Furthermore, proteinase K treatment of brush borders completely digested all CS3 binding structures. It also seems that these proteins are glycosylated and that CS3 binds to their carbohydrate portion, as indicated by the fact that sodium *meta*-periodate treatment of the brush border membranes abolished the binding of CS3 completely. Additionally, three different lectins bound to the 120- to 140-kDa proteins, namely, the *D. stramonium* agglutinin, *G. nivalis* agglutinin, and *M. amurensis* agglutinin, further corroborating the assumption that these proteins contain carbohydrates. Interestingly, only one of these lectins, the *M. amurensis* agglutinin, which has specificity for sialic acid α2-3 bound to penultimate galactose residues of oligosaccharides (32), was able to inhibit the binding of CS3 to the intestinal glycoproteins. This 130-kDa lectin is larger than the other two lectins, which might imply that its inhibitory effect was due to steric hindrance. However, despite DSA having a similar molecular mass (86 kDa) and exhibiting stronger binding to the 120- to 140-kDa structures, this lectin was totally incapable of inhibiting the binding of CS3. It seems likely that MAA recognized sialic acid-containing galactoproteins in the rabbit intestine which could also act as binding structures for CS3. However, it is possible that the specificities and binding sequences of both plant and bacterial lectins were different. The fact that we were able to inhibit the binding of CS3 to the rabbit proteins by using the mucin-type glycopeptides derived from human meconium (22) indicates that CS3-binding structures from human intestine can compete with those of rabbit origin.

In a previous study we have shown that all the CS components of CFA/II, including CS3, bound to asialo-GM1 in thin-layer chromatograms (24). In order to test whether the carbohydrate moiety of this glycolipid could compete with the carbohydrate chains of the rabbit intestinal galactoproteins for the binding of CS3, we attempted to inhibit the binding of CS3-expressing bacteria to rabbit proteins with asialo-GM1. We could show that not only asialo-GM1 but also GM1 and GM2 were able to inhibit CS3 binding at equimolar concentrations. However, in thin-layer chromatograms, we could not detect any binding of CS3 to GM1 or GM2 (24). An explanation for these apparently contradictory results may be that sialic acid blocks the binding site of CS3 in the carbohydrate chain when the glycolipids are fixed to a solid surface but not when they are in solution. Moreover, the results from the present study strongly support the idea that sialic acid is not part of the binding site for CS3 since asialo-GM1 and GM1 were found to be equally potent inhibitors. Furthermore, since it is known that human intestinal mucin does not contain sialic acid in α2-3 linkage (26) but contains it only in α2-6 linkage, which was not encountered in the 120- to 140-kDa structures, and since the mucin-derived glycopeptides that we employed inhibited the binding of CS3, this lends further support to our finding that sialic acid in α2-3 linkage is not a component of the CS3-binding structure.

Major evidence that the CS3-binding site could be found within the carbohydrate portion of asialo-GM1 was that a polyclonal antibody against asialo-GM1 totally blocked the binding of CS3-expressing bacteria to the rabbit intestinal proteins. *E. coli* LT is known to bind to GM1 and to galactoproteins in the rabbit intestine (8). LT did not bind to the 120- to 140-kDa bands recognized by CS3 but instead bound to a protein of even higher molecular mass. It is thought that LT (and cholera toxin, for that matter) recognizes the terminal galactose residue in conjunction with the sialic acid residue of the oligosaccharide chain of GM1 (8). This suggests that this specific combination of sugars is not found in the 120- to 140-kDa proteins recognized by CS3. More importantly, the finding that GM1 and GM2 were equally good CS3 inhibitors suggests that the GalNAcβ1-4Gal disaccharide was the critical carbohydrate moiety for the binding of CS3 in both glycoproteins and glycolipids.

The finding that CS3-expressing bacteria bound to electroblotted GalNAcβ1-4Gal coupled to BSA further lends support to our belief that the GalNAcβ1-4Gal disaccharide is the binding epitope for CS3. Interestingly, the neoglycoprotein had to be separated electrophoretically by SDS-PAGE and then transferred to nitrocellulose in order to function as binding site for CS3. When the neoglycoprotein was directly blotted onto the nitrocellulose membrane (dot blot), the CS3 lectin did not seem to recognize it. This is not surprising, since in the latter case it is probable that the disaccharide is not exposed at all and/or is not in the optimal conformation for recognition by CS3. It is well known that proteins separated by SDS-PAGE after transfer to nitrocellulose often regain their original conformation and expose hydrophilic components such as sugar sequences.

The observation that the neoglycoprotein containing the GalNAcβ1-4Gal disaccharide did not inhibit the binding of CS3-expressing bacteria in the immunoblot assay is somewhat surprising. However, these results are analogous to those of Jagannatha et al. (9) concerning the binding structures for enteropathogenic *E. coli* (EPEC) displaying a localized adherent binding pattern to tissue-cultured cells. Interestingly, they identified the GalNAcβ1-4Gal sequence of asialo-GM1 as being the binding epitope for EPEC. Additionally, even though they could show that EPEC bound specifically to a neoglycoprotein containing this disaccharide, they were unable to inhibit the binding of EPEC to asialo-GM1 in a microtiter plate assay using the same neoglycoprotein. Their explanation for the failure of the receptor analog to inhibit the binding of EPEC was that this binding constituted low-affinity binding, requiring multivalent receptor analogs for effective inhibition (9, 11). However, even though the disaccharide was unable to inhibit CS3 binding, we were able to show in electron microscopic studies, using the same approach as that of Bühler et al. (3), that the GalNAcβ1-4Gal disaccharide covalently bound to BSA localized around CS3-expressing bacteria but not around corresponding mutant bacteria lacking CS3.

The finding that CS3 could bind to both glycolipids and glycoproteins suggests that the CS3-binding epitopes are similar in these two glycoconjugates. It is known that the carbohydrate moieties of glycolipids and glycoproteins differ quite extensively in the core region but exhibit great similarities in the terminal carbohydrate sequences (27). The fact that MAA inhibited CS3 binding to the glycoproteins and the knowledge that MAA binds to terminal carbohydrate residues of oligosaccharides (32) suggest that CS3 recognizes terminal sugar sequences in the rabbit intestinal glycoproteins.

The GalNAcβ1-4Gal disaccharide has also been implicated as a binding structure for non-*E. coli* bacteria, with tropism for nonintestinal tissues: Krivan et al. (14) have shown that many pulmonary pathogens, such as *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*, bind to this same sequence in glycolipids. Thus, the GalNAcβ1-4Gal disaccharide seems to be important for the adhesiveness and probably also the colonizing capacity of a diverse group of pathogenic bacteria at different mucosal surfaces in the human body. To our knowledge, this disaccharide has hitherto been described in only two glycoproteins of human origin, i.e., the Tamm-Horsfall urinary glycoprotein (4) and as part of the rare Cad blood group determinant (2).

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