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Receptor Structure for F1C Fimbriae of Uropathogenic *Escherichia coli*

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F1C fimbriae are correlated with uropathogenic *Escherichia coli* strains. Although F1C fimbriae mediate binding to kidney tubular cells, their receptor is not known. In this paper, we demonstrate for the first time specific carbohydrate residues as receptor structure for F1C-fimbria-expressing *E. coli*. The binding of the F1C fimbriated recombinant *E. coli* strain HB101(pPIL110-54) and purified F1C fimbriae to reference glycolipids of different carbohydrate compositions was evaluated by using thin-layer chromatography (TLC) overlay and solid-phase binding assays. TLC fimbrial overlay analysis revealed the binding ability of purified F1C fimbriae only to glucosylceramide (GlcCer), β1-linked galactosylceramide 2 (GalCer2) with nonhydroxy fatty acids, lactosylceramide, globotriaosylceramide, paragloboside (nLc_4Cer), lactotriaosylceramide, gangliotriaosylceramide (asialo-GM_2 [GgO_3Cer]) and gangliotetraosylceramide (asialo-GM_1 [GgO_4Cer]). The binding of purified F1C fimbriae as well as F1C fimbriated recombinant *E. coli* strain HB101(pPIL110-54) was optimal to microtiter plates coated with asialo-GM_2 (GgO_3Cer). The bacterial interaction with asialo-GM_1 (GgO_4Cer) and asialo-GM_2 (GgO_3Cer) was strongly inhibited only by disaccharide GalNAcβ1-4Galβ linked to bovine serum albumin. We observed no binding to globotetraosylceramide or Forssman antigen (Gb_5Cer) glycosphingolipids or to sialic-acid-containing gangliosides. It was demonstrated that the presence of a GalCer or GlcCer residue alone is not sufficient for optimal binding, and additional carbohydrate residues are required for high-affinity adherence. Indeed, the binding efficiency of F1C fimbriated recombinant bacteria increased by 19-fold when disaccharide sequence GalNAcβ1-4Galβ is linked to glucosylceramide as in asialo-GM_2 (GgO_3Cer). Thus, it is suggested that the disaccharide sequence GalNAcβ1-4Galβ of asialo-GM_2 (GgO_3Cer) which is positioned internally in asialo-GM_1 (GgO_4Cer) is the high-affinity binding epitope for the F1C fimbriae of uropathogenic *E. coli*.

One of the indispensable steps in several infections caused by *Escherichia coli* and other gram-negative bacteria is the specific adherence to host cell surface carbohydrates linked to glycoproteins or to glycolipids. This phenomenon is known to be mediated either by nonfimbrial adhesins or by hair-like heteropolymeric bacterial surface appendages called fimbriae or pili (4, 8–10, 19, 20, 28).

Accumulating data argue that the glycolipids present on eukaryotic cell surfaces play a significant role in cell-cell and cell-ligand interactions (3, 43). Several different types of fimbrial adhesins have been described to mediate attachment of bacteria to the carbohydrate sequence of glycolipids present on host cell surfaces (7, 16, 18, 26, 30, 35, 39, 48, 49).

Urinary tract infections in humans are strongly associated with *E. coli* producing P, type 1, S, and F1C fimbriae. P fimbriae are defined by their ability to mediate binding to the Galα1-4Gal saccharide in glycolipids of the globoseries (13, 14, 26, 47). Type 1 fimbriae bind specifically to mannose residues (9, 31). The S fimbria super family has been described to consist of S fimbriae, F1C fimbriae, and S- and F1C-related fimbriae (34, 40, 42). S fimbriae bind to receptors containing sialic acid sugar moieties. They have the capacity to agglutinate human and bovine erythrocytes (24, 33, 52). Their binding to brain glycolipids has also been demonstrated (39).

Another member of this family, namely F1C fimbria, has been described as a nonhemagglutinating adherence factor and is expressed by approximately 14% of the *E. coli* known to cause urinary tract infections and 7% of *E. coli* fecal isolates (11, 32, 37, 38, 45). A cluster of eight genes (*foc*) is necessary for the biogenesis of F1C fimbriae (21, 22, 40, 50, 51). The F1C fimbrial complex is composed of the major subunit protein FocA (16 kDa) and the minor subunits FocF (17 kDa), FocG (15 kDa), and FocH (30 kDa) (40). These fimbriae share high sequence homology to the major and minor subunits of the S fimbriae (11). *E. coli* harboring F1C fimbriae have been reported to bind to epithelial cells in the distal tubules and collecting ducts as well as to endothelial cells of human kidney and bladder (53). Their binding specificity has also been suggested to be similar to that of S fimbriae (27). However, their exact receptor specificity has not yet been identified.

In the present study, we identify glycolipid receptors for purified F1C fimbriae as well as for F1C fimbriated recombinant *E. coli* HB101(pPIL110-54) by using thin-layer chromatography (TLC) overlay and solid-phase binding assays.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* K-12 strain HB101 used in this study was transformed with the plasmid pPIL110-54 (pACYC184 plasmid vector containing the complete *foc* gene cluster cloned from the uropathogenic *E. coli* strain AD110) (40, 51). Recombinant bacteria were grown overnight (with shaking) in liquid broth supplemented with the appropriate antibiotic (50 μg of chloramphenicol per ml).

Reagents and chemicals. Purified glycolipids, saccharides, and enzymes used were obtained from Sigma, Deisenhofen, Germany. Recombinant β-*N*-acetyl-

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hexosaminidase was from New England Biolabs, Beverly, Mass. Carbohydrates conjugated to bovine serum albumin (BSA) and disaccharides used as inhibitors were purchased from Dextra Laboratories (Reading, United Kingdom); Toronto Research Chemicals, North York, Canada; and Glycorex, Lund, Sweden. All chemicals used were of analytical grade.

Antisera. Polyclonal antibodies against purified F1C fimbriae were raised in chicken (Nano-Tools, Freiburg, Germany). Antiserum was adsorbed with HB101 host strain before use.

Seroagglutination. The capacity of bacteria to express F1C fimbriae was assayed by agglutinating bacteria by using chicken polyclonal anti-F1C antibody as described earlier (20). Briefly, diluted (1,000 times) antiserum (10 μl) was mixed with the same volume of liquid bacterial culture grown for 16 h (with shaking), and agglutination was observed. Nonfimbriated strain HB101 carrying plasmid vector pACYC184 was used as a negative control. Fimbriated bacteria did not show agglutination with phosphate-buffered saline (PBS).

Purification of F1C fimbriae. F1C fimbriae were harvested from recombinant strain HB101 (pPIL110-54) by using commercial blendor (Omnimixer; Waring) and were purified essentially as described by Khan and Schifferli (20). Moreover, like other fimbriae (20), F1C fimbriae were heat extractable. Fimbriated bacteria from broth cultures were pelleted by centrifugation, were suspended in 0.5 mM Tris-HCl (pH 7.4) containing 75 mM NaCl, and were treated at 60°C for 30 min in a shaking water bath. Bacteria were removed by centrifugation, and F1C fimbrial preparations were concentrated from the supernatant by using an ultrafiltration pressure cell (YM100 membranes; Amicon, Beverly, Mass.).

Biotinylation of bacteria. Overnight cultures of bacteria were pelleted at 1,940 × *g* for 15 min. After being washed three times with PBS bacteria were resuspended in PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.6) to a final concentration of 5×10^8 bacteria/ml. To this, an equal volume of PBS-$CaCl_2$-$MgCl_2$ containing 0.2 to 0.3 mg of sulfo-NHS-LC–biotin (sulfosuccinimidyl-6-[biotinamido]-hexanoate) (Pierce Chemical Company) per ml was added. Bacterial surface proteins were allowed to biotinylate for 2 h by incubating the mixture at room temperature with gentle shaking. The bacteria were collected, washed four times, resuspended to a final concentration of 2×10^9 bacteria/ml in PBS (pH 7.4) containing 1% BSA, and stored at 4°C. This preparation was used for 3 days.

TLC fimbriae overlay assay. TLC was performed as described earlier (12, 17, 18) with a solvent system involving chloroform-methanol-water (60:35:8). Briefly, pure glycolipids (Sigma) were separated for analytical purpose on high-performance TLC (HPTLC) aluminium-backed silica gel 60 plates (Merck, Darmstadt, Germany). Glycolipids were stained with orcinol spray (Sigma). For the overlay assay, the plates were sequentially treated for 2 min with polyisobutylmethacrylate (0.1% in hexane), were blocked with 2% BSA in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.4], 154 mM NaCl) for 90 min at room temperature, were washed once with TBS, were overlaid with pure F1C fimbriae (20 μg/ml in 1% BSA-TBS) for 2 h, and were washed three times with TBS–0.05% Tween 20 and once with TBS. Fimbrial antigen was detected sequentially with anti-F1C fimbrial chicken polyclonal antibody and horseradish peroxidase-conjugated anti-chicken antibody (Sigma) by conventional Western blot technique by using the DAB peroxidase substrate tablet set (Sigma) as described earlier (20).

Neuraminidase treatment of disialogangliosides separated by HPTLC. The disialoganglioside fraction containing *O*-acetylated derivatives of the gangliosides (GD3, disialosylparagloboside [DSPG], disialosyllacto-*N*-nor-hexaosylceramide [DSnHC], and disialosyllacto-*N*-nor-octaosylceramide [DSnOC]) purified from human leucocytes (23) were separated on five glass-backed HPTLC plates (Merck) in parallel and were fixed with polyisobutylmethacrylate as described above. One plate was used for fimbrial overlay assay and the other one was immunostained as described earlier (23) by using CDw60 monoclonal antibody M-T21, which is specific for GD3, 9-*O*-acetylated GD3, and terminally disialyated gangliosides of the neolacto series such as DSPG and DSnHC. In order to remove sialosyl residues linked to gangliosides, three plates were incubated overnight at 37°C with neuraminidase (10 mU) per ml of *Arthrobacter ureafaciens* (Boehringer GmbH, Mannheim, Germany) in 0.1% $CaCl_2$, were washed three times with PBS, and were blocked with 2% BSA-PBS. After the washing of blocked plates, one plate was overlaid with the monoclonal antibody 1B2 specific for the terminal Galβ1-4GlcNAc-carbohydrate structure found in glycolipids of the neolacto series and was further processed as described earlier (23). The second plate was overlaid with purified fimbriae. The third plate was used as a control where neither fimbriae nor monoclonal antibody 1B2 was overlaid. They were further processed as described above.

Periodate oxidation. Microtiter wells containing either asialo-GM_1 (GgO_4Cer) or asialo-GM_2 (GgO_3Cer) were washed twice with PBS, incubated with 10 mM sodium metaperiodate in PBS (pH 7.1) at 37°C for 90 min (31), washed four times with PBS, blocked with 2% BSA-PBS at 37°C for 2 h, and then overlaid with twofold serially diluted bacteria as described below. Bacterial binding was determined as mentioned below.

Solid-phase binding and binding inhibition assay. Polyvinyl chloride plates (Falcon; Becton Dickinson) were coated with glycolipids per well, (2 μg of glucosylceramide, 2 μg of galactosylceramide 1 and 2-[hydroxylated and nonhydroxylated fatty acids], 2 μg of sulfatide, 2 μg of lactosylceramide [LacCer], 2 μg of Gb_3Cer, 2 μg of GM1, 2 μg of asialo-GM_1 (GgO_4Cer), 1 μg of asialo-GM_2 (GgO_3Cer), 2 μg of paragloboside) in chloroform-methanol (1:9 [vol/vol]). The solvent was allowed to evaporate overnight at room temperature, and the wells were blocked with 2% BSA-PBS (pH 7.37) for 2 h at 37°C and were washed three times with PBS. For each glycolipid, a serial dilution of F1C fimbriated *E. coli* HB101(pPIL110-54), control strain HB101(pACYC184), or purified F1C fimbriae was first tested to determine the numbers of bacteria and the quantity of purified fimbriae required to obtain 50% binding. The number of fimbriated HB101(pPIL110-54) and nonfimbriated control strain HB101(pACYC184) was determined by measuring absorbance at a wavelength of 550 nm, with a standardized chart correlating absorbance with viable counts. After removal of the blocking solution and washing, 100 μl of serially diluted bacterial suspensions (2×10^8 bacteria in the first well) or purified fimbrial solution (10 μg of fimbriae in the first well) in PBS–0.5% BSA was added to each well, and plates were incubated for 2 h at 37°C. After wells were washed as previously described, rabbit anti-*E. coli* polyclonal antibody (Biodesign International, Kennebunk, Maine) in PBS–0.5% BSA (1:2,000) (or for wells with fimbriae, anti-F1C chicken antibody [polyclonal; 1:400]) was added for 1 h at 37°C (100 μl/well). Following another washing step, peroxidase-conjugated goat anti-rabbit immunoglobulin G (Dako, Hamburg, Germany) or anti-chicken immunoglobulin G whole molecule (Sigma) in PBS–1% BSA (1:2,000) was added for 1 h at 37°C (100 μl/well). Following a final wash, the bound enzyme was detected by the addition of 100 μl of substrate (Pierce ImmunoPure TMB Substrate Kit) per well for 5 to 30 min. The reaction was stopped by adding 100 μl of 2 M H_2SO_4 to each well. Absorbance was measured at a wavelength of 450 nm with an enzyme-linked immunosorbent assay (ELISA) reader. Similar experiments were also performed by using biotinylated bacteria, and binding was detected with horseradish peroxidase-conjugated streptavidine (Pierce Chemical Company) and its substrate as above. The control wells were treated in the same manner except that blank control wells had no bacteria or fimbriae.

The binding inhibition assay was performed only with asialo-GM_1 (GgO_4Cer)- and asialo-GM_2 (GgO_3Cer)-coated microtiter plates. For the binding inhibition assays, bacteria at the number (3×10^7/well) giving 50% binding were preincubated with twofold serially diluted carbohydrates for 1 h at room temperature. The mixtures were transferred to the glycolipid-containing microtiter plates, and bacterial binding was determined as described above. Percentages of inhibition were calculated by the following equation: percent inhibition = $\{ 1 - [(A_{450}$ of the test well $- A_{450}$ of the blank control well) / (A_{450} of the 100% binding control well $- A_{450}$ of the blank control well)]$\} \times 100$. For this calculation, the 100% binding control wells had no carbohydrate inhibitors and the blank control well had neither bacteria nor inhibitors. Each strain was tested in three separate experiments, and in each experiment, 30 determinations of bacterial or fimbrial adherence were performed in parallel.

To establish the binding affinities of *E. coli* HB101(pPIL110-54), control strain HB101(pACYC184), and purified fimbriae to different glycolipids, twofold serially diluted glycolipids were dried in microtiter plates as mentioned above. After blocking and washing were carried out as described above, bacteria (3×10^7/well) or purified fimbriae (1.5 μg/well) giving 50% binding was incubated as described above. Bacterial or fimbrial binding was determined as described above.

Enzymatic treatment of asialo-GM_1 (GgO_4Cer). Purified asialo-GM_1 (GgO_4Cer) (Sigma) was immobilized on ELISA plates and was sequentially treated with bovine β-galactosidase (β-Gal) (Sigma) and then with recombinant β-*N*-acetylhexosaminidase (New England Biolabs). Asialo-GM_1 (GgO_4Cer) was suspended in methanol (10 μg/ml), and 100 μl of the suspension was added to wells of microtiter plates. After evaporation of methanol, immobilized glycolipids were incubated with 0.4 U of β-Gal in Tris buffer [10 mM Tris, 1.7 M $(NH_4)_2SO_4$, 10 mM $MgCl_2$, pH 7.3] for 2 h at 37°C. The wells were washed with PBS, and the glycolipids were further treated with 10 U of β-*N*-acetylhexosaminidase in citrate buffer (50 mM sodium citrate, pH 4.5) per well for 6 h at 37°C in a shaking water bath. After performing washing and blocking steps, bacterial or fimbrial binding was determined as described above.

RESULTS

Analysis of F1C fimbrial binding to purified glycolipids. The efficiency of F1C fimbrial binding to purified glycolipids was studied by using the HPTLC overlay assay (Table 1). Purified fimbriae bound to glucosylceramide (GlcCer), galactosylceramide 2 (nonhydroxylated fatty acids), lactosylceramide (LacCer), paragloboside (nLc_4Cer), lactotriaosylceramide (Lc_3Cer), asialo-GM_1 (GgO_4Cer), asialo-GM_2 (GgO_3Cer), and globotriaosylceramide (Gb_3Cer) (Table 1) but did not bind to sulfatide (SFT), globotetraosylceramide (Gb_4Cer), the Forssman antigen (Table 1), GM_1 (Fig. 1), GM_2, GM_3, GD_2, GD_3 (Table 1), DSPG, DSnHC, DSnOC (see Fig. 3), or ceramide (Cer) (Table 1). A weak binding was observed with GlcCer and GalCer2 (Fig. 1). F1C fimbriae bound better to LacCer, Gb_3Cer, Lc_3Cer (data not shown), and nLc_4Cer (Fig. 1). Binding to GalCer1 containing hydroxylated ceramide portion was even weaker than GlcCer or GalCer2 (Table 1). Optimal binding of

TABLE 1. Glycolipids screened for the binding of purified F1C fimbriae on thin-layer chromatograms and F1C fimbriated recombinant bacteria on ELISA plates

Name[a]	Binding		Structure
	TLC[b] overlay	ELISA[c]	
Ceramide	−	−	Cer
Galactosylceramide (GalCer1)	±	±	Galβ1-1Cer (hydroxylated fatty acids)
Galactosylceramide (GalCer2)	+	+	Galβ1-1Cer (nonhydroxylated fatty acids)
Sulfatide (SFT)	−	−	SO_3Galβ1-1Cer
Galabiosylceramide	−	ND	Galα1-4Galβ1-1Cer
Glucosylceramide (GlcCer)	+	+	Glcβ1-1Cer
Lactosylceramide (LacCer)	++	++	Galβ1-4Glcβ1-1Cer
Globotriaosylceramide (Gb_3Cer)	++	++	Galα1-4Galβ1-4Glcβ1-1Cer
Globotetraosylceramide (Gb_4Cer)	−	−	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer
Forssman antigen (Gb_5Cer)	−	ND	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-4Glcβ1-1Cer
Disialosylparagloboside (DSPG)	−	ND	Neuα2-8NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc1-1Cer
Disialosyllacto-*N*-nor-hexaosylceramide (DSnHC)	−	ND	Neuα2-8NeuAcα2-3(Galβ1-4GlcNAc)$_2$β1-3Galβ1-4Glc1-1Cer
Paragloboside (nLc_4Cer)	++	++	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer
Lactotriaosylceramide (Lc_3Cer)	++	++	GlcNAcβ1-3Galβ1-4Glcβ1-1Cer
Gangliotriaosylceramide (asialo-GM_2, GgO_3Cer)	++++	++++	GalNAcβ1-4Galβ1-4Glcβ1-1Cer
Gangliotetraosylceramide (asialo-GM_1, GgO_4Cer)	+++	+++	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer
GM_1	−	−	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer
GM_2	−	−	GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer
GM_3	−	−	NeuAcα2-3Galβ1-4Glcβ1-1Cer
GD_2	−	−	GalNAcβ1-4Gal(3-2NeuAc8-2αNeuAc)β1-4Glcβ1-1Cer
GD_3	−	−	NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer

[a] Glycolipids are abbreviated according to the recommendations made in reference 15. Purified glycolipids were obtained from Sigma.
[b] Binding to glycolipids separated on HPTLC is graded as follows: ++++, optimal binding; +++, suboptimal binding; ++, strong binding; +, weak binding; ±, very weak binding; −, no binding.
[c] Binding to glycolipids immobilized on ELISA plates is graded as follows: ++++, absorbance of >3.00 at 450 nm; +++, absorbance of >2.00 at 450 nm; ++, absorbance of >1.00 at 450 nm; +, absorbance of >0.5 at 450 nm; ±, absorbance of >0.2 at 450 nm; and ND, not done.

purified F1C fimbriae or F1C fimbriated recombinant bacteria to asialo-GM_2 (GgO_3Cer) and asialo-GM_1 (GgO_4Cer) on HPTLC plates was observed (Fig. 1 and 2).

These results indicate that carbohydrate sequences linked to asialo-GM_1(GgO_4Cer), asialo-GM_2(GgO_3Cer), paragloboside (nLc_4Cer) lactotriaosylceramide (Lc_3Cer), lactosylceramide (LacCer), and globotriaosylceramide (GB_3Cer) carry the epitope required as a receptor for F1C fimbrial adhesin complex. This further suggests that the presence of β-linked galactose and/or glucose in the absence of sialic acid may be the minimum requirement for F1C fimbrial binding.

Glycolipid binding specificity of F1C fimbriae and fimbriated bacteria. The ability of purified fimbriae and fimbriated bacteria with selected glycolipids was studied in microtiter plate assay. Both fimbriae and bacteria bound to glucosylceramide (GlcCer), galactosylceramide (GalCer1 and -2), lactosylceramide (LacCer), paragloboside (nLc_4Cer), globotriaosylceramide (Gb_3Cer), asialo-GM_1 (GgO_3Cer), and asialo-GM_2 (GgO_4Cer) in a dose-dependent manner (data not shown).

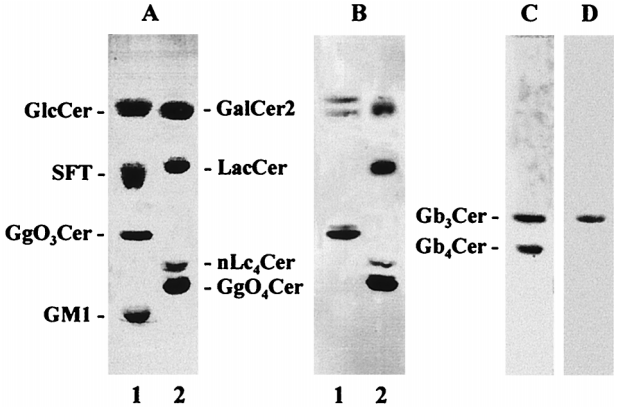

FIG. 1. HPTLC-separated glycolipids and overlay analysis of the binding of purified F1C fimbriae. Chromatograms of glycolipids separated on HPTLC silica gel 60 and stained with orcinol (A and C), probed with purified F1C fimbriae, and immunostained with anti-fimbrial antibodies and horseradish peroxidase-conjugated anti-chicken antibodies (B and D) are shown. (A) Lane 1, (from top to bottom) glucosylceramide (4 μg, GlcCer), sulfatide (4 μg, SFT), asialo-GM_2 (GgO_3Cer) (2 μg), and sialylated GM_1 (4 μg, GM1); lane 2, (from top to bottom), galactosylceramide 2 (4 μg, GalCer2), lactosylceramide (3 μg, LacCer), paragloboside (1 μg, nLc_4Cer), and asialo-GM_1 (GgO_4Cer) (2 μg). (B) Lane 1, fimbrial binding to GlcCer (upper double band) and to asialo-GM_2 (GgO_3Cer) (lower double band); lane 2, fimbrial binding from top to bottom, GalCer2, LacCer, nLc_4Cer, and asialo-GM_1 (GgO_4Cer). (C) Lane contains (from top to bottom) globotriaosylceramide (4 μg, Gb_3Cer) and globotetraosylceramide (4 μg, Gb_4Cer). (D) Lane contains fimbriae bound to globotriaosylceramide (Gb_3Cer).

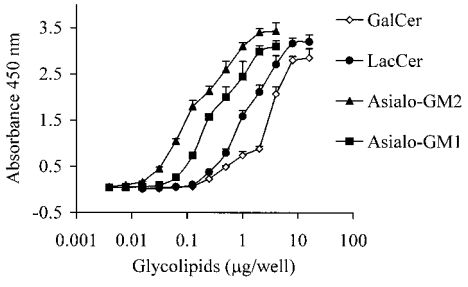

FIG. 2. Dose-dependent binding of biotinylated F1C fimbriated recombinant *E. coli* (3×10^7/well) to purified glycolipids immobilized in microtiter wells. The twofold serially diluted glycolipids in 50 μl of chloroform-methanol (1:9), asialo-GM_1 (GgO_4Cer), asialo-GM_2 (GgO_3Cer), LacCer, and GalCer2 were added to microtiter wells. Bound bacteria were detected with horseradish peroxidase-conjugated streptavidine as described in Materials and Methods. Data represent the average of triplicate determinations.

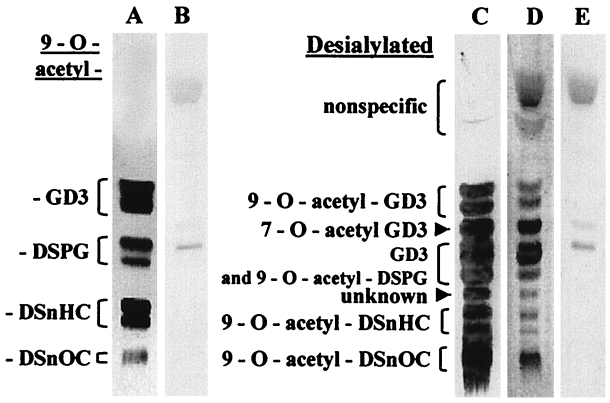

FIG. 3. Binding of purified F1C fimbriae prior to and after *A. ureafaciens* neuraminidase treatment to the disialoganglioside fraction of unseparated human leucocytes. An aliquot of the disialoganglioside fraction was manually loaded and separated simultaneously on five small HPTLC plates (glass backed) and was fixed with polyisobutylmethacrylate as described in Materials and Methods. The glycolipids were immunostained with the CDW60 monoclonal antibody M-T21 specific for *O*-acetylated disialogangliosides (A) showing doublets of the 9-*O*-acetylated forms of GD3, DSPG, DSnHC, and DSnOC. The same glycolipids as mentioned in the legend of Fig. 1 were probed with purified F1C fimbriae (B). After neuraminidase treatment, the resulting asialogangliosides were immunostained with the monoclonal antibody IB2 specific for glycolipids containing an external Galβ1-4GlcNAc structure (C) and were probed with purified F1C fimbriae in a way similar to that shown in panel B (D). The strongest bands visible in panel D are supposed to originate from 7-*O*-acetylated GD_3 and GD_3, respectively, which are known to migrate between 9-*O*-acetyl GD_3 and 9-*O*-acetyl DSPG. These two gangliosides were not visualized by M-T21 (A), but their presence was deduced by IB2 stain (C). A control plate was neuraminidase treated and probed with PBS containing no fimbriae but 1% BSA (E).

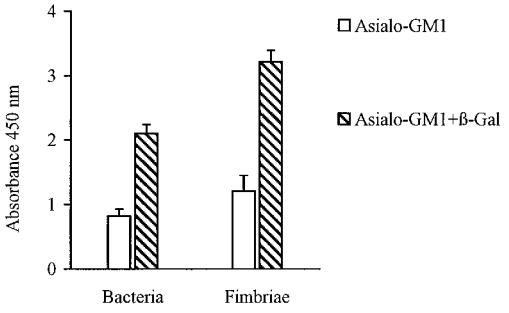

FIG. 4. Effect of enzymatic sequential treatment of asialo-GM_1 (GgO_4Cer). Asialo-GM_1 (GgO_4Cer) (1 μg/well) was coated in microtiter wells and treated with bovine β-Gal. The ability of purified F1C fimbriae (1.5 μg/well) and biotinylated F1C fimbriated recombinant *E. coli* (3×10^7/well) to bind to asialo-GM_1 (GgO_4Cer) and to β-Gal-treated asialo-GM_1 (GgO_4Cer), i.e., to asialo-GM_2 (GgO_3Cer) was determined by ELISA. The binding of fimbriae or bacteria was determined as described in the legends of Fig. 1 and 2. Results were compared with the binding of the purified F1C fimbriae and F1C fimbriated bacteria to asialo-GM_1 (GgO_4Cer). Significant increase (two- to threefold) in binding due to the generation of asialo-GM_2 (GgO_3Cer) with β-Gal was noted. Data represent the average of quadruplicate determinations.

Neither purified fimbriae nor fimbriated bacteria showed any binding to glycolipids containing sialic acid residues (Table 1). Even sialic acid residue linked to internal galactose of GM_1 was able to block the binding, probably due to steric hinderance (Fig. 1). Pretreatment of the glycolipids with sodium metaperiodate almost completely eliminated the binding (data not shown).

To examine the relative affinity of purified fimbriae and fimbriated bacteria, serially diluted glycolipids were coated on ELISA plates, and the binding assay was performed with either F1C fimbriated bacteria (3×10^7/well) or purified F1C fimbriae (1.5 μg/well). The binding was found to be saturable, with 50% binding occurring at 0.350 μg (asialo-GM_1 [GgO_4Cer]), 0.178 μg (asialo-GM_2 [GgO_3Cer]), 1.5 μg (LacCer), and 3.4 μg (GalCer2) (Fig. 2). Together with the results of sodium metaperiodate treatments, these data strongly suggest that the carbohydrate residues of glycolipids may be the binding site for F1C fimbriae.

Treatment of glycolipids with neuraminidase or glycosidases. Purified fimbriae did not bind to disialoganglioside fraction containing *O*-acetylated derivatives of the gangliosides (GD_3, disialosylparagloboside, disialosyllacto-*N*-nor-hexaosylceramide, and disialosyllacto-*N*-nor-octaosylceramide) purified from human leucocytes and separated on HPTLC plates (Fig. 3B). After the treatment of the separated disialogangliosides with neuraminidase, the binding of fimbriae could be observed (Fig. 3D). The removal of terminal sialic acid residues was confirmed by using specific monoclonal antibody 1B2 to Galβ1-4GlcNAc carbohydrate sequences (Fig. 3C).

To further confirm the specificity of F1C fimbrial binding, asialo-GM_1 (GgO_4Cer) was sequentially degraded from its nonreducing end. Terminal galactose residues were removed by using β-galactosidase. Removal of terminal galactose residue from asialo-GM_1 (GgO_4Cer) resulted in an approximately two- to threefold increase in binding of purified fimbriae as well as fimbriated bacteria compared with that of asialo-GM_1 (GgO_4Cer) (Fig. 4). *N*-Acetylhexosaminidase did not digest GalNAcβ1-4β residues efficiently (data not shown). However, a small decrease in the rate of binding could be observed.

Carbohydrate inhibition studies. In order to obtain information about the carbohydrate receptor structure for F1C adhesin, the ability of saccharides corresponding to β-Gal, lactose, and GalNAcβ1-4Galβ recognition sites to inhibit the F1C ligand binding to asialo-GM_1 (GgO_4Cer) and asialo-GM_2 (GgO_3Cer) was studied (Table 2). Attachment of HB101 (pPIL110-54) to either glycolipid was inhibited most effectively (>60%) with 1 mg of neoglycoprotein (GalNAcβ1-4Galβ-spacer-BSA) per ml, representing 0.26 mM of GalNAcβ1-4Galβ disaccharide. The other mono- and disaccharides used as inhibitors reduced the bacterial adhesion to about 50% at 10 mM concentration (Table 2). At 1 mM, none of these sugars

TABLE 2. Effect of saccharides on the binding of F1C fimbriated recombinant *E. coli* HB101(pPIL110-54) to immobilized asialo-GM_1 (GgO_4Cer) or asialo-GM_2 (GgO_3Cer)

Saccharide	Inhibition (mean % of control)		
	Conc (mM)	Asialo-GM_1 (GgO_4Cer)	Asialo-GM_2 (GgO_3Cer)
D-Galactose	10	53	57
	1	12	10
Galβ1-3GalNAc	10	51	37
	1	8	6
Galβ1-4GlcNAc	10	51	41
	1	10	7
GalNAcβ1-4Gal-spacer-BSA[a]	0.26	64	69
	0.026	40	45
N-Acetyl-D-galactosamine	10	47	47
	1	12	9
Lactose	10	48	41
	1	11	8

[a] Molarity of disaccharide GalNAcβ1-4Gal linked to neoglycoprotein according to the company's information. Binding of nonfimbriated control HB101(pACYC 184) strain or fimbriated bacteria HB101(pPIL110-54) suspended in buffer only was used as a control value and was represented as 0.0 and 100%, respectively.

inhibited adhesion to a significant degree. In contrast, the GalNAcβ1-4Gal containing neoglycoprotein inhibited the adhesion even at a concentration of only 0.026 mM to a similar extent as the other sugars at more than 380-fold higher concentration (Table 2). These results confirm that disaccharide GalNAcβ1-4Galβ specifically binds to F1C fimbriae. The fact that some saccharides were not efficient inhibitors reflects a multivalency effect in the binding of F1C fimbriae to asialo-GM_2 (GgO_3Cer) or asialo-GM_1 (GgO_4Cer).

DISCUSSION

Binding of bacteria to glycolipids either separated by TLC (16, 49) or immobilized on microtiter plates appears to be a convenient way of detecting and characterizing carbohydrate receptor(s). In this study, we identified for the first time ligand-receptor interactions between the F1C fimbrial adhesin complex of uropathogenic *E. coli* and reference glycolipids of different carbohydrate compositions (Table 1). This was accomplished by performing binding assays with purified F1C fimbriae or F1C fimbriated recombinant *E. coli* strain HB101(pPIL110-54) and a panel of naturally occurring purified glycolipids resolved by TLC or immobilized in microtiter wells (Table 1 and Fig. 1, 2, and 3).

The binding affinity expressed as the amount of glycolipid required to achieve 50% maximal binding in the microtiter plates was the same for both F1C fimbriae and fimbriated *E. coli* being asialo-GM_2 (GgO_3Cer) (0.18 μg) > asialo-GM_1 (GgO_4Cer) (0.35 μg) > LacCer (1.50 μg) > GalCer2 (3.40 μg) (Fig. 2). The twofold difference in binding affinity of the F1C fimbriae to LacCer compared to GalCer2 suggested that a disaccharide sequence is needed for more efficient binding of F1C fimbriae. The binding of F1C adhesin increased by a factor of 8 after the addition of a GalNAcβ1-4β residue to LacCer as in asialo-GM_2 (GgO_3Cer) (Fig. 2). However, the addition of Galβ1-3β residue to GalNAcβ1-4β in asialo-GM_2 (GgO_3Cer) approximately doubles the quantity of glycolipid (asialo-GM_1 [GgO_4Cer]) to reach 50% binding of F1C fimbriae (Fig. 2). This must be due to the presence of the β-linked galactose residue at the nonreducing end of asialo-GM_1 (GgO_4Cer), which may partially inhibit the accessibility of the rest of the glycolipid containing the "real" receptor structure. Alternatively, F1C fimbriae might also bind to some extent Galβ residues terminally linked to asialo-GM_2 (GgO_3Cer) which would interfere with binding to the internal sequence of asialo-GM_1 (GgO_4Cer) (GalNAcβ1-4Galβ).

This notion is supported by the finding that preincubation of F1C fimbriated recombinant *E. coli* strain HB101(pPIL110-54) with 10 mM free disaccharide (Galβ1-3GalNAc) corresponding to the nonreducing end of asialo-GM_1 (GgO_4Cer) or with the same concentration of free D-galactose reduced the binding to asialo-GM_1 (GgO_4Cer) by approximately 50% (Table 2). Moreover, maximum inhibition was only achieved with a very low amount (1 mg/ml and 0.1 mg/ml of GalNAcβ1-4Gal containing neoglycoprotein equivalent to 0.26 mM and 0.026 mM of disaccharide GalNAcβ1-4Galβ (Table 2). The inhibitory effect of neoglycoprotein containing disaccharide GalNAcβ1-4Galβ was 38- to 380-fold more potent than that of the mono- and disaccharides tested.

This indicates that terminally linked β-Gal alone, as in asialo-GM_1 (GgO_4Cer) and internally positioned β-Gal alone or terminally linked β-*N*-acetylgalactosamine alone, as in asialo-GM_2 (GgO_3Cer) are not sufficient for effective binding of purified F1C fimbriae or F1C fimbriated bacteria. This is supported by the fact that bacteria did not bind to galabiosylceramide, which contains a single internally linked Galβ residue, or to globotetraosylceramide (Gb_4Cer), which contains a terminal GalNAcβ residue (Table 1, Fig. 1). They also did not bind to Forssman glycosphingolipid, which contains the GalNAcα1-3GalNAc sequence (Table 1). Hence, it appears that for the high-affinity binding of F1C fimbriae, the disaccharide sequence GalNAcβ1-4Galβ is required, and the minimum carbohydrate moiety of glycolipids needed for F1C fimbrial binding is the β1-linked galactose or glucose, provided they are presented in the correct conformation and configuration.

However, as has been noted for other adhesin systems, the presence of the minimum receptor structure in glycolipids does not necessarily correlate with binding (16). For example, despite containing β-Gal in their oligosaccharide sequence, GM_1, GM_2, GM_3, GD_2, GD_3, DSPG, DSnHC, and DSnOC do not show any affinity towards F1C fimbrial adhesin (Table 1 and Fig. 3B). This might be due to steric hinderance from sialyl residues linked to those glycolipids, preventing the access of fimbriae to the binding epitope on the glycolipids. Furthermore, purified F1C fimbriae or fimbriated bacteria did not bind to GM_1 but did bind very efficiently to asialo-GM_1 (GgO_4Cer) or to asialylated GD_3, asialylated DSPG, asialylated DSnHC, and asialylated DSnOC (Fig. 1B and Fig. 3C).

The findings that sialyl residues abolish binding corroborate a previously reported study which showed the inability of several pulmonary pathogenic bacteria to bind to gangliosides (25), although they bind to the receptor structure GalNAcβ1-4Galβ of asialo-GM_1 (GgO_4Cer) and asialo-GM_2 (GgO_3Cer). In addition, the inability of bacteria to recognize glycolipids such as globotetraosylceramide (Gb_4Cer) and Forssman antigen, which do not carry sialic acid residues, could be attributed to the conformational restrictions on the presentation of specific carbohydrate receptors due to the saccharide sequence and the configuration of the oligosaccharide moieties of those glycolipids.

In addition, the findings of glycosidase treatment showing that the removal of terminal Galβ1-3 residue from asialo-GM_1 (GgO_4Cer) increased the binding by approximately two- to threefold demonstrated again the importance of the internal GalNAcβ1-4Gal disaccharide sequence of asialo-GM_1 (GgO_4Cer) or the terminal GalNAcβ1-4Gal disaccharide sequence of asialo-GM_2 (GgO_3Cer) for high-affinity binding of F1C fimbriae. The specificity for internal sequences is similar to that reported for the P fimbrial adhesin, which recognizes terminal or internal Galα1-4Gal sequences in glycosphingolipids (5) but is different from K88 fimbriae of enterotoxigenic *E. coli* and pH6 antigen of *Yersinia pestis* (35, 36).

These observations support a role for the F1C fimbrial adhesin as a mediator of bacterial adherence. They also suggest that GalCer- or GlcCer-sensitive adherence may be the mechanism of low-affinity transient binding of bacteria. For low-affinity binding, these data further suggest the requirement of the terminal Galβ at the disaccharide level in the case of LacCer binding. Part of the internal Glcβ apparently is also involved in the binding epitope since this residue is described to be quite exposed in the conformation responsible for a correct presentation of the epitope (1). Furthermore, the inhibitory effect of neoglycoprotein containing disaccharide GalNAcβ1-4Galβ together with the binding affinity and specificity for glycolipids (Table 1) suggests the involvement of disaccharide GalNAcβ1-4Galβ sequence as a high-affinity receptor for F1C fimbriae.

The poor binding of the F1C fimbrial adhesin to GalCer1 containing hydroxylated fatty acids and comparatively better binding to GalCer2 which contains nonhydroxylated fatty acids may contribute to the nonoptimal presentation of the relevant

epitope. In support of this, it was reported that the Gal headgroup of GalCer with hydroxylated fatty acid is in an L-like conformation in relation to ceramide. In contrast, the Glc headgroup of GlcCer with nonhydroxylated fatty acid projects straight up (29, 46). However, this could not be resolved using TLC overlay assay or solid-phase binding assay. Moreover, an increased affinity of ligands for glycoceramides with higher levels of hydroxylation has been described in other systems (1, 2, 16, 18, 35, 36, 39, 41, 49). This dependence on the ceramide structure for bacterial binding has been reported to be limited at the two sugar level, which disappeared upon elongation of the saccharide chain (48).

Several other fimbrial adhesins of bacteria have also been shown to be able to bind to glycolipids (18, 35, 39, 44, 47). For instance, the *Pseudomonas aeruginosa* fimbrial receptor was determined to be the carbohydrate sequence GalNAcβ1-4Gal of asialo-GM_1 (GgO_4Cer) or asialo-GM_2 (GgO_3Cer) (6, 44). Moreover, a protein distinct from fimbriae present on the surface of *Neisseria gonorrhoeae* has also been shown to mediate the binding of gonococci to LacCer, Gb_3Cer, asialo-GM_2 (GgO_3Cer) and asialo-GM_1 (GgO_4Cer) (48).

The data presented in this paper demonstrate the high-affinity binding of F1C fimbriae to the GalNAcβ1-4Galβ sequence of glycolipids, i.e., asialo-GM_1 (GgO_4Cer) and asialo-GM_2 (GgO_3Cer). An additional binding to carbohydrate structures GlcNAcβ1-3Galβ, Galβ1-4Glc, Gal, and Glc of glycolipids may indicate functional low-affinity receptor sites. Studies are in progress to demonstrate the relevance of this binding for uropathogenecity of F1C fimbriated *E. coli*.

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