Identification of carbohydrate structures that are possible receptors for *Neisseria gonorrhoeae*

(bacterial binding/glycolipids/lactose epitope/adhesion/overlay assay)

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Communicated by Stanley Falkow, January 11, 1988

ABSTRACT Different strains and isogenic variants of *Neisseria gonorrhoeae* were assayed for their ability to bind glycolipids extracted from various sources. Among a large number of reference glycolipids, binding was observed only to lactosylceramide [Gal(β 1–4)Glc(β 1–1)Cer], isoglobotriaosylceramide [Gal(α 1–3)Gal(β 1–4)Glc(β 1–1)Cer], gangliotriaosylceramide [GalNAc(β 1–4)Gal(β 1–4)Glc(β 1–1)Cer], and gangliotetraosylceramide [Gal(β 1–3)GalNAc(β 1–4)Gal(β 1–4)Glc(β 1–1)Cer]. The latter two glycolipids bound gonococci with the highest affinity. Lactosylceramide and gangliotriaosylceramide were found in glycolipid preparations from ME180 cells, an epithelial cell line derived from a human cervical carcinoma, and thus are possible receptors for gonococci. The gonococcal surface component that bound the above glycolipids is a protein distinct from pilin and protein II.

The attachment of a pathogen to host tissues is an important step in the initiation of an infection. An increasing number of studies indicate the importance of animal cell surface carbohydrates as attachment sites for microbial ligands (1, 2). Many of these are present as glycolipids. There is evidence that eukaryotic cell receptors for *Neisseria gonorrhoeae* (the gonococcus) contain sugar residues (1–5). However, no specific receptor structure has been identified, in part because of methodological difficulties (6). Piliated strains of gonococci attach to epithelial cells more readily than their nonpiliated counterparts, and binding of the gonococcus to human epithelial cells is thought to be mediated by pilin (7), the major subunit of the pilus fiber. In addition, other bacterial surface components are thought to be adhesins (6, 8).

We have used a recently developed binding assay to examine in detail gonococcal–host cell interactions. Glycosphingolipids were separated on thin-layer chromatograms (9, 10), and radiolabeled gonococci were incubated with the chromatograms to detect specific bacterial binding. Using this binding assay, we have defined epithelial cell glycolipids recognized by various clinical isolates of *N. gonorrhoeae*. Experiments using isogenic strains of *N. gonorrhoeae* MS11 that differ only in their state of piliation indicate that the bacterial adhesin recognizing these glycolipids is neither pilin nor protein II (PII).

MATERIALS AND METHODS

Bacterial Strains and Radiolabeling. Piliated (P^+) and nonpiliated (P^-) variants of *N. gonorrhoeae* strains MS11 (11), ML1, and LP1 (12) have been described. In the B2 deletion variant of MS11, both pilin expression sites lack the

structural gene and promoter sequences (11). This strain produces no pilin mRNA or protein. PII variants of strain FA1090 were obtained from J. G. Cannon (University of North Carolina; ref. 8). The UM and plasmid-free KH4318 strains were obtained from the Department of Microbiology, University of Umea. *Escherichia coli* J96 (13), a uropathogenic isolate, produces Pap pili, which are associated with pyelonephritis.

For all assays, bacteria were grown overnight on supplemented GCB (Difco) plates at 37°C in 5% CO₂/95% air. Cells were metabolically labeled by addition of [³⁵S]methionine (Amersham, 600 Ci/mmol; 150 μ Ci per plate; 1 Ci = 37 GBq), diluted with GCB broth, and directly layered onto 50-mm agar plates streaked with bacteria.

Glycolipids. Total acid and nonacid glycolipids obtained from the sources given in Table 1 were prepared by standard methods (14–16). Brain gangliosides (nos. 18 and 19 in Table 1) were isolated by DEAE-Sepharose chromatography (17), followed by chromatography on Iatrobead (6RS-8060, Iatron Laboratories, Tokyo) columns using chloroform/methanol/5 M NH₃ in water (17) as eluant. Most of the individual nonacid glycolipids were prepared as acetylated derivatives (14) by Iatrobead column chromatography using chloroform/methanol (14) gradients and, after deacetylation (14), using chloroform/methanol/water gradients (14). Glycolipid 8 in Table 1 was obtained by degradation of 180 mg of glycolipid 18 in 30 ml of 0.1 M HCl at 100°C for 60 min, followed by Iatrobead column chromatography. Glycolipid 12 was obtained from Fuc(α 1–2)Gal(β 1–4)GlcNAc(β 1–3)Gal(β 1–4)Glc(β 1–1)Cer (Cer = ceramide) of dog small intestine (18) by hydrolysis with bovine kidney α -fucosidase (Boehringer Mannheim) in which repeated additions of enzyme (1.5 units total) were made for 24 hr at 37°C to 4 ml of 0.1 M acetate buffer (pH 4.5) containing 10 mg of taurodeoxycholate. The structures of the isolated glycolipids were confirmed by mass spectrometry (sequence) and NMR spectroscopy (anomer) after derivatization, and by gas chromatography following degradation (18, 19). The total nonacid glycolipids of ME180 cells (20) were prepared as described elsewhere (14).

Binding Assays. The chromatogram overlay assay was performed as described earlier (9, 10). Briefly, glycolipids were separated on silica gel 60 coated on aluminum sheets (HPTLC nano plates, Merck) with chloroform/methanol/water, 60:35:8 (vol/vol), as solvent. The dried plate was dipped for 1 min into 0.5% polyisobutylmethacrylate in diethyl ether, dried, and immersed for 2 hr in phosphate-buffered saline (50 mM sodium phosphate/150 mM NaCl, pH 7.3) containing 2% (wt/vol) bovine serum albumin. The plate

Abbreviations: PII, protein II; Cer, ceramide.

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Table 1. Binding of piliated and nonpiliated variants of *N. gonorrhoeae* MS11 to immobilized glycolipids

No.	Glycolipid Structure†	Binding of variants*		Source (ref.)‡
		P+	P-	
1	Gal(β1-1)Cer(h)	—	—	Various sources (15)
2	Glc(β1-1)Cer(h)	—	—	Various sources (15)
3	Gal(β1-4)Glc(β1-1)Cer	—	—	Human erythrocyte (15)
4	Gal(β1-4)Glc(β1-1)Cerh	+	+	Dog small intestine (21)
5	GlcNAc(β1-3)Gal(β1-4)Glc(β1-1)Cer	—	—	Malignant melanoma§
6	Gal(α1-3)Gal(β1-4)Glc(β1-1)Cerh	+	+	Dog small intestine (21)
7	Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer	—	—	Human erythrocyte (15)
8	GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cer	+	+	Human brain (16)
9	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cerh	+	+	Mouse small intestine (22)
10	GalNAc(β1-3)Gal(α1-3)Gal(β1-4)Glc(β1-1)Cer	—	—	Rat intestinal carcinoma (23)
11	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc(β1-1)Cerh	—	—	Human meconium (19)
12	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(β1-1)Cerh	—	—	Dog small intestine (18)
13	GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer	—	—	Human erythrocyte (15)
14	GalNAc(α1-3)GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer	—	—	Dog small intestine (21)
15	Fuc(α1-2)Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc(β1-1)Cerh	—	—	Mouse large intestine (22)
16	Gal(β1-3)GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cerh	—	—	Vero cells (24)
17	GalNAc(α1-3)[Fuc(α1-2)]Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)Glc(β1-1)Cerh	—	—	Dog small intestine (18)
18	Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer	—	—	Human brain (16)
19	NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc(β1-1)Cer	—	—	Human brain (16)

*The binding pattern to the carbohydrate structures listed above was identical for all gonococcal variants. The thin-layer plate (see Fig. 1) was used for the binding; + means a distinct darkening of the autoradiogram, while — means no darkening, both at a level of 1–2 μg of glycolipid (compare Fig. 1). The binding pattern is very similar but deviates somewhat from a group of other lactosylceramide-binding bacteria (ref. 25; also K.-A.K. and N.S., unpublished data).

†Cer, a ceramide with a nonhydroxy fatty acid and a dihydroxy long-chain base; Cerh, a ceramide with a 2-hydroxy fatty acid and/or a trihydroxy base; Cer(h), both types of ceramide existed.

‡The reference at the preparation source reports the structure and/or the isolation procedure.

§K.-A.K., unpublished data.

was then overlaid for 2 hr with labeled gonococci (2 ml of 10⁷ cpm/ml; 10⁸ cells per ml), washed five times with phosphate-buffered saline, dried, and autoradiographed on XAR-5 x-ray film (Eastman).

To establish binding curves, serial dilutions of glycolipids in methanol (50 μl) were dried overnight in microtiter wells (10). After binding sites were blocked with 2% bovine serum albumin in phosphate-buffered saline, the wells were incubated with labeled gonococci for 4 hr (50 μl per well; 2.5 × 10⁶ cpm; ≈10⁷ cells). After five washes with phosphate-buffered saline, the wells were dried, cut out, and measured for radioactivity in a scintillation counter. Curves were approximated from triplicate determinations (<10% SD).

RESULTS AND DISCUSSION

Total acid and nonacid glycolipids, obtained from the sources listed in Table 1, were chromatographed on thin-layer plates and fixed. Fig. 1a shows three lanes of one such chromatogram after spray detection with anisaldehyde. Fig. 1b shows an autoradiogram of an identical plate after it was incubated with metabolically labeled *N. gonorrhoeae* MS11A (P⁺). A pattern identical to that in Fig. 1b was observed when the plate was incubated with a P[−] variant (B2) of MS11A (data not shown). These results indicate that both P⁺ and P[−] gonococci bound to the same carbohydrate structures when exposed to a large number of reference glycolipids. Binding was limited to molecular species in the two- and four-sugar region. Labeled cells did not bind to other tested glycolipids even though the latter were each at concentrations of 1 μg or more. The minimum sugar recognized by the bacterium was lactose in free lactosylceramide (Table 1, glycolipid 4). The other three glycolipids bound by gonococci had various saccharide extensions on lactose: Gal(α1-3), isoglobotriaosylceramide (no. 6); GalNAc(β1-4), gangliotriaosylceramide (no. 8); and Gal(β1-3)GalNAc(β1-4), gangliote-

traosylceramide (no. 9). Thus, lactose [Gal(β1-4)Glc], the core sugar in these glycolipids, probably carries at least part of the binding epitope recognized by a gonococcal adhesin. Recognition by bacteria of internally placed sugars on glycolipids has been demonstrated previously for the Pap adhesin of uropathogenic *E. coli*, the minimum sequence

FIG. 1. Detection of receptor-active glycolipids by binding ³⁵S-labeled *N. gonorrhoeae*, strain MS11 A (P⁺), to glycolipids resolved in a thin-layer chromatogram. (a and b) Three lanes are shown after spray detection with anisaldehyde (a) and after autoradiography following overlay with labeled gonococci (b). Lanes: 1 and 2, total nonacid glycolipid mixtures (20–40 μg) isolated (14) from mouse (22) and dog (21) small intestine, respectively; 3, 1 μg each of glycolipids 3, 8, 9, and 17 (Table 1). Numbers on spots refer to Table 1, and numbers between a and b indicate the approximate number of sugars on the separated glycolipids (no. 9 migrates atypically; see ref. 22). Autoradiography was for 70 hr. (c) Autoradiogram (70 hr) following overlay of labeled gonococci on a chromatogram with 20 μg of total nonacid glycolipid isolated from ME180 cells. The arrows indicate positive bands with mobilities corresponding to lactosylceramide, no. 4 (upper arrow), and gangliotriaosylceramide, no. 8 (lower arrow).

recognized being Gal(α 1-4)Gal (26). Table 1 summarizes the results of our binding studies.

The relative affinity of binding of gonococci to the glycolipids was next examined. Microtiter wells were coated with dilutions of each glycolipid and labeled MS11A (P^+) were incubated in these wells. Fig. 2 shows that the bacteria bound best to the three- and four-sugar glycolipids (gangliotetraosylceramide, no. 8; and gangliotetraosylceramide, no. 9), with half-maximal binding occurring at a glycolipid concentration of ≈ 100 ng per well. In this assay, lactosylceramide was bound poorly. The extension of lactosylceramide with GalNAc(β 1-4) (no. 8) increased the binding affinity, in contrast to an extension with Gal(α 1-3) (no. 6). Identical binding affinities were observed when the experiment was repeated with labeled MS11 B2 (P^-) cells (data not shown). The relative affinities of gonococcal-receptor binding are analogous to those obtained for receptor binding by the Pap adhesin of uropathogenic *E. coli*. In the latter case, the *E. coli* bound with higher affinity to a glycolipid with a GalNAc(β 1-3) extension to Gal(α 1-4), than to one with Gal(α 1-4) alone (26).

Gonococci failed to bind to most of the other lactose-containing glycolipids (Table 1). This may be due to steric hindrance from additional sugars, which prevents the adhesin from recognizing or having access to the binding epitope on the glycolipid (27, 28). This has been analyzed by molecular modeling methods (28). There is in addition a peculiar dependence on the ceramide structure for binding (Table 1; data not shown). At the two-sugar level (free lactosylceramide), only species with hydroxy fatty acid and/or trihydroxy base (di- or trihydroxylated ceramide) were recognized, while species with nonhydroxy fatty acid and dihydroxy base (monohydroxylated ceramide) were not (Table 1). This strict dependence on ceramide structure disappeared on elongation of the saccharide chain. Thus, gangliotetraosylceramide of mouse intestine (Table 1, no. 9, di- and trihydroxylated ceramide) and gangliotetraosylceramide derived from human brain gangliosides, having mainly stearic acid and sphingosine (monohydroxylated ceramide), apparently bound gonococci equally well both on thin-layer chromatograms and in microtiter wells (data not shown). Similarly, the three-sugar species gangliotetraosylceramide (no. 8), prepared from brain gangliotetraosylceramide, was as effective for binding as mouse gangliotetraosylceramide (Table 1, Figs. 1b and 2). For globotriaosylceramide, which is not recognized by gonococci, the results are consistent both in the case of monohydroxylated ceramide (Table 1, no. 7) and di- or trihydroxylated ceramide (human kidney, data not shown).

FIG. 2. Quantitative curves from binding of *N. gonorrhoeae* MS11 A (P^+) to glycolipids coated in microtiter wells. Numbers at the curves refer to glycolipids listed in Table 1. Nonpiliated variants gave similar curves. Irregular values appear above ≈ 200 ng of glycolipid, at which point the wells have become saturated with a monolayer of glycolipid.

This interesting relation to the neighboring groups (ceramide or sugar) may be a different presentation of the actual lactose epitope at the assay surface and probably in the membrane. One may note that the more hydroxylated ceramide is typical of epithelial cells and appears to be absent from cells of other type (29). Of relevance are earlier conformation analyses of two monoglycosylceramides: x-ray crystallography revealed a shovel-like bend and the glycosidic oxygen in the case of a species with 2-hydroxy fatty acid (30), while NMR data suggested a "straight" conformation for a species with nonhydroxy fatty acid (31). Finally, gonococci did not bind brain gangliosides (Table 1, nos. 18 and 19) in our thin-layer assay, in contrast to previous reports based on other methods (ref. 3; see also the discussion in ref. 6).

Attempts to inhibit gonococcal binding to gangliotetraosylceramide (Table 1, no. 9) in the microtiter well assay with free lactose or multivalent lactose covalently coupled to bovine serum albumin (Swedish Sugar, Arlov, Sweden) were unsuccessful. This may be explained by a low affinity of gonococci for the lactose epitope and/or a need for proper presentation of the lactose epitope before binding can occur. In comparison, the binding of uropathogenic *E. coli* to Gal(α 1-4)Gal can be inhibited by free receptor oligosaccharide (32). However, it must be noted that the half-maximal binding to receptor glycolipids in microtiter wells is 10- to 100-fold lower for these *E. coli* than for gonococci (ref. 26; unpublished results), suggesting a higher affinity interaction in the case of *E. coli* binding.

Hemagglutination has been used as an assay for bacteria-receptor interactions (32) because of its relative ease and because it affords an examination of such interactions under more natural conditions. We next studied the ability of gonococci to hemagglutinate erythrocytes coated with the proper glycolipid. Guinea pig erythrocytes coated with gangliotetraosylceramide (Table 1, no. 9) induced hemagglutination (Table 2). In contrast, gonococci did not hemagglutinate uncoated erythrocytes or those coated with globoside. In addition, uropathogenic *E. coli* J96 did not hemagglutinate gangliotetraosylceramide-coated erythrocytes, although they did hemagglutinate erythrocytes coated with globoside, its receptor. These data support our previous observations on the specific binding of gonococci to gangliotetraosylceramide *in vitro*.

We also examined the presence of the relevant glycolipids on human epithelial cells. Total nonacid glycolipids were prepared from ME180 cells (a human epithelial cell line derived from a cervical carcinoma), separated on thin-layer plates, and assayed for the ability of MS11A (P^+) and MS11B2 (P^-) variants to bind. Both variants exhibited the same binding pattern (Fig. 1c). In these experiments gonococci bound two structures corresponding in mobility to di- and triglycosylceramide. These are likely lactosylceramide (Table 1, no. 4) and gangliotetraosylceramide (no. 8), since the

Table 2. Agglutination of glycolipid-coated guinea pig erythrocytes by *N. gonorrhoeae* MS11 and by *E. coli* J96

Bacteria	PBS	Gangliotetraosylceramide (no. 9)	Globoside (no. 13)
MS11 A(P^+)	—	++	—
MS11 B2(P^-)	—	++	—
J96 (<i>E. coli</i>)	—	—	+++

Guinea pig erythrocytes [1% in phosphate-buffered saline (PBS), pH 7.4] were incubated (32) with glycolipid (0.1 mg/ml) at 37°C for 3 hr. The cells were washed three times in phosphate-buffered saline and used fresh. For hemagglutination, 10 μ l of coated erythrocytes (4%) and bacteria (10^9 cells per ml), both suspended in phosphate-buffered saline, were mixed on a glass slide. At higher glycolipid concentrations (0.5 mg/ml), gangliotetraosylceramide may cause spontaneous hemagglutination.

other three-sugar binder, isoglobotriaosylceramide (no. 6), is not known to exist in human tissues. Thus, the putative receptor glycolipids defined by the thin-layer assay are present in relevant host cells.

The molecular nature of the gonococcal surface component(s) responsible for binding the above glycolipids was initially examined by using isogenic variants of strain MS11 (11, 12). The P⁺ and P⁻ variants bound the same carbohydrate structures (Table 1) with the same affinity (Fig. 2), suggesting that the bacterial surface ligand(s) interacting with the glycolipids is not pilin, the major subunit of the pilus. This adhesin is a protein, as gentle proteinase K treatment of MS11A (P⁺) abolished the ability of these cells to bind glycolipids in the thin-layer assay. PII⁻ variants of strain FA1090 and MS11 exhibited the same glycolipid binding pattern (Table 3), indicating that the bacterial adhesin is not PII. Furthermore, this adhesin is most probably not encoded by the cryptic plasmid because a cryptic plasmid-free strain tested positive in our assay.

CONCLUSIONS

This study identifies a specific glycolipid-binding property of *N. gonorrhoeae* that may be involved in the attachment of this pathogen to host epithelial cells. Binding is mediated by a gonococcal surface protein distinct from pilin and PII. Our findings are consistent with the notion that in bacterial pilus systems, notably *pap* (13, 33) and type 1 of *E. coli* (34, 35), specificity of receptor binding is determined by minor surface proteins and not by the major pilin subunit. Recently, the Pap adhesin has been shown to be associated with the pilus structure (36).

Studies demonstrate that piliated gonococci are more virulent (6) and attach more readily to epithelial cells (6) than do their nonpiliated counterparts. Our *in vitro* system used to detect carbohydrate receptors, although no less valid, is devoid of other factors that would affect binding of bacteria

Table 3. Binding to the thin-layer chromatograms of gonococcal isolates with different outer membrane protein profiles

Strain	Phenotype	Binding to glycolipids
MS11-A	P ⁺ PII ⁻ PIBahjk	+
MS11-B4.1	P ⁻ PII ⁻	+
MS11-B2	P ⁻ PII ⁻	+
MS11-B1	P ⁻ PII ⁻	+
MS11-C2	P ⁺ PII ⁻	+
MS11-C10	P ⁺ PII ⁻	+
MS11-A	P ⁺ PII ⁻ + proteinase K	-
UM	P ⁺ PII? PIAedghih	+
UM	P ⁻ PII?	+
FA1090	P ⁻ PII ⁻	+
FA1090	P ⁻ PIIa	+
FA1090	P ⁻ PIIb	+
FA1090	P ⁻ PIIc	+
FA1090	P ⁻ PIId	+
FA1090	P ⁻ PIIe	+
FA1090	P ⁻ PIIf	+
ML1	P ⁺	+
LP1	P ⁺	+
KH4318	P ⁺ PII ⁺ PIBack (cryptic plasmid free)	+

For proteinase K treatment of MS11, bacteria were incubated with 2.5 µg of proteinase K per ml for 30 min at 37°C. Plate counts of bacteria before and after treatment indicated no loss of viability. The glycolipid binding properties were tested by using the chromatogram binding assay as shown in Fig. 1. A positive binding (+) indicated a binding pattern identical to that of Table 1. PI, protein I.

to a topologically complex cell surface in a more natural setting. The gonococcal pilus structure is clearly important to the *in vivo* attachment process and, in this regard, is similar to the binding requirements of the *E. coli* type 1 system (34, 35). Adhesion studies with pilin as well as adhesin mutants and synthetic receptor analogues may elucidate the exact nature of gonococcal interactions with eucaryotic carbohydrate receptors *in vivo*.

This work was supported by grants from the Swedish Medical Research Council (3967 and 4769) and from the National Institutes of Health (RO1 AI22152).

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