Characterization of the Binding of Actinomyces naeslundii (ATCC 12104) and Actinomyces viscosus (ATCC 19246) to Glycosphingolipids, Using a Solid-phase Overlay Approach*

(Received for publication, December 19, 1989)

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Actinomyces naeslundii (ATCC 12104) and Actinomyces viscosus (ATCC 19246) were radiolabeled externally (185I) or metabolically (35S) and analyzed for their ability to bind glycosphingolipids separated on thin layer chromatograms or coated in microtiter wells. Two binding properties were found and characterized in detail. (i) Both bacteria showed binding to lactosylceramide (LacCer) in a fashion similar to bacteria characterized earlier. The activity of free LacCer was dependent on the ceramide structure; species with 2-hydroxy fatty acid and/or a trihydroxy base were positive, while species with nonhydroxy fatty acid and a dihydroxy base were negative binders. Several glycolipids with internal lactose were active but only gangliotriaosylceramide and gangliotetraosylceramide were as active as free LacCer. The binding to these three species was half-maximal at about 200 ng of glycolipid and was not blocked by preincubation of bacteria with free lactose or lactose-bovine serum albumin. (ii) A. naeslundii, unlike A. viscosus, showed a superimposed binding concluded to be to terminal or internal GalNAcβ and equivalent to a lactose-inhibitable specificity previously analyzed by other workers. Terminal Galβ was not recognized in several glycolipids, although free Gal and lactose were active as soluble inhibitors. The binding was half-maximal at about 10 ng of glycolipid.

A glycolipid mixture prepared from a scraping of human buccal epithelium contained an active glycolipid with sites for both binding specificities.

Specific adherence as a determinant of tissue selectivity of bacterial colonization was early suggested within oral microbiology including studies with Actinomyces naeslundii and Actinomyces viscosus (for a review, see Ref. 1), which have been regarded as potential pathogens in oral infections (2-4). Both A. naeslundii and A. viscosus constitute typical members of the tooth plaque but differ from each other in that A. naeslundii is more prominent at oral epithelial surfaces but relatively less common at tooth surfaces (5). The molecular basis for this situation is not fully understood, although it has recently been observed that there are two antigenically distinct types of fimbriae (type 1 and type 2), which differ both in their adhesive properties and their distribution between typical members of the two Actinomyces species (6, 7). Type 2 fimbriae, present on most strains of both species, are associated with a lactose-sensitive binding and mediate adherence to streptococci of tooth plaque (8-10), agglutination of human erythrocytes (11, 12), as well as adhesion to oral epithelial cells (13, 14). Type 1 fimbriae, on the other hand, are thought to mediate attachment of bacteria to the salivary pellicle coating the tooth surface, and these fimbriae appear on most A. viscosus strains but not on typical A. naeslundii strains (6, 7).

The lactose sensitivity associated with type 2 fimbriae has been examined by inhibition studies with various soluble saccharides (15 and references therein), by a model aggregation system based on beads coated with various glycoconjugates (16), and more recently by the overlay technique developed by us and used in the present work (17). The general conclusion from these studies has been a recognition of terminal Galβ residues. However, owing to limited access to diverse natural sequences, the specificity has not been worked out in detail.

We have previously used a direct binding of bacteria to glycolipids on thin layer chromatograms (18, 19) and characterized carbohydrate receptors for uropathogenic Escherichia coli (20, 21) and a group of bacteria classified as lactose binders (22-26), including N. gonorrhoeae (25). Using this approach, we now demonstrate that A. naeslundii (ATCC 12104), unlike A. viscosus (ATCC 19246), exhibits a high affinity binding to glycolipids with the sequence GalNAcβ, and that such compounds probably represent receptors on human erythrocytes and oral epithelial cells for A. naeslundii. For both these Actinomyces strains, we also identified an unrelated low affinity binding to lactosylceramide (LacCer). At present, we do not know the biological role of this low affinity binding, although receptor glycolipids were detected in oral epithelial cells.

MATERIALS AND METHODS

Preparation of Glycolipids—Buccal epithelium was collected from one of us (N. S.) by scraping with a spatula every other day for 2 weeks. The collected material was stored frozen in methanol until it was extracted, first twice with 20 ml of methanol at 70°C for 30 min, and finally three times with 20 ml of chloroform/methanol (2:1, by volume) for 30 min. From this and other tissue extracts (Table I), total neutral and acidic glycolipids were purified by mild alkaline degradation, dialysis, silicic acid, and DEAE-cellulose column chromatography of acetylated and native substances, as previously described (27). Individual glycolipid species were purified by repeated

2 The glycolipid nomenclature and abbreviations used in this paper follow the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN for lipids: Eur. J. Biochem. (1977) 79, 11-21 and J. Biol. Chem. (1982) 257, 3347-3351). It is assumed that Gal, GLe, GalNAc, GlcNAc, and NeuAc are of the D configuration. Fuc of the L configuration, and that all sugars present are in the pyranose form. The abbreviations used are: PBS, phosphate-buffered saline; RSA, bovine serum albumin.
continuous and stepwise gradient column chromatography of acetylated and native substances, as described in detail elsewhere (26).

To generate compound no. 26, a frog brain di- and triasialoganglioside fraction containing compound no. 32 (28) was treated with Newcastle disease virus, which specifically hydrolyzes NeuAcα2→3 Gal linkages but does not hydrolyze NeuAcα2→6GalNAc linkages (29), and the product was then purified (26). The glycosidase (9) was added and the incubation continued for an additional 18 h. The mixture was then desalted on a reverse-phase Sep-Pak column (Waters, Milford, MA) reconditioned with 20 ml of chloroform/methanol (2:1, by volume). The column was eluted with 10 ml of PBS, then with 5 ml of methanol, and finally with 10 ml of chloroform/methanol (2:1, by volume). The combined methanol/chloroform fractions were dried and further purified (26).

To generate compounds nos. 23 and 24, 2 mg each of nos. 29 and 30 were incubated in 5 ml of 0.05 M HCl at 90 °C for 2 h. The reaction mixture was then partitioned by adding 18 ml of chloroform and 4 ml of methanol to each followed by thin layer chromatography analyses using various solvent systems, including chloroform/methanol/ H2O (60:40:9, by volume), chloroform/methanol 5 N HCl in H2O (60:40:9, by volume) and chloroform/methanol, 0.2% CaCl2 in H2O (60:40:9, by volume). These analyses all revealed the generation of two compounds, constituting about 5–10% of each fraction, and which both migrated slightly above their precursor glycolipids but not in the position of H-5 type 1 or H-5 type 2 (i.e. nos. 19 and 20 of Table I).

Identity of Isolated Fractions—Individual glycolipids were initially assessed for purity and quantity by thin layer chromatography and various staining reagents (30–32). Chemical structures (Table I) were confirmed by mass spectrometric and proton NMR spectroscopic analyses of permethylated and permethylated-reduced substances (33–39), and through gas chromatographic analyses of the alditol acetates obtained after acid hydrolysis of the native (40) and derivatized glycolipids (41).

Bacterial Strains and Labeling—A comparative study was made of A. naeslundii, strain ATCC 12104 isolated from the human sinus, and A. viscosus, strain ATCC 19246, isolated from a case of human cervico-facial actinomycosis. For all assays, the bacteria were cultured anaerobically in PYG broth media or on Brucella agar plates for 3 days, harvested, and washed three times with PBS, pH 7.3. For binding to glycolipids, bacteria were labeled either externally with 14C-labeled Bolton-Hunter reagent (42), as described earlier (18, 26) or metabolically by adding [35S]methionine (600 μCi/mmol, 150 μCi/plate, Amersham Corp.) diluted with 50 μl of broth media onto 50-mm glass coverslips, incubated with bacteria (26). Labeled bacteria were washed three times in PBS and used immediately. The two labeling procedures gave the same results and incorporated about the same amount of radioactivity, which was 0.05–0.1 cpm/bacterium.

Binding to Solid-phase Glycolipids—The thin layer chromatogram (18, 19) and microtiter well (19) binding assays were conducted as described in detail elsewhere (26). Glycolipid chromatograms, pre-treated with plastic and bovine serum albumin (BSA), were overlaid with labeled bacteria (2 ml of 106 cpm/ml, 105 cells/ml), washed five times with PBS, dried, and exposed to x-ray film (KAR-5, Eastman, Kodak) for 70 h. Glycolipids in methanol (50 μl) were dried overnight onto open glass microscope slides followed by blocking with BSA. The wells were then incubated with radiolabeled bacteria (50 μl/well with 5 × 106 cpm and 5 × 105 cells) for 4 h, washed five times with PBS, dried, cut out, and measured for radioactivity.

Hemagglutination and Sugar Inhibition Experiments—For hemagglutination, 10 μl of fresh bacteria (105 cells/ml) and 10 μl of human erythrocytes were suspended in PBS, pH 7.3, were mixed in 10 μl of test solutions of sugars or compounds of interest and incubated for 20 min in 37 °C water bath. The blood group typing was performed in duplicates using 0.1% saline. A positive result was used to denote glycolipid bands refer to the structures listed in Table I. The vertical numbering to the left indicates the approximate number of sugar residues in glycolipids with a corresponding position. Thin layer plates with non-acid glycolipid fractions of various tissues. Shown are representative autoradiograms of the binding of each strain and the total glycolipid pattern of the test fractions as visualized by the anisaldehyde reagent. Total non-acid glycolipids (20 μg) used were from human erythrocytes of blood group A (lane A), human meconium of blood group B (lane B), monkey intestine of species Macaca cynomolagus (lane C), dog small intestine (lane D), rabbit small intestine (lane E), and guinea pig small intestine (lane F). The numbers used to denote glycolipid bands refer to the structures listed in Table I. The vertical numbering to the left indicates the approximate number of sugar residues in glycolipids with a corresponding position. Thin layer plates with non-acid glycolipid fractions of various tissues were obtained from Sigma, (lactose-O-CTE)n-BSA (2-2-carboxyethylthioethylglycosides) coupled in amide linkage to Lys; 20–30 mol/mole of BSA) and GalNAcβ1-3Gala-0-ethyl were purchased from Sockerbolaget, Arlöv, Sweden.

RESULTS

Binding of bacteria to glycolipids separated on thin layer chromatograms is a convenient way of detecting and characterizing receptors based on carbohydrate (18–26). The ability of reference glycolipids of different carbohydrate and lipophilic compositions to attach A. naeslundii (strain ATCC 12104) and A. viscosus (strain ATCC 19246) was investigated in detail (Figs. 1 and 2 and Table I). Both a lactose- and a GaINAcβ-based binding property were identified.

Detection of Glycolipids Mediating in Vitro Adherence of A. naeslundii and A. viscosus—Screening various mixtures of glycolipids (Fig. 1), A. viscosus bound selectively to certain molecular species of LacCer and to Galβ3 (nos. 4 and 7 of Fig. 1 and Table I). Besides attaching to these compounds, A. naeslundii bound several additional glycolipids ranging in size from about three to eight sugars (e.g. nos. 12 and 17). Several major glycolipids (e.g. nos. 16 and 36), including monobenzoxycelerylamides (nos. 1 and 2), were negative, even though present in amounts of 2 μg or more, documenting the selectivity of these interactions. The binding pattern noted for both bacteria is accounted for by a recognition of terminally (no. 4) and internally located lactose (no. 7), and the second and superimposed binding restricted to A. naeslundii by a recognition of GaINAcβ (see below).

Apparent Specificity of A. viscosus and A. naeslundii for LacCer—The glycolipid binding pattern of A. viscosus (Table I) is typical of most of the bacteria previously classified as lactose binders (22–26). The features of this binding were recently analyzed in detail for a strain of Propionibacterium granulosum (26).

As seen in Table I and Fig. 3, LacCer (no. 4) is among the most active glycolipids (nos. 4, 9, and 13) and carries the

A. naeslundii
A. viscosus
Anisaldehyde

FIG. 1. Binding of 125I-labeled A. viscosus (ATCC 19246) and A. naeslundii (ATCC 12104) to thin layer chromatograms with non-acid glycolipid fractions of various tissues. Shown are representative autoradiograms of the binding of each strain and the total glycolipid pattern of the test fractions as visualized by the anisaldehyde reagent. Total non-acid glycolipids (20 μg) used were from human erythrocytes of blood group A (lane A), human meconium of blood group B (lane B), monkey intestine of species Macaca cynomolagus (lane C), dog small intestine (lane D), rabbit small intestine (lane E), and guinea pig small intestine (lane F). The numbers used to denote glycolipid bands refer to the structures listed in Table I. The vertical numbering to the left indicates the approximate number of sugar residues in glycolipids with a corresponding position. Thin layer plates with non-acid glycolipid fractions of various tissues were obtained from Sigma, (lactose-O-CTE)n-BSA (2-2-carboxyethylthioethylglycosides) coupled in amide linkage to Lys; 20–30 mol/mole of BSA) and GalNAcβ1-3Gala-0-ethyl were purchased from Sockerbolaget, Arlöv, Sweden.

Materials and Methods.
Binding of Bacteria to Glycolipids

Besides the lactose-based recognition (i.e. nos. 4 and 7) A. naeslundii, unlike A. viscosus, recognized more slow-migrating bands (≥4 sugars) corresponding to GalNAcβ-containing glycolipids (Table I). For instance, the reactive band in the four sugar region of all samples (lanes A–F) in Fig. 1 reflects recognition of the GalNAcβ terminus of Gb4α (no. 12). The series of glycolipids recognized in the rat intestinal sample (Fig. 2, lane G) are Galα1–3 repetitive structures all terminating in a GalNACβ residue (no. 28). The relatively low amount of glycolipid needed for this binding was first noted when A. naeslundii stained several compounds not visible with the chemical detection reagent (marked with X in Fig. 1). When glycolipids coated in microtiter wells were used (Fig. 3), this binding was detectable at about 1 ng/well and was half-maximal at 10 ng/well. By comparison, these values are 10–100-fold lower than those observed for the lactose-based bindings (Fig. 3). Additional evidence for two chemically distinct and unrelated binding sites was provided by the sugar inhibition experiments presented below and by the finding that a certain batch of A. naeslundii was found to express the GalNAcβ-based activity only (Fig. 2). This batch of A. naeslundii failed to interact with LacCer (no. 4) and Gb3b (no. 7), although the binding to the GalNAcβ-containing glycolipids remained unaffected (see nos. 12 and 17).

The binding of A. naeslundii to serial dilutions of Gb4α (no. 12) in thin layer chromatograms was detectable at 1–10 ng, and at 500 ng it was maximal (data not shown). When this larger amount was used for the qualitative testing of the pure and structurally defined compounds shown in Table I, the most intensively stained glycolipids (marked +++) all contained internally or terminally located GalNAcβ. The glycolipid binding properties already attributed to the lactose-based specificity (compare with A viscosus) showed much weaker staining intensities at this concentration (marked with + and ++). So, except for these less active compounds, glycolipids lacking the GalNAcβ structure did not bind the bacteria.

The specificity for GalNAcβ in glycolipids may be summarized as follows. The demand for a β-anomerity was first deduced from the inactivity of the Forssman hapten (no. 16) and of blood group A active glycolipids (nos. 29 and 30). These compounds (i.e. nos. 29 and 30) also remained inactive after removal of their fucosyl residues (nos. 23 and 24). Lack of binding to substances with a terminal Galβ residue (i.e. nos. 1, 5, 14, 15, and 35) implied a substantial specificity for the acetamido group at C-2. The importance of the stereochemistry at C-4 was indicated from the results with GlcNACβ (nos. 10 and 15), which only showed a weak activity (+), probably attributable to the lactose-based specificity (compare with A viscosus). In addition, the hydroxyls at C-3 and C-6 of GalNAcβ may be substituted with either sialic acid (e.g. nos. 32 and 33) or NeuAcα (no. 26), respectively, without abolishing its binding activity.

According to the data in Table I, it appears that the GalNAcβ structure contains the whole recognition site. There is no difference in activity between compounds nos. 9 and 13 (Fig. 3), indicating that the terminal Galβ1–3 substituent of substances nos. 13 and 18 does not contribute to the interaction. Similarly, the equivalent activity of compounds terminating in a GalNAcβ1–4Gal sequence (no. 9), as compared with those with a GalNAcβ1–3Gal terminus (nos. 11, 12, 17, and 28), suggests that the interaction is also independent of the penultimate Gal.

Even though it was substituted with both Galβ1–3 and NeuAcα2–6, the epitope on GalNAcβ was apparently available to the bacteria (no. 26). However, further extensions of the Galβ1–3GalNAcβ terminus with either sialic acid (e.g. nos. 32

Fig. 2. Incidental failure of A. naeslundii (ATCC 12104) to bind to LacCer present in thin layer chromatograms with non-acid glycolipid mixtures of various tissues. II is the autoradiogram showing the binding of this particular batch of 111I-labeled A. naeslundii, and I the total glycolipid pattern as chemically detected with anisaldehyde. Total non-acid glycolipids (20 μg) used were from human erythrocytes of blood group A (lane A), human meconium of human erythrocytes of blood group B (lane B), monkey small intestine of species Macaca cynomolgus (lane C), dog small intestine (lane D), rabbit small intestine (lane E), guinea pig small intestine (lane F), and rat small intestine (lane G). The numbering of glycolipid bands refers to the structures listed in Table I. The numbers to the left of II indicate the number of sugar residues in the glycolipids with corresponding position. The thin layer plate was treated for bacterial binding and autoradiographed (70 h) as described in “Materials and Methods.”

the smallest sugar head group. It is also clear that the Galβ terminus of other glycolipids (nos. 18, 21, 25, and 26), including Galβ-Cer (no. 1), do not react with A. viscosus, supporting a requirement for lactose. Nor did glycolipids lacking the lactose sequence bind the bacteria (nos. 2 and 3). Some sugar additions to lactose completely abolished the binding (e.g. Galα1–4 for nos. 8 and 12), whereas others retained activity (e.g. Galα1–3 of no. 7, GlcNACβ1–3 of no. 10, and GalNAcβ1–4 of no. 9). This indicates that A. viscosus also accommodates lactose that is placed internally in the chain. However, only GalNAcβ1–4, as present in nos. 9 and 13, allowed binding at a level of free lactose. Also, the size of sugar additions at the Gal of lactose critically affected the accessibility of the binding site (Table I). This was evident when comparing compound no. 9 with 19, no. 7 with 11, and no. 10 with 35. Another finding from Fig. 1 is that binding to LacCer is clearly dependent on the ceramide structure. The bacteria bound to LacCer with a trihydroxy base and/or hydroxy fatty acid (no. 4, lane D) but did not bind to the faster migrating LacCer species of human erythrocytes with a dihydroxy base and nonhydroxy fatty acid (no. 5, lane A).

Studies with selected glycolipids absorbed in microtiter wells (Fig. 3) confirmed both the preferential binding of A. viscosus to certain species of LacCer (nos. 4 and 7) and the equal activity of LacCer (no. 4) and its GalNAcβ-substituted counterparts (nos. 9 and 13). In this system, the shift from a non-adhesive to an adhesive surface occurred between 200 and 500 ng/well (with a half-maximal binding at 200 ng/well).

As shown in Fig. 1, A. naeslundii also bound to LacCer (no. 4) and compound no. 7 but not to LacCer of human erythrocytes (no. 5). Additional reactive bands (e.g. nos. 12 and 17), which did not bind A. viscosus, were attributable to the second and superimposed recognition of GalNAcβ (see below). The results nevertheless indicated that A. naeslundii had a lactose-based specificity very similar, if not identical, to that of A. viscosus.

Apparent Additional Specificity of A. naeslundii for GalNAcβ
and 37) or blood group AB0 determinants (e.g., nos. 19, 33, and 34) blocked the binding. The Forsmann hapten (GalNAcβ1-GalNAcβ1) was also inactive (no. 16). On the other hand, sialic acid residues linked to the internal Gal of substance no. 13 did not introduce steric hindrances, as judged by the strong activity of certain brain gangliosides (e.g., nos. 25 and 31). Finally, when tested against a mixture of acidic glycolipids prepared from human erythrocytes, A. naeslundii bound several bands within the 5–12 sugar interval (data not shown).

Sugar Inhibition Studies—By preincubation of the two actinomycetes with free soluble sugars, we investigated the ability of saccharides corresponding to the identified lactose and GalNAcβ recognition sites to inhibit the glycolipid binding of Fig. 1 (Table II).

As earlier observed for other lactose-binding bacteria (23–26), both lactose (up to 10 mg/ml) and multivalent lactose-BSA (5 mg/ml, ~25 mol/mol) failed to interfere with bacterial binding to LacCer. On the other hand, GalNAcβ1 3Galα0 ethyl at the concentration of 1 mg/ml, as well as free lactose, 2-

TABLE I

Results from binding of A. naeslundii (ATCC 12104) and A. viscous (ATCC 19246) to pure and structurally defined glycolipids on a thin-layer chromatogram.

<table>
<thead>
<tr>
<th>No.</th>
<th>Structure*#</th>
<th>A. viscous</th>
<th>A. naeslundii</th>
<th>Symbol</th>
<th>Source (Ref.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Galβ1-Cer(b)</td>
<td>–</td>
<td>–</td>
<td>Most tissues (52)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Glcβ1-Cer(b)</td>
<td>–</td>
<td>–</td>
<td>Most tissues (52)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Galα1-4Galβ1-Cer(b)</td>
<td>–</td>
<td>–</td>
<td>Human meconium (53)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Galβ1-3Galα1-4Glcβ1-Cer(b)</td>
<td>–</td>
<td>+</td>
<td>LacCer</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Galβ1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Human erythrococytes (52)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>NeuAc-2-3Galα1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Human erythrococytes (52)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Galβ1-3Galα1-4Glcβ1-Cer(b)</td>
<td>(+)</td>
<td>(+)</td>
<td>Glb3b</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Galα1-4Galβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Glbα</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>GalNAcβ1-3Galβ1-Cer</td>
<td>+</td>
<td>++</td>
<td>Gb03</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>GalNAcβ1-3Galβ1-4Glcβ1-Cer</td>
<td>(+)</td>
<td>(+)</td>
<td>Malig. melanoma</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>GalNAcβ1-3Galα1-4Glcβ1-Cer</td>
<td>–</td>
<td>++</td>
<td>Rat colon carcinoma (55)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>GalNAcβ1-3Galα1-Cer</td>
<td>–</td>
<td>++</td>
<td>Gbα</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Galβ1-3Glcβ1-Cer(b)</td>
<td>(+)</td>
<td>(+)</td>
<td>Human meconium (53)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Galβ1-4GalNAcβ1-3Galα1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Human meconium (53)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Galβ1-4GalNAcβ1-3Galα1-4Glcβ1-Cer</td>
<td>(+)</td>
<td>(+)</td>
<td>Glb3b</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>GalNAcβ1-3GalNAcβ1-3Galα1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Human meconium (53)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>GalNAcβ1-3GalNAcβ1-3Galα1-4Glcβ1-Cer</td>
<td>–</td>
<td>++</td>
<td>Human erythrococytes (60)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Galβ1-3GalNAcβ1-Cer(b)</td>
<td>–</td>
<td>++</td>
<td>High cells (61)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Fuco-2-Galβ1-3GalNAcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Mouse liver (58)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Fuco-1-4Galβ1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Dog small intestine (59)</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Galα1-4(Fuco-1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Dog small intestine (59)</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>NeuAc-2-3Galα1-4Glcβ1-3Galα1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Human erythrococytes (52)</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>GalNAcβ1-3Galβ1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Dog small intestine (59)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>GalNAcβ1-3Galβ1-3Galα1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Human meconium (53)</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Galβ1-3GalNAcβ1-4(Fuco-3-Galα1)</td>
<td>–</td>
<td>+</td>
<td>Human brain (56)</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Galβ1-3(Fuco-2-6)GalNAcβ1-4Glcβ1-Cer</td>
<td>–</td>
<td>++</td>
<td>Frog brain (28)</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>(Galα1-3)Galβ1-Cer</td>
<td>–</td>
<td>++</td>
<td>Rat small intestine (57)</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>GalNAcβ1-3Galα1-3Galα1-4Glcβ1-Cer</td>
<td>–</td>
<td>++</td>
<td>Rat small intestine (57)</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>GalNAcβ1-3(Fuco-2-6)Galβ1-Cer</td>
<td>–</td>
<td>++</td>
<td>Rat small intestine (57)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>GalNAcβ1-3(Fuco-1-4Galβ1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Dog small intestine (59)</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>Galβ1-3GalNAcβ1-4(Fuco-2-8)Galα1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Human meconium (53)</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>NeuAc-2-3Galβ1-3Galα1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Human brain (56)</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>NeuAc-2-3Galβ1-3(Fuco-2-6)Galβ1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Frog brain (28)</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>NeuAc-2-3Galβ1-3(Fuco-2-6)Galβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Dog small intestine (59)</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>GalNAcβ1-3(Fuco-2-6)Galβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Human meconium (53)</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>GalNAcβ1-3(Fuco-2-6)Galβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Human kidney (63)</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>(Gal or GalNAcβ1-3(Fuco-2-6)Galβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Various sources (61, 74)</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>(Gal or GalNAcβ1-3(Fuco-2-6)Galβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Various sources (61, 74)</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>NeuAc-2-3Galα1-3Galα1-4Glcβ1-Cer</td>
<td>–</td>
<td>–</td>
<td>Human brain (56)</td>
<td></td>
</tr>
</tbody>
</table>

* The receptor-active GalNAcβ and Galβ1–4Glc sequences have been underlined with a solid and dotted bar, respectively.

* Cer indicated ceramide containing nonhydroxy fatty acid and dihydroxy long-chain base; Cerh indicated the presence of hydroxy fatty acids and/or trihydroxy base; Cer indicates ceramide containing nonhydroxy fatty acid and dihydroxy long-chain base; Cerh indicates that both types of ceramide were present in the fraction tested.

* A main source and a reference is given for each compound. In general, the same compound of several sources was tested.

* Prepared by acidic hydrolysis of the predominant human brain ganglioside (no. 25) as described in Ref. 25.

* Prepared by neuraminidase treatment of substance no. 31 as described under “Materials and Methods.”

* Defined by guest on September 13, 2017 http://www.jbc.org/ Downloaded from...
completely inhibited the binding of A. naeslundii to the GalNAcβ-based glycolipids of Fig. 1. Lactose was, however, at least 10 times less potent, requiring 10 mg/ml.

As expected from the finding that A. naeslundii, but not A. viscosus, attached to thin layer chromatograms of human erythrocyte glycolipids (Fig. 1, lane A and above), only A. naeslundii agglutinated these cells (Table II). GalNAcβ1-3Galα-0-ethyl inhibited this hemagglutination down to 50 μg/ml; free lactose was 10-fold less potent, while both Gal and GalNAc were even less efficient (two to three times), and lactose-BSA was negative when 2 mg/ml was used.

**DISCUSSION**

This study identifies and characterizes a low affinity lactose and a high affinity GalNAcβ binding property of Actinomyces, both of which may contribute to the intra-oral distribution and establishment of this genus. This was accomplished by binding of radiolabeled A. naeslundii (strain ATCC 12104) and A. viscosus (strain ATCC 19246) to a panel of naturally occurring glycolipids resolved on thin layer chromatograms (Fig. 1) or coated in microtiter wells (Fig. 3). Our study also provides evidence that glycolipids with the GalNAcβ sequence serve as human erythrocyte and epithelial cell surface receptors for *Actinomyces*. Interestingly, lactose of LacCer is also recognized by several other commensal and pathogenic bacteria in man (22-26). Such a broadly distributed binding to a common cell-surface carbohydrate structure has earlier been demonstrated only for the many bacteria classified as mannose binders (43).

Among the bacteria hitherto classified as lactose binders, we have noted both variations in detailed specificities (24, 26) and a typical mode of binding (22, 23) recently characterized in detail for P. granulosum (26). The binding characteristics for A. viscosus, however, appeared very similar to those for P. granulosum and are therefore only briefly discussed here. A. viscosus bound to both terminally and internally located lactose (underlined in Table I), although most of the lactose-containing glycolipids were negative, as explained by steric hindrances from proximal sugars. There was a clear dependence on the ceramide structure with regard to the binding to free LacCer as only those species with 2-hydroxy fatty acid and/or a trihydroxy base (the epithelial type of ceramide) was active (compare nos. 4 and 5 in Fig. 1 and Table I). This has previously been explained by influences from the ceramide structure on the conformation of the lactose head group (24, 26). The relatively large amount of LacCer needed for binding (half-maximal binding at ~200 ng/well in Fig. 3), together with the inability of free lactose to work inhibitorily (Table II), is explained by a low affinity at individual binding sites (23-26). A similar situation was recently found for the Shiga toxin and its recognition of Galβ1-4Gal (45).

*A. naeslundii* exhibited two superimposed binding properties of which one was very similar, if not identical, to that of *A. viscosus*. This was initially indicated by the binding of both species to LacCer and Gb3β (nos. 4, 5, and 7 in Fig. 1 and Table I). In support of this, preincubation of *A. naeslundii*...
with GalNAcβ1-3Galα1-0-ethyl prevented the binding specified by GalNAcβ but not the binding to LacCer (Table II). Also, one unique batch of A. naeslundii failed to bind LacCer (nos. 4 and 7), although its binding to the GalNAcβ-containing glycolipids was unaffected (Fig. 2). One explanation for this is that the lactose-binding component of the bacteria was selectively turned off.

A. naeslundii also recognized GalNAcβ when substituted with Galβ1-3 or NeuAcα2-6 (Table I). However, extensions of the Galβ1-3GalNAc terminus with either blood group ABO-determinants or sialic acid apparently introduced steric hindrances, as judged by their blocking effect on binding. Recognition by microbial ligands of internally located sequences has been demonstrated earlier for uropathogenic E. coli (20), the Shiga toxin (45), and other lactose-binding bacteria (24–26). The very similar activity of some glycolipids with different neighboring groups to GalNAcβ substantiates our conclusion of a small adhesin-combining site that does not extend beyond GalNAcβ (compare no. 9 with 13, no. 9 with 12). The β-anomerity, the acetamido group, and the axial hydroxyl at C-4 were essential, and the hydroxyls at C-3 and C-6 may carry substitutions. The affinity of binding was relatively high, as indicated by the detection level at 1–10 ng of glycolipid, amounts that are 10–100 times lower than those needed in the case of LacCer (Fig. 3) and that free sugars inhibited the binding to thin layer chromatograms (Table II) as well as hemagglutination (Table II and below).

In view of the sugar inhibition with free lactose (Table II), the proposed binding to GalNAcβ in glycolipids is attributable to the lactose-sensitive lectin activity reported earlier for Actinomyces (see Introduction). Although GalNAcβ1-3Galα1-0-ethyl was about 10 times more potent than lactose, both saccharides selectively inhibited the binding of A. naeslundii to GalNAcβ-containing glycolipids on thin layer plates. Consistent with this is that A. naeslundii, unlike A. viscosus, efficiently agglutinated human erythrocytes (Table II) and that the inhibitory effect of GalNAcβ1-3Galα1-0-ethyl was again 10 times more potent than that of lactose, which in turn was two to three times better than Gal and GalNAc. These data, together with the noted specificity for glycolipids (Table I), suggest that the lactose sensitivity of Actinomyces is specified by GalNAcβ in internal or terminal positions rather than by terminal Galβ residues as earlier claimed (see Introduction). McIntire et al. (15) recently proposed a lectin of A. naeslundii (ATCC 12104) with specificity for Galβ1-3GalNAc, on the basis of the 50-fold lower efficiency of Galβ1-4GalNAc in inhibiting the aggregation of this strain with Streptococcus sanguis S34. Alternatively, the Galβ1-4 substituent could induce a steric hindrance, since our data suggest a critical role of the axial hydroxyl at C-4 of GalNAcβ. Also, the extension of terminal GalNAcβ with Galβ1-3 did not, in our case, improve the binding (compare nos. 9 and 13 in Fig. 3). On the other hand, perhaps the ATCC 12104 strain used by these workers differs in detailed sugar specificity, thereby also explaining why Brennan et al. (17) were unable to show binding of strain ATCC 12104 to GalGO3 (no. 9). Whether the GalNAcβ sequence is the interaction site on S. sanguis S34 remains to be determined, although it should be noted that the site has been traced to a hexasaccharide with 2 GalNAc residues (46). In addition, the ability of this lectin to accommodate free lactose and Gal is of interest in principle, since A. naeslundii apparently failed to associate with certain glycolipids that have these sugars in the terminal position (nos. 1, 5, and 21), as well as with lactose-BSA (Table II). The presentation and activity of terminal sugars in glycolipids and glycoproteins may, therefore, be largely affected by their internal structures. This observation, together with the earlier hypothesis of a lectin with specificity for terminal Galβ residues (15 and references therein), emphasizes that sugar inhibition studies may be misleading if used to predict membrane-bound receptor sequences.

As expected from the hemagglutination pattern seen in Table II, A. naeslundii, in contrast to A. viscosus, bound both acid and non-acid human erythrocyte glycolipids (lane A in Fig. 1, and under "Results"). The two receptor-active non-acid glycolipids are known to contain the sequence GalNAcβ (nos. 12 and 17 in Table I) and one is the predominant erythrocyte glycolipid Gb4a (no. 12), known to be accessible at the surface of these cells (47). Based on these data and the sugar inhibition experiments (Table II), we propose that glycolipids with the sequence GalNAcβ serve as the human erythrocyte receptors for Actinomyces. At present, we cannot exclude that protein-bound GalNAcβ sequences may also be receptor sites (48). However, it should be noted that GalNAcβ in glycoproteins has so far been found only as part of the Sid blood group antigen (49). Even though the hemagglutination induced by A. naeslundii was a strong and immediate event (Table II), most other strains of A. viscosus and A. naeslundii require prior priming of human erythrocytes with neuraminidase for lactose-sensitive hemagglutination (11, 12). This may mean that there is another lectin which recognizes a slightly different epitope masked by sialic acids in the native erythrocytes. Alternatively, if the amounts of lectins on these strains are lower than for A. naeslundii (ATCC 12104), the neuraminidase treatment may expose additional GalNAcβ sites necessary for a clear hemagglutination.

A scraping of human buccal epithelium, a target tissue for adhesion of A. viscosus and A. naeslundii (5), was preliminary analyzed for the presence of receptor-active glycolipids (Fig. 4). A. viscosus (ATCC 19246) bound one of the major glycolipids in this material. In view of the strict specificity of this strain for lactose (Table I), this glycolipid probably contains an active lactose site. Since it is also a lactose binder (Table I), A. naeslundii (ATCC 12104) stained the same glycolipid but apparently much more strongly. This glycolipid therefore...
probably also contains an active GalNAcP site, and, on this basis and in view of its mobility, may possibly be Gg04 (no. 13). These results possibly imply that, in the oral epithelium, there are two populations of receptor-sites for Actinomycetes, a low affinity lactose site and a high affinity GalNAcP site. Adhesion of A. naeslundii ATCC 12104 to buccal cells is, at least partially, inhibitable with lactose (13) and appears to be pilus-independent. LacCer binding was recently identified for oral epithelium glycolipid may indicate functional low affinity site and a high affinity GalNAcP site. The nonfimbrial appearance of the lactose-binding component. A GalNAcP specificity are also important with regard to the principal interest in view of the fact that this bacterial genus predominance of Actinomyces in the dental plaque is of particular interest in view of the fact that this bacterial genus may be a key intermediate for the colonization of “periodontopathogenic" Bacteroides (51). On this basis, one may consider the possible use of synthetic receptor analogues to prevent such a series of events.

REFERENCES

Characterization of the binding of Actinomyces naeslundii (ATCC 12104) and Actinomyces viscosus (ATCC 19246) to glycosphingolipids, using a solid-phase overlay approach.
N Strömberg and K A Karlsson