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Specific Binding of *Haemophilus influenzae* to Minor Gangliosides of Human Respiratory Epithelial Cells

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Gangliosides are sialylated glycosphingolipids that serve as receptors for various bacteria. To investigate endogenous gangliosides of human respiratory epithelial cells as potential receptors for *Haemophilus influenzae*, three strains, including nontypeable *H. influenzae* (NTHI) 1479, and isogenic fimbriated (f^+) and nonfimbriated (f^0) *H. influenzae* type b 770235, were ^3H labeled and overlaid on two-dimensional thin-layer chromatography (TLC) plates containing either purified HEp-2 gangliosides or murine brain gangliosides. NTHI 1479 bound exclusively to two distinct minor ganglioside doublets, with mobilities near that of G_{M1}. These minor gangliosides comprised only 14.2 and 9.4% of the total, respectively. NTHI 1479 also bound to a distinct ganglioside of human macrophages whose chromatographic mobilities closely resemble those of one of the NTHI-binding gangliosides of HEp-2 cells. *H. influenzae* type b 770235 f^+ and f^0 each bound to a different minor HEp-2 ganglioside doublet, with proportionately weaker affinity for a major ganglioside doublet. Remarkably, none of the three strains bound to any murine brain gangliosides. Moreover, when 80 to 90% of sialic acid residues were enzymatically removed from HEp-2 gangliosides, NTHI 1479 binding was proportionately impaired, compared with untreated controls. Our findings support a role for specific gangliosides of specific cells as receptors for *H. influenzae* strains. Our findings further demonstrate that individual minor gangliosides possess unique biological properties.

Haemophilus influenzae is an important and common bacterial organism causing pneumonia and bronchitis, especially in the elderly and patients suffering from chronic lung diseases (18, 19). Adherence is a prerequisite to respiratory cell invasion. While fimbriae provide one means of adherence to respiratory epithelial cells, nonfimbriated organisms, including nonfimbriated nontypeable *H. influenzae* (NTHI), also adhere to respiratory epithelial cells, by alternative means (14, 15, 24, 26). Both NTHI and type b *H. influenzae* adhere to a variety of target cells, including erythrocytes, buccal epithelial cells, and HEp-2 cells (9, 26). HEp-2 cells are particularly appealing as target cells for the study of respiratory pathogens, since they are derived from human respiratory epithelial cells.

Gangliosides are sialylated glycosphingolipids found in all eukaryotic cell membranes (41). These molecules play an important role in immunomodulation of cellular growth and differentiation (7). Specific gangliosides are receptors for bacteria and bacterial products (6, 41). For example, gangliosides serve as functional receptors for the heat-labile enterotoxin of *Escherichia coli* and for the β subunit of cholera toxin. Gangliosides also serve as receptors for bacterial organisms such as enterotoxigenic *E. coli* (22) and *Pseudomonas aeruginosa* (2). In several biological systems, cell surface gangliosides are postulated to adhere to specific binding molecules on the surfaces of other cells, via their oligosaccharide portions (29).

Evidence also supports a role for gangliosides as receptors for *H. influenzae*. In one study, van Alphen et al. showed that the gangliosides G_{M1}, G_{M2}, G_{M3}, and G_{D1a} specifically inhibited hemagglutination secondary to fimbriated *H. influenzae*, while asialo-G_{M1} had no effect (35). These results contrast with earlier findings in which *H. influenzae* bound to desialylated lipids, including fucosylasialo-G_{M1}, asialo-G_{M1}, and asialo-G_{M2}, but not to sialylated glycosphingolipids (13). Thus, the relative importance of sialic acid in this interaction was called into question. None of these earlier studies evaluated ganglioside interactions with NTHI. It is noteworthy that both earlier studies used commercially available gangliosides, purified from bovine brain and other exogenous sources, rather than gangliosides of receptor cells. Earlier studies in our laboratory determined that endogenous macrophage gangliosides were immunologically far more potent inhibitors of lectin-induced T-lymphocyte and B-lymphocyte mitogenesis than were equal concentrations of exogenous brain gangliosides (5).

Since this evidence suggests that gangliosides of different tissues have potentially different functional properties, we investigated the abilities of NTHI, and of fimbriated and nonfimbriated *H. influenzae* type b, to bind to specific gangliosides of HEp-2 cells, compared with gangliosides of murine brain origin. We further performed experiments to determine the importance of sialic acid in binding.

MATERIALS AND METHODS

Materials. Minimum essential medium, fetal bovine serum, and trypsin-EDTA were purchased from Gibco (Grand Island, N.Y.); brain heart infusion was purchased from Becton Dickinson (Cockeysville, Md.); and bovine hemin and NAD were purchased from Sigma Chemical Co. (St. Louis, Mo.). L-[4,5-^3H] leucine was purchased from Amersham Life Science (Arlington Heights, Ill.). *Arthrobacter ureafaciens* neuraminidase was purchased from EY Laboratories (San Mateo, Calif.).

Solvents were of standard analytical high-performance liquid chromatography quality (Baker Chemical Co., Phillipsburg, N.J.). High-performance thin-layer chromatographic (TLC) plates (10 by 10 cm) were purchased from E. Merck (Darmstadt, Germany). Murine brain gangliosides were prepared from C3H/HeN mice, as previously described (3).

Bacterial strains. The bacterial strains used included NTHI 1479, which has been characterized in related studies in our laboratories (17). Isogenic strains of *H. influenzae* type b, 770235f^+ (fimbriated) and its nonfimbriated variant, 770235f^0, were generously provided by Loek van Alphen, University of Amsterdam, Amsterdam, The Netherlands. Adult human erythrocytes (AnWj antigen

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positive) were used to determine the presence or absence of fimbriae by hemagglutination, as described previously (34).

Cell culture. Human laryngeal cancer (HEp-2) cells were purchased from the American Type Culture Collection (Rockville, Md.). Peripheral human blood monocyte-derived macrophages were purified as previously detailed (3). In brief, buffy coat suspensions were obtained from human immunodeficiency virus-seronegative donors at the Red Cross of Western New York. Mononuclear cells were purified by density centrifugation. Macrophages were purified by adherence to glass petri dishes over 10 to 12 days. Purity by nonspecific esterase staining was 97 to 99%.

Purification of gangliosides. HEp-2 cells were plated at 5×10^4 cells/ml in glass petri dishes and grown to confluence in minimum essential medium supplemented with 10% fetal bovine serum. The culture medium was then discarded, and nonadherent cells were removed with two washes of 10 ml of phosphate-buffered saline (PBS) (Whittaker M. A. Bioproducts, Walkersville, Md.) and then were washed once with 5 ml of 0.31 M pentaerythritol (Sigma Chemical Co.). Total lipid extract was obtained by flooding petri dishes with chloroform-methanol (1:1 [vol/vol]) and sonicating the cells. Gangliosides were then purified from the total lipid extract as previously described (3). In brief, ganglioside-containing acidic lipids were eluted through a 3-ml column of DEAE–Sephadex A-25 (Sigma Chemical Co.), dried by rotary evaporation, and hydrolyzed with 0.1 N NaOH at 37°C for 90 min. Samples were neutralized with 0.1 N HCl and desalted on a reverse-phase silica gel column (SepPak; Waters Associates, Waltham, Mass.). Samples were applied to a 2- to 3-ml column of Iatrobeads 6RS-8060 (Iatron Laboratories, Tokyo, Japan) in chloroform-methanol (85:15 [vol/vol]). After elution of less polar lipids, the total ganglioside fraction was eluted with chloroform-methanol (1:2 [vol/vol]) and dried by rotary evaporation. Total lipids were extracted, and gangliosides were purified from human macrophages in culture by the same methods. Purified gangliosides were run in two dimensions on TLC plates in chloroform–methanol–0.25% KCl (50:45:10) (solvent 1) and, after the TLC plate was rotated 90° counterclockwise, in chloroform–methanol–0.25% KCl in 2.5 N NH_4 (50:40:10) (solvent 2). Distinct ganglioside peaks were visualized by heating the TLC plates to 92 to 94°C after spraying with resorcinol reagent (32).

Adherence of ^{3}H-labeled NTHI to gangliosides. TLC plates of both HEp-2 and murine brain gangliosides were immersed in 0.4% polyisobutylmethacrylate (Polysciences, Warrington, Pa.) in chloroform-hexane (1:5 [vol/vol]) for 1 min (27). Plasticized TLC plates were preincubated with 0.1% gelatin (Bio-Rad, Richmond, Calif.) for 30 min and then were washed with PBS. Whole bacteria were inoculated in brain heart infusion supplemented with bovine hemin and NAD, both at concentrations of 10 mg/μl. One millicurie of [^{3}H]leucine (specific activity, 167 mCi/mmol) was added to bacteria to achieve a final concentration of 250 μCi/ml. Whole bacteria were grown to late logarithmic phase. Optical density at 600 nm was plotted against time at 1-h intervals, to confirm the phase of growth (0.5 to 0.8 Å). The suspension was centrifuged at $5{,}000 \times g$ for 10 min. Supernatant was discarded, and bacteria were resuspended in PBS and washed four additional times. Labeled bacteria were resuspended in 4 ml of PBS. Incorporation of [^{3}H]leucine into whole bacterial proteins was documented by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the preparation.

In every experiment, the suspension of ^{3}H-labeled bacteria was divided into two equal volumes of 2 to 2.5 ml each. One volume was placed on a TLC plate of HEp-2 cell gangliosides. The other volume was placed on a TLC plate of murine brain gangliosides. Each TLC plate contained approximately equal quantities (3 to 6 μg of lipid-bound sialic acid) of gangliosides. Both were simultaneously incubated for 60 min at room temperature (8, 16). Both plates were then vigorously washed with 0.1% Triton X-100 in PBS. TLC plates were then sprayed with En^{3}Hance spray (New England Nuclear, Boston, Mass.) and covered with plastic wrap. TLC plates were then placed on XAR-5 film (Eastman Kodak, Rochester, N.Y.) and left at −70°C for 2 to 14 days. X-ray film was hypersensitized in order to optimize visualization of even minor images, as previously described (37). After the autoradiographs were developed, the original TLC plate was treated with chloroform-hexane (1:5 [vol/vol]) to remove residual polyisobutylmethacrylate. Gangliosides were visualized by spraying with resorcinol reagent and heating, as described earlier. Each autoradiograph was superimposed on the TLC plate from which it was derived, and each area of radiographic intensity was precisely matched with the corresponding ganglioside peak of the TLC plate.

Neuraminidase treatment. To determine optimal conditions for desialylation with *A. ureafaciens* neuraminidase, HEp-2 gangliosides containing 10 to 20 μg of sialic acid were divided into two equal volumes and coincubated with *A. ureafaciens* neuraminidase at varied concentrations (20 to 400 mU/ml), or with 0.1 M sodium acetate buffer, for 2 to 18 h at 37°C. Final preparations were loaded onto TLC plates, sprayed with resorcinol reagent, and scanned by densitometer. A comparison of the intensities of resorcinol-positive contents was made and utilized to calculate the percent desialylation as (1 − volume of resorcinol-positive peaks of enzyme-treated sample/volume of resorcinol-positive peaks of buffer-treated sample) × 100. Volume of peaks was quantitatively determined by densitometry, based upon area and light absorbance. Under the conditions chosen (below), the percent desialyation was calculated at 87 to 93%.

Once optimal conditions were established, HEp-2 cell gangliosides containing approximately 10 μg of lipid-bound sialic acid were divided into two equal volumes and placed in two individual tubes. One sample was incubated with *A. ureafaciens* neuraminidase (200 mU/ml) in 0.1 M sodium acetate buffer (at pH 4.8 and 37°C) for 18 h (27). The other sample was simultaneously treated under identical conditions, with buffer substituted for neuraminidase. The reaction was stopped by using 2 ml of 0.1 N NaOH, and both tubes were placed in ice. The pH was titrated to 4 to 5 by using 0.1 N HCl. Both samples were individually loaded onto a 2-ml SepPak column and desalted, as described earlier. Purified glycolipids were then spotted onto separate TLC plates. Gangliosides were visualized by spraying both plates with resorcinol and heating to 92 to 94°C as described earlier. Relative intensities of residual resorcinol-positive spots were determined by densitometric scanning as described below.

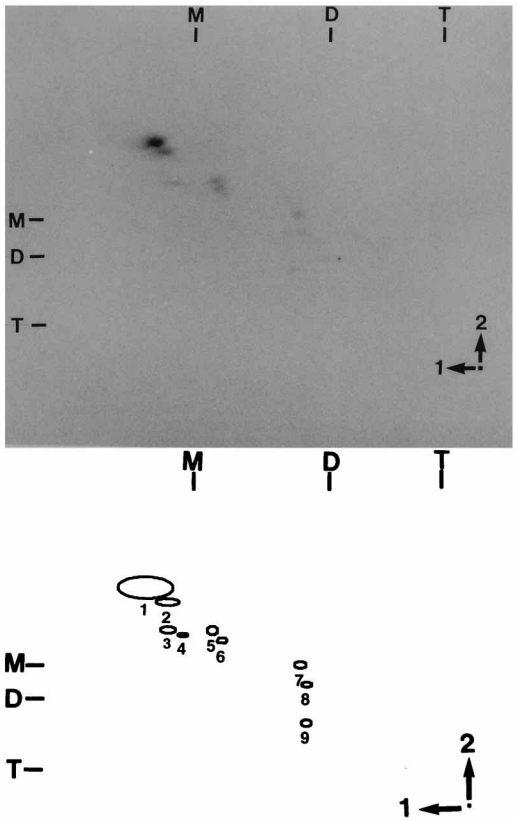

FIG. 1. Thin-layer chromatogram of HEp-2 cell gangliosides. Gangliosides were extracted from HEp-2 cells and analyzed on a two-dimensional thin-layer chromatogram (upper panel). A schematic diagram of the TLC plate (lower panel) indicates individual peaks designated by numbers that correspond to those given in Table 1. Nine individual ganglioside peaks could be reproducibly visualized. Arrows and numbers in the lower right corner indicate the directions of the first and second solvent runs. The origin is denoted by a dot in the lower right corner. Chromatographic standards: M, G_{M1} (II^{3}NeuAc-GgOse$_4$Cer); D, G_{D1a} (IV^{3}NeuAc,II^{3}NeuAc-GgOse$_4$Cer); T, G_{T1b} [IV^{3}NeuAc-II^3(NeuAc)$_2$-GgOse$_4$Cer]. Ganglioside nomenclature is according to Svennerholm (33).

Densitometric analysis. Quantitative analysis of autoradiographs and TLC plates was performed with a two-dimensional analytical scanning system (Molecular Dynamics, Sunnyvale, Calif.) as previously described (4). Results are expressed as relative percentages of the total to allow for possible small differences in background and quantity of material between individual TLC plates.

Sialic acid quantitation. Ganglioside-bound sialic acid was quantitated by densitometric scanning and compared to known samples of 0.2 to 3.2 μg of *n*-acetylneuraminic acid (Sigma Chemical Co.) (12). Results were further confirmed by a spectrophotometric assay conducted according to the resorcinol method of Svennerholm (32).

RESULTS

Characterization of HEp-2 cell gangliosides. Purified HEp-2 gangliosides were analyzed by two-dimensional TLC (Fig. 1). Nine distinct resorcinol-positive peaks were reproducibly identified and were quantitated by densitometric scanning (Table 1). Faint peaks that could not be reproducibly identified were

TABLE 1. Relative percentages of HEp-2 cell gangliosides[a]

Peak no.	Relative %
1	51.9
2	11.8
3	7.5
4	6.7
5	2.6
6	6.9
7	3.9
8	4.6
9	4.1

[a] Relative percentages of ganglioside peaks of the thin-layer chromatogram shown in Fig. 1 were determined by densitometric scanning. Each value is expressed as a percentage of the total ganglioside content of the thin-layer chromatogram to compensate for possible small differences in total quantity of gangliosides applied to individual TLC plates. Numbers assigned to peaks (left column) correspond to those of the schematic diagram in Fig. 1. Ganglioside peaks that could not be reliably reproduced on TLC plates were not included.

not included in our measurements. The major doublet, peaks 1 and 2, comigrated with G_{M3} (II^3NeuAc-LacCer) and comprised 63.7% of the total (Table 1). Peaks 3 and 4 migrated slightly ahead of G_{M1} (II^3NeuAc-GgOse$_4$Cer) standards; peaks 5 and 6 ran closely behind G_{M1} in the first solvent and near G_{M1} in the second solvent. Peaks 7, 8, and 9 ran close to the region of G_{D1a} (IV^3NeuAc,II^3NeuAc-GgOse$_4$Cer) standards. HEp-2 gangliosides were quantitated by densitometric scanning and contained 15.9 ± 3.3 ng of lipid-bound sialic acid/10^6 cells. Concordant results were obtained by resorcinol assay. Accrual of sufficient material to have 3 to 6 μg of ganglioside-bound sialic acid for each TLC plate required 10 to 20 petri dishes of confluent HEp-2 cells per TLC plate.

NTHI 1479 binding to HEp-2 gangliosides. To determine the binding specificity of NTHI to HEp-2 and murine brain gangliosides, ^{3}H-labeled NTHI was overlaid on TLC plates to develop autoradiographic images. In each experiment, the same sample of ^{3}H-NTHI was divided into two equal volumes. One volume was overlaid on a TLC plate of HEp-2 gangliosides; the other was simultaneously overlaid on a TLC plate of murine brain gangliosides. In each experiment, both TLC plates were simultaneously placed on a single sheet of X-ray film. TLC plates of HEp-2 and murine brain gangliosides were then sprayed with resorcinol and superimposed on autoradiographic images. ^{3}H-labeled NTHI 1479 bound with greatest affinity to peaks 3 and 4 and with weaker affinity to peaks 5 and 6 (Fig. 2). HEp-2 ganglioside peaks 3 and 4 together comprise only 14.2% of the total HEp-2 gangliosides, thus accounting for approximately 2.3 ng of sialic acid/10^6 cells. While individual peaks of doublets were easily distinguished on high-resolution TLC plates, borders of corresponding peaks overlapped and were somewhat less distinct on autoradiographs. NTHI 1479 did not bind to the more abundant gangliosides of peaks 1 and 2, which comprised 63.7% of the total. ^{3}H-labeled NTHI 1479 did not bind to any murine brain gangliosides. Results were reproduced in four separate experiments.

Binding of *H. influenzae* type b 770235 to HEp-2 gangliosides. To determine the specificity of binding of *H. influenzae* type b, isogenic f^+ and f^0 strains of *H. influenzae* type b were labeled with [^{3}H]leucine. As in previous experiments, the same sample of ^{3}H-labeled *H. influenzae* was equally divided and overlaid on TLC plates of HEp-2 and murine brain gangliosides (Fig. 3). The f^0 strain bound to peaks 5 and 6, with less intense binding to peaks 1 and 2. The f^+ strain bound expressly to peaks 5 and 6 (data not shown). The comparative intensity of binding to peaks 5 and 6 is out of proportion to their relative abundance on the TLC plate compared with that of peaks 1 and 2. Peaks 5 and 6 comprise 9.5% of the total, thus accounting for approximately 1.5 ng of sialic acid/10^6 cells. Neither strain bound at all to peaks 3 and 4, the major binding peaks for NTHI. Moreover, as with NTHI 1479, neither strain of *H. influenzae* type b bound to murine brain gangliosides (Fig. 3). Each experiment was reproduced in triplicate.

Binding of NTHI 1479 to neuraminidase-treated HEp-2 gangliosides. In order to determine whether sialic acid was essential for binding to occur, experiments were performed with HEp-2 gangliosides that were treated with *A. ureafaciens* neuraminidase. We first determined the efficacy of *A. ureafaciens* neuraminidase for desialylating HEp-2 gangliosides. Independent experiments were performed in which approximately 10 to 20 μg of HEp-2 gangliosides was divided into two equal volumes which were simultaneously incubated with either neuraminidase or buffer, as detailed earlier. Both products were spotted onto separate TLC plates and were then developed with resorcinol spray, as a specific means of detecting sialic acid residues. TLC plates were scanned and compared densitometrically. Compared with buffer-treated controls, neuraminidase-treated samples consistently had residual resorcinol-positive peaks that were 10 to 20% of the volume of controls. Thus, we determined that *A. ureafaciens* neuraminidase cleaved 87 to 93% of sialic acid moieties from HEp-2 gangliosides. The conditions established to achieve this result (see Materials and Methods) were used for later experiments.

Prior to incubation with ^{3}H-labeled NTHI, the presence of desialylated glycolipids on TLC plates was confirmed by visualization, by exposure to iodine vapor (23) or spraying with orcinol reagent (30). Under optimal conditions (described above), ^{3}H-labeled NTHI was overlaid on TLC plates containing neuraminidase-treated or buffer-treated HEp-2 ganglio-

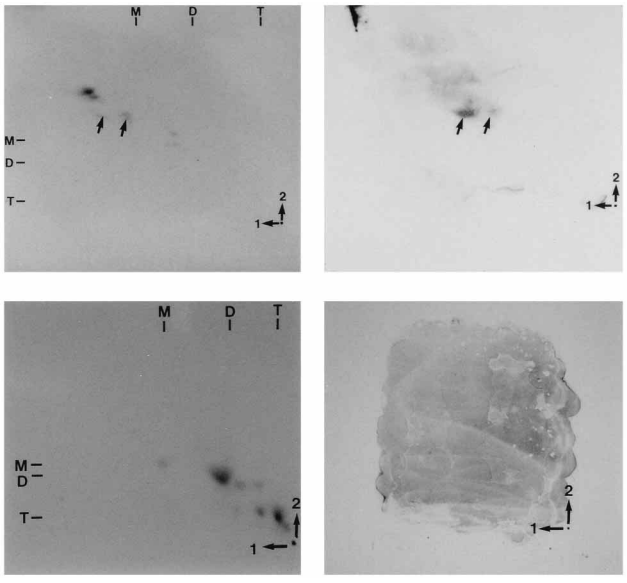

FIG. 2. Autoradiograph of ^{3}H-labeled NTHI 1479 binding to HEp-2 cell gangliosides. Autoradiographs (right panels) were made from TLC plates (left panels) of HEp-2 cell gangliosides (top panels) and murine brain gangliosides (bottom panels) treated with ^{3}H-labeled NTHI 1479. In each experiment, the same sample of [^{3}H]leucine-labeled NTHI 1479 was divided into two equal volumes and each half was simultaneously overlaid on TLC plates containing either HEp-2 or murine brain gangliosides. Autoradiographic images corresponded precisely with peaks 3 and 4 and peaks 5 and 6 of the TLC plate, indicated by arrows. Positions of ganglioside standards and directions of solvent runs are as specified in the legend to Fig. 1.

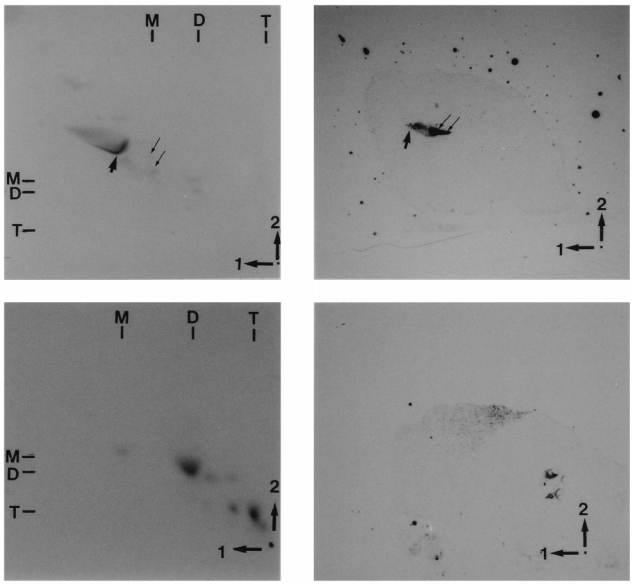

FIG. 3. Autoradiographs of binding of ^{3}H-labeled *H. influenzae* type b 770235 to HEp-2 cell gangliosides. Autoradiographs (right panels) were made from TLC plates (left panels) of HEp-2 gangliosides (upper panels) and murine brain gangliosides (lower panels) treated with ^{3}H-labeled isogenic *H. influenzae* type b f^+ and f^0 mutants. Results shown are from one experiment with the f^0 strain. Peaks 5 and 6 are indicated by small arrows; peaks 1 and 2 are indicated by large arrows. In some experiments, polymethacrylate-coated TLC plates, developed with resorcinol reagent, developed a background artifact. Although the plates were interpretable for analysis, this made accurate photographic reproduction difficult. Therefore, a duplicate TLC plate of gangliosides that was not polymethacrylate coated was created. Since minor differences in chromatographic migration may occur on different TLC plates, spots of the demonstration TLC plate were carefully matched with those of the original TLC plate, relative to identical ganglioside standards of each dimension on each TLC plate. Positions of ganglioside standards and directions of solvent runs are as specified in the legend to Fig. 1.

sides. The binding of NTHI 1479 to neuraminidase-treated HEp-2 gangliosides was only 15 to 21% of the binding of the same quantity of ^{3}H-labeled NTHI to buffer-treated control (Fig. 4). In each of these experiments, binding of ^{3}H-labeled NTHI was limited to peaks 3 and 4. Binding to peaks 5 and 6 was not seen, even in buffer-treated samples.

Binding of NTHI 1479 to human macrophage gangliosides. Besides binding to respiratory epithelial cells, *H. influenzae*, including NTHI, also binds to macrophages (10, 20). To determine whether ^{3}H-labeled NTHI 1479 would bind to specific gangliosides of other human cells, ^{3}H-labeled NTHI was overlaid on TLC plates of human macrophage gangliosides. ^{3}H-labeled NTHI bound with greatest affinity to a minor doublet that migrates in the monosialoganglioside region of the TLC plate (Fig. 5). ^{3}H-labeled NTHI also bound less avidly to a large doublet, previously identified as G_{M3}. As with binding to HEp-2 gangliosides, the intensity of binding to the minor ganglioside peak was out of proportion to its relative overall abundance on TLC plates compared with the major ganglioside doublet. As before, no binding of NTHI 1479 occurred on simultaneously run TLC plates of murine brain gangliosides.

DISCUSSION

While gangliosides are implicated as receptors for *H. influenzae*, no previous studies have evaluated NTHI interactions with gangliosides, and conflicting data exist regarding the importance of sialylation in *H. influenzae*-glycosphingolipid interactions. Furthermore, gangliosides of previous studies were derived from non-receptor cell sources. This investigation is the first to demonstrate ganglioside-binding properties of NTHI and the first to demonstrate binding of any strains of *H. influenzae* to endogenous gangliosides of receptor cells. Though present in minute quantities, cell membrane gangliosides play important roles as receptors of specific bacteria and bacterial products (1, 2, 6).

Related studies indicate that macrophage gangliosides, despite being present in minute quantities, possess unique structural (11, 38) and immunologic (5, 40) attributes, compared with gangliosides of other tissues. The choice of cells used as source material for glycolipids for binding studies of bacteria may also be crucial. Earlier studies of binding of *H. influenzae* to gangliosides, which showed seemingly conflicting results, utilized commercially available gangliosides derived from exogenous sources, including brain tissue (13, 35). In each of our experiments, none of the three strains of *H. influenzae* bound to any murine brain gangliosides. Yet individual gangliosides both of HEp-2 cells and of human macrophages provided important and consistent binding foci. As can be seen on chromatograms (Fig. 2 and 3), murine brain gangliosides consist of more heavily sialylated species compared with gangliosides of HEp-2 cells and of human macrophages. Gangliosides of the last two cell types have in common a large doublet that comigrates with G_{M3} and several less abundant peaks. These data support the idea that individual gangliosides do not necessarily share the same biological properties.

The affinity of NTHI 1479 to two relatively minor ganglio-

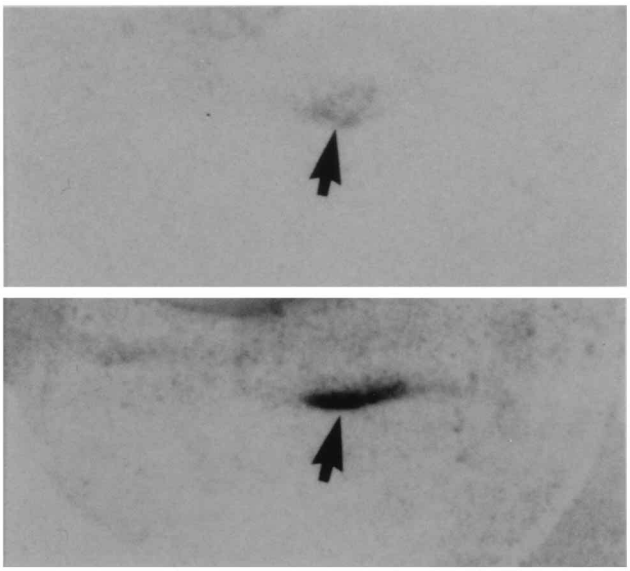

FIG. 4. Autoradiograph of binding of NTHI 1479 to neuraminidase-treated HEp-2 cell gangliosides. Autoradiographs were made of ^{3}H-labeled NTHI 1479, overlaid on TLC plates containing equal samples of HEp-2 cell gangliosides that had been treated with *A. ureafaciens* neuraminidase (upper panel) or with buffer (lower panel). Each autoradiograph shows the area of binding from one experiment. In each experiment, both samples were derived from a single sample of HEp-2 gangliosides that was divided into two equal parts. After neuraminidase treatment, desialylated glycolipids were visualized by exposure to iodine vapors or spraying with orcinol before treatment with ^{3}H-labeled NTHI. The same sample of [^{3}H]leucine-labeled NTHI 1479 was also divided into two equal volumes and overlaid on each TLC plate at the same time. Binding was retained, limited to peaks 3 and 4 (arrows), with less intensity in the neuraminidase-treated sample. Development of the TLC plate showed residual faint resorcinol-positive spots that corresponded with the autoradiograph image of the neuraminidase-treated sample. To correct for differences in background, quantitation of each autoradiographic image was corrected against its own measured background.

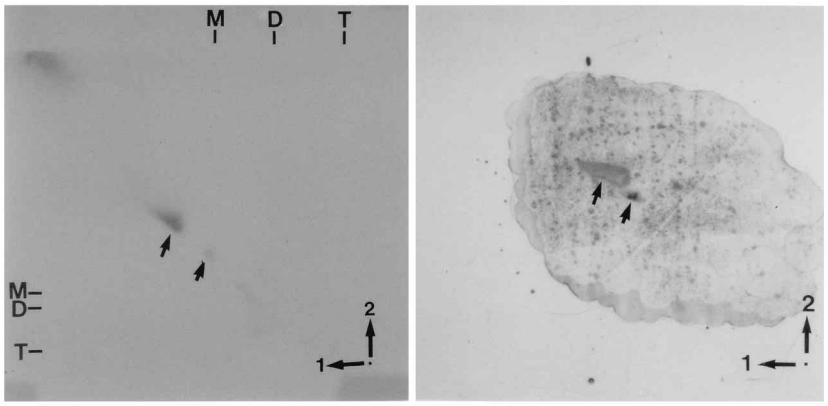

FIG. 5. Autoradiograph of NTHI 1479 binding to human macrophage gangliosides. An autoradiograph (right panel) was made of a TLC plate (left panel) of human macrophage gangliosides overlaid with ^{3}H-labeled NTHI 1479. The most intense autoradiographic binding sites were at a minor ganglioside doublet (right arrow). Binding to the larger G_{M3} ganglioside doublet (left arrow) also occurred, but with far less intensity than to the smaller ganglioside peak. Positions of ganglioside standards and directions of solvent runs are as specified in the legend to Fig. 1.

side doublets (peaks 3 and 4, with weaker binding to peaks 5 and 6) contrasts with the absence of binding to the far more abundant peaks 1 and 2, indicating relative specificity of binding. Furthermore, the major focus of binding of both strains of *H. influenzae* type b was a different minor ganglioside doublet (peaks 5 and 6) of HEp-2 cells. Finally, none of these strains bound to any concomitantly treated murine brain gangliosides in any experiments. Despite the paucity of material (15.9 ng of sialic acid/10^6 cells), it was critical to our studies to use endogenous gangliosides of HEp-2 cells rather than those of murine brain.

Several features of our methodology were critical to our findings. Two-dimensional TLC provided a distinct advantage in distinguishing ganglioside peaks 3 and 4 of HEp-2 cells, which bind NTHI, from peaks 5 and 6, which bind *H. influenzae* type b. The greater resolution offered by this method was recently used to distinguish G_{M1b} from G_{M1b}-GalNAc in a murine macrophage cell line (39). Since both HEp-2 ganglioside doublets comigrated in the first solvent, all four would have been superimposed upon one another and would have been indistinct had we performed only one-dimensional chromatography. Thus, our finding that NTHI 1479 bound to different HEp-2 cell gangliosides than did *H. influenzae* type b 770235 was dependent upon the improved resolution offered by two-dimensional TLC.

It was also critical to our studies to use *A. ureafaciens* neuraminidase to achieve optimal cleavage of sialic acid moieties. *A. ureafaciens* neuraminidase is more effective at removal of sialic acid from gangliosides than neuraminidase of *Vibrio cholerae* (28), used in other studies (35). Even so, under our most stringent conditions, even *A. ureafaciens* neuraminidase still left 10 to 20% of HEp-2 ganglioside sialic acid residues uncleaved. Our finding that desialylation of the gangliosides abrogated the binding of NTHI 1479 to HEp-2 cell gangliosides indicates that the binding of NTHI 1479 to HEp-2 cell gangliosides is dependent upon the presence of sialic acid. However, sialic acid alone is not adequate for binding NTHI 1479, since the organism did not bind to the more heavily sialylated gangliosides of murine brain or to other HEp-2 gangliosides, which are also sialylated. Since conditions of incubation alone, without neuraminidase, were sufficient to disturb the integrity of binding to peaks 5 and 6, our conclusions regarding the importance of ganglioside-bound sialic acid are limited to peaks 3 and 4.

It is noteworthy that NTHI 1479 bound avidly to minor peaks of both HEp-2 and macrophage gangliosides. The chromatographic mobilities of the NTHI-binding ganglioside peaks of both cells are very similar, although not perfectly identical, indicating that these ganglioside peaks may share common structural attributes. Based upon chromatographic mobilities and published structural studies of human mononuclear cell gangliosides, we suspect that the NTHI-binding ganglioside doublet of human macrophages is sialosylparagloboside (11). This will ultimately require purification and structural confirmation.

Earlier studies demonstrated a role for macrophages in binding and phagocytosis of unencapsulated *H. influenzae* (20, 36) and further demonstrated that NTHI was more readily phagocytosed by human alveolar macrophages than was *H. influenzae* type b (10). In one study, the interaction of adhesion between macrophages and NTHI was promoted by the expression of high-molecular-weight proteins by NTHI (21). However, the adherent component of the receptor cell remains unknown. Our data provide evidence that specific macrophage gangliosides may be one component of this interaction.

Related studies of St. Geme et al. also showed that two high-molecular-weight proteins of NTHI mediated adherence to epithelial cells, and that the introduction of the genes encoding both high-molecular-weight proteins into *E. coli* increased adherence to epithelial cells (31). Our data further support a fimbria-independent mechanism of binding of NTHI. NTHI 1479 may contain these high-molecular-weight proteins, since ^{3}H-labeled bands with molecular sizes greater than 120 kDa were consistently visible in autoradiographs of sodium dodecyl sulfate-polyacrylamide gel electrophoresis preparations of this organism.

van Alphen et al. also demonstrated that gangliosides inhibit fimbria-mediated hemagglutination of erythrocytes and adherence to epithelial cells by *H. influenzae* type b 770235 f^+ and found sialic acid critical to this interaction as well (35). Our studies demonstrate a role for ganglioside-*H. influenzae* binding in nonfimbriated and nontypeable strains also. In particular, sialic acid appears essential to the interaction between gangliosides and NTHI. Sialylated oligosaccharides of nasopharyngeal mucin also bind specific outer membrane proteins of NTHI (25). Previous evidence suggests that, under some conditions, asialogangliosides function as potential binding sites for some respiratory pathogens (13). In part, these differ-

ences may be due to the use of different strains of bacteria, to differences in culture conditions between different studies, and to the use of gangliosides of a different cell source in the current study. Our findings do not permit us to conclude that the ganglioside-binding differences observed between *H. influenzae* type b and NTHI would necessarily apply to all strains, particularly since the organisms included as NTHI are themselves relatively heterogeneous (18). Such a determination would require further study.

In summary, our studies support a role for specific individual gangliosides of specific human cells as receptors for *H. influenzae*. Ongoing studies will determine the exact structural epitopes of each of the ganglioside doublets responsible for binding individual *H. influenzae* strains.

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REFERENCES

1. **Backenson, P. B., J. L. Coleman, and J. L. Benach.** 1995. *Borrelia burgdorferi* shows specificity of binding to glycosphingolipids. Infect. Immun. **63:**2811–2817.
2. **Baker, N., G. C. Hansson, H. Leffler, G. Riise, and C. Svanborg-Edén.** 1990. Glycosphingolipid receptors for *Pseudomonas aeruginosa*. Infect. Immun. **58:**2361–2366.
3. **Berenson, C. S., M. A. Patterson, M. A. Pattoli, and T. F. Murphy.** 1996. A monoclonal antibody to human macrophage gangliosides inhibits macrophage migration. J. Leukocyte Biol. **59:**371–379.
4. **Berenson, C. S., M. A. Patterson, J. A. Miqdadi, and P. Lance.** 1995. *n*-Butyrate mediation of ganglioside expression of human and murine cancer cells demonstrates relative cell specificity. Clin. Sci. **88:**491–499.
5. **Berenson, C. S., and J. L. Ryan.** 1991. Murine peritoneal macrophage gangliosides inhibit lymphocyte proliferation. J. Leukocyte Biol. **50:**393–401.
6. **Fishman, P. H., T. Pacuszka, and P. A. Orlandi.** 1993. Gangliosides as receptors for bacterial enterotoxins. Adv. Lipid Res. **25:**165–187.
7. **Hakomori, S.** 1990. Bifunctional role of glycosphingolipids: modulators for transmembrane signaling and mediators for cellular interactions. J. Biol. Chem. **265:**18713–18716.
8. **Hansson, G. C., K. A. Karlsson, G. Larson, N. Stromberg, and J. Thurin.** 1985. Carbohydrate-specific adhesion of bacteria to thin-layer chromatograms: a rationalized approach to the study of host cell glycolipid receptors. Anal. Biochem. **146:**158–163.
9. **Jallat, C., A. Darfeuille-Michaud, and B. Joly.** 1991. Adhesive properties of *Haemophilus influenzae* to different human cells. Pathol. Biol. **39:**140–146.
10. **Jonsson, S., D. M. Musher, and E. C. Lawrence.** 1987. Phagocytosis and killing of *Haemophilus influenzae* by alveolar macrophages: no differences between smokers and nonsmokers. Eur. J. Respir. Dis. **70:**309–315.
11. **Kiguchi, K., C. B. Henning-Chubb, and E. Huberman.** 1990. Glycosphingolipid patterns of peripheral blood lymphocytes, monocytes, and granulocytes are cell-specific. J. Biochem. **107:**8–14.
12. **Kniep, B., C. Claus, J. Peter-Katalinic, D. A. Monner, W. Dippold, and M. Nimtz.** 1995. 7-0-Acetyl G_{D1a} in human T-lymphocytes is detected by a specific T-cell activating monoclonal antibody. J. Biol. Chem. **270:**30173–30180.
13. **Krivan, H. C., D. D. Roberts, and V. Ginsburg.** 1988. Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAcβ1-4Gal found in some glycolipids. Proc. Natl. Acad. Sci. USA **85:**6157–6160.
14. **Lampe, R. M., E. O. Mason, Jr., S. L. Kaplan, C. L. Umstead, M. D. Yow, and R. D. Feigin.** 1982. Adherence of *Haemophilus influenzae* to buccal epithelial cells. Infect. Immun. **35:**166–172.
15. **Loeb, M. R., E. Connor, and D. Penny.** 1988. A comparison of the adherence of fimbriated and nonfimbriated *Haemophilus influenzae* type b to human adenoids in organ culture. Infect. Immun. **56:**484–489.
16. **Magnani, J. L., D. F. Smith, and V. Ginsburg.** 1980. Detection of gangliosides that bind cholera toxin: direct binding of ^{125}I-labeled toxin to thin layer chromatograms. Anal. Biochem. **109:**399–402.
17. **Mandrell, R. E., R. McLaughlin, Y. Abu Kwaik, A. Lesse, R. Yamasaki, B. Gibson, S. M. Spinola, and M. A. Apicella.** 1992. Lipooligosaccharides (LOS) of some *Haemophilus* species mimic human glycosphingolipids, and some LOS are sialylated. Infect. Immun. **60:**1322–1328.
18. **Murphy, T. F., and M. Apicella.** 1987. Nontypeable *Haemophilus influenzae*: a review of clinical aspects, surface antigens, and the human immune response to infection. Rev. Infect. Dis. **9:**1–15.
19. **Murphy, T. F., and S. Sethi.** 1992. Bacterial infection in chronic obstructive pulmonary disease. Am. Rev. Respir. Dis. **146:**1067–1083.
20. **Noel, G. J., S. K. Hoiseth, and P. J. Edelson.** 1992. Type b capsule inhibits ingestion of *Haemophilus influenzae* by murine macrophages: studies with isogenic encapsulated and unencapsulated strains. J. Infect. Dis. **166:**178–182.
21. **Noel, G. J., S. J. Barenkamp, J. W. St. Geme III, N. Haining, and D. M. Mosser.** 1994. High-molecular-weight surface-exposed proteins of *Haemophilus influenzae* mediate binding to macrophages. J. Infect. Dis. **169:**425–429.
22. **Ono, E., K. Abe, M. Nakazawa, and M. Naiki.** 1989. Ganglioside epitope recognized by K99 fimbriae from enterotoxigenic *Escherichia coli*. Infect. Immun. **57:**907–911.
23. **Palumbo, G., and F. Zullo.** 1987. The use of iodine staining for the quantitative analysis of lipids separated by thin layer chromatography. Lipids **22:**201–205.
24. **Read, R. C., R. Wilson, A. Rutman, V. Lund, H. C. Todd, A. P. R. Brain, P. K. Jeffery, and P. J. Cole.** 1990. Interaction of nontypeable *Haemophilus influenzae* with human respiratory mucosa *in vitro*. J. Infect. Dis. **163:**549–558.
25. **Reddy, M. S., J. M. Bernstein, T. F. Murphy, and H. S. Faden.** 1996. Binding between outer membrane proteins of nontypeable *Haemophilus influenzae* and human nasopharyngeal mucin. Infect. Immun. **64:**1477–1479.
26. **Sable, N. S., E. M. Connor, C. B. Hall, and M. R. Loeb.** 1985. Variable adherence of fimbriated *Haemophilus influenzae* type b to human cells. Infect. Immun. **48:**119–123.
27. **Saito, M., N. Kasai, and R. K. Yu.** 1985. *In situ* immunological determination of basic carbohydrate structures of gangliosides on thin-layer plates. Anal. Biochem. **148:**54–58.
28. **Schauer, R., R. W. Veh, M. Sander, A. P. Corfield, and H. Wiegandt.** 1980. "Neuraminidase-resistant" sialic acid residues of gangliosides. Adv. Exp. Med. Biol. **125:**283–294.
29. **Schengrund, C.-L.** 1995. Evidence that molecules on the surface of one cell can adhere to the oligosaccharide portion of gangliosides on the surface of another cell. Biol. Signals **4:**1–13.
30. **Sewell, A. C.** 1979. An improved thin-layer chromatographic method for urinary oligosaccharide screening. Clin. Chim. Acta **92:**411–414.
31. **St. Geme, J. W., III, S. Falkow, and S. J. Barenkamp.** 1993. High-molecular-weight proteins of nontypeable *Haemophilus influenzae* mediate attachment to human epithelial cells. Proc. Natl. Acad. Sci. USA **90:**2875–2879.
32. **Svennerholm, L.** 1957. Quantitative estimation of sialic acids. Biochim. Biophys. Acta **24:**604–611.
33. **Svennerholm, L.** 1963. Chromatographic separation of human brain gangliosides. J. Neurochem. **10:**613–623.
34. **van Alphen, L., J. Poole, L. Geelen, and H. C. Zanen.** 1987. The erythrocyte and epithelial cell receptors for *Haemophilus influenzae* are expressed independently. Infect. Immun. **55:**2355–2358.
35. **van Alphen, L., L. Geelen-van den Broek, L. Blaas, M. van Ham, and J. Dankert.** 1991. Blocking of fimbria-mediated adherence of *Haemophilus influenzae* by sialyl gangliosides. Infect. Immun. **59:**4473–4477.
36. **Williams, A. E., D. J. Maskell, and E. R. Moxon.** 1991. Relationship between intracellular survival in macrophages and virulence of *Haemophilus influenzae* type b. J. Infect. Dis. **163:**1366–1369.
37. **Yohe, H. C., C. S. Berenson, C. L. Cuny, and J. L. Ryan.** 1990. Altered B-lymphocyte membrane architecture indicated by ganglioside accessibility in C3H/HeJ mice. Infect. Immun. **58:**2888–2894.
38. **Yohe, H. C., C. L. Cuny, L. J. Macala, M. Saito, W. J. McMurray, and J. L. Ryan.** 1991. The presence of sialidase-sensitive sialylgangliotetraosyl ceramide (G_{M1b}) in stimulated murine macrophages. J. Immunol. **146:**1900–1908.
39. **Yohe, H. C., L. J. Macala, G. Giordano, and W. J. McMurray.** 1992. G_{M1b} and G_{M1b}-GalNAc: major gangliosides of murine-derived macrophage-like WEHI-3 cells. Biochim. Biophys. Acta **1109:**210–217.
40. **Yohe, H. C., and J. L. Ryan.** 1986. Ganglioside expression in macrophages from endotoxin-responder and nonresponder mice. J. Immunol. **137:**3921–3927.
41. **Yu, R. K., and M. Saito.** 1989. Structure and localization of gangliosides, p. 1–34. *In* R. U. Margolis and R. K. Margolis (ed.), Neurobiology of glycoconjugates. Plenum Publishing Corporation, New York, N.Y.

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