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Binding of *Pseudomonas aeruginosa* to Neutral Glycosphingolipids of Rabbit Corneal Epithelium

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^{35}S-labeled *Pseudomonas aeruginosa* isolates were shown to bind to neutral glycosphingolipids (NGSLs) of rabbit corneal epithelia in culture by a thin-layer chromatogram overlay procedure. The lipids of the corneal epithelial cells grown in culture were extracted and partitioned into a chloroform-rich lower phase containing NGSLs and an aqueous upper phase containing gangliosides. By using a dot-blot assay, at least six times more radiolabeled *P. aeruginosa* isolates were shown to bind to the lipids in the lower phase compared with those in the upper phase. Thin-layer chromatography of the lower-phase lipids followed by staining with an orcinol spray revealed at least 10 NGSL components and several fast-migrating, nonglycosylated neutral lipid components (including cholesterol). ^{35}S-labeled *P. aeruginosa* was shown to bind to NGSL components 1, 2, 5, 6, and 9. *P. aeruginosa*-reactive NGSL components 6 and 9 migrated with chromatographic mobilities similar to those of the standards ceramide trihexoside (CT) and ceramide monohexoside, respectively. Components 1 and 2 migrated slightly ahead of asialo G_{M1}, and component 5 migrated faster than globoside but slower than CT. Among the various standards tested, *P. aeruginosa* bound to asialo G_{M1} and, to a lesser extent, to ceramide dihexoside and CT but not to G_{M1}, G_{D1A}, G_{M3}, or ceramide monohexoside. It remains to be determined whether any of the five *P. aeruginosa*-reactive NGSL components of corneal epithelium identified in this study plays a role in the development of corneal infection. However, we have previously shown that component 9, one of the five *P. aeruginosa*-reactive NGSL components identified in this study, is present in significantly greater amounts in migrating epithelia than it is in nonmigrating epithelia (N. Panjwani, G. Michalopoulos, J. Song, G. Yogeeswaran, and J. Baum, Invest. Ophthalmol. Vis. Sci., in press). This may prove to be of biological significance because it is generally believed that traumatized (migrating) epithelia are more susceptible to infection than normal (nonmigrating) epithelia are.

Pseudomonas aeruginosa is one of the most common pathogens associated with bacterial corneal ulcers (15) and is the major cause of corneal infection in wearers of extended-wear contact lenses (2, 3, 8, 23, 26). Furthermore, the rapidity of corneal degradation and the risk of perforation that occur 24 to 48 h following the onset of infection makes *P. aeruginosa* keratitis one of the most dangerous of all corneal infections.

The adherence of bacteria to the host tissue is generally recognized as the first important step in the pathogenesis of a corneal infection (9, 16). The mechanisms by which *P. aeruginosa* adheres to the cornea and causes infection have not been fully defined. Although corneal injury is believed to be a major predisposing factor to infection (14, 24), it has been discovered recently (18) that *P. aeruginosa* keratitis may occur in the absence of overt corneal trauma, particularly in the wearers of extended-wear contact lenses. Adherence of *P. aeruginosa* to the injured corneas of mice and rats appears to be mediated by *N*-acetylmannosamine (13) and mannose (27) receptors, respectively. In studies of nonocular tissues, cell surface glycosphingolipids of host tissues have been reported to act as receptors for a number of pathogenic bacteria, including *P. aeruginosa* (19, 20), uropathogenic *Escherichia coli* (4, 22), *Actinomyces naeslundii* (5), *Propionibacterium granulosum* (11), *Neisseria gonorrhoeae* (6), and *Pseudomonas cepacia* (19). In the present study we demonstrate that *P. aeruginosa* derived from a clinical corneal ulcer binds to the neutral glycosphingolipids (NGSLs) extracted from rabbit corneal epithelial cells grown in culture.

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MATERIALS AND METHODS

Isolation of glycosphingolipids from rabbit corneal epithelium in culture. Corneal epithelial cells were grown in tissue culture (17) by using rabbit eyes purchased from Pel-Freez Biologicals (Rogers, Ark.). We used sparse cultures, which we designated as migrating epithelial cells, and confluent cultures, which we designated as nonmigrating epithelial cells. Throughout this study, primary cell cultures were used and cells from 25 to 35 dishes (diameter, 100 mm) were pooled for each preparation. The procedure to prepare glycosphingolipids from rabbit corneal epithelial cells has been described in detail elsewhere (N. Panjwani, B. Clark, M. Cohen, M. Barza, and J. Baum, Invest. Ophthalmol. Vis. Sci., in press). Briefly, by using a modified extraction procedure (25) of Folch et al. (7), a lower phase that contained NGSLs and an upper phase that contained gangliosides were prepared from rabbit corneal epithelial cells in culture. Since the lower phase was expected to contain phospholipids and cholesterol in addition to NGSLs, it was saponified to hydrolyze and eliminate phospholipids. Cholesterol was not removed from the lower phase prior to use because it migrated with the solvent front during thin-layer chromatography (TLC) and, therefore, did not interfere with the analyses of bacterial binding to NGSLs.

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Radiolabeling of bacteria. *P. aeruginosa* 100 and *Staphylococcus aureus* 286, each of which was derived from a clinical bacterial corneal ulcer, and *E. coli* ATCC 8677 were used. To radiolabel the bacteria, a fraction of the bacterial suspension containing 3.3×10^7 CFU was incubated in 50 ml of 5% Mueller-Hinton broth containing 200 μCi of [^{3}H]adenine (20 Ci/mmol; Du Pont Co., Wilmington, Del.) for 18 to 24 h. To radiolabel the bacteria with ^{35}S, a fraction of a bacterial suspension containing 1.2×10^8 CFU was used and incubation was performed with 500 μCi of [^{35}S]methionine (660 Ci/mmol; Du Pont Co.). At the end of the labeling period, the radiolabeled bacteria were washed four times with phosphate-buffered saline (PBS) by centrifugation to remove free isotope, suspended in PBS, and combined with unlabeled bacteria to adjust the specific activity of the bacteria to 1 cpm/100 CFU.

Bacterial binding to glycosphingolipids of rabbit corneal epithelia. (i) Dot-blot assay. To determine whether *P. aeruginosa* bound to glycosphingolipids that were extracted in the upper phase, the lower phase, or both, triplicate fractions from the two phases, each representing 2 mg of cell protein, were suspended in a 20- to 25-μl volume of chloroform-methanol (2:1) and spotted onto strips (5 by 10 cm) of aluminum-backed TLC plates (HPTLC, Alufolien, Kieselgel 60; EM Science, Cherry Hill, N.J.). For background values, an equivalent volume of solvent was spotted onto the strips. The plates were allowed to air dry and were dipped in 0.5% polyisobutylmethacrylate for 1 min to prevent detachment of the silica gel from the plate during subsequent procedures. The plates were then incubated for 90 min in 2% bovine serum albumin in PBS to block nonspecific binding prior to incubation with 7.5 ml of ^{3}H- or ^{35}S-labeled *P. aeruginosa* (1 cpm/100 CFU) for 90 min. To remove the unbound bacteria, the corners of the plates were bent to 90 degrees, and the plates were then washed upside down six times in 300 ml of PBS. After the plates were allowed to air dry, the spots were scraped off the TLC plates and were analyzed for radioactivity in a β-scintillation counter.

(ii) Thin-layer chromatogram overlay assay. To identify *P. aeruginosa*-reactive NGSLs, the thin-layer chromatogram overlay procedure described by Hansson and colleagues (11) was used. For this procedure, duplicate fractions of the lower phase, each representing 2 mg of cell protein, were chromatographed on an aluminum-back TLC plate by using a solvent system consisting of chloroform-methanol-H_2O (65:25:4). Following chromatography, one lane of each sample was stained with an orcinol spray (21), and the other lane was treated with 0.5% polyisobutylmethacrylate and was then incubated with 2% bovine serum albumin and ^{35}S-labeled bacteria by the procedure described above for the dot-blot assay. After incubation with ^{35}S-labeled bacteria, the plates were washed extensively with PBS as described above, air dried, sprayed with En^{3}Hance (Dupont, NEN Research Products, Boston, Mass.), and then subjected to fluorography. We obtained six glycosphingolipid standards, glucosylceramide (ceramide monohexoside [CM]); lactosylceramide (ceramide dihexoside [CD]); and asialo G_{M1}, G_{M1}, G_{M3}, and G_{D1A}, from Sigma Chemical Co. (St. Louis, Mo.). In addition, two standards, globoside and ceramide trihexoside (CT), were prepared from human erythrocytes by one of the authors (F. B. Jungalwala) by the method of Hakomori and colleagues (10).

To estimate the approximate amounts of various corneal NGSL components observed on the thin-layer chromatogram, 0.25, 0.50, 1.0, 1.5, and 2.0 μg of each of the various NGSL standards and fractions of the lower-phase samples of nonmigrating epithelial cell cultures representing 1.5, 2.0, and 3.0 mg of original cell protein were chromatographed on a thin-layer plate. After orcinol staining, the density of each component was measured with a laser densitometer (Ultroscan XL; LKB Instruments, Inc., Piscataway, N.J.). Four NGSL standard curves, those for asialo G_{M1}, CT, CD, and CM, were constructed, and approximate amounts of various orcinol-positive corneal NGSLs were then determined from the appropriate standard curves. The sugar contents of all the standards and the lower-phase samples were measured by using an orcinol reagent as described by Balazs and associates (1). The molecular weights of various standards were calculated on the basis of their sugar contents. The amount of cholesterol in the lower phase was measured by the procedure of Zlatkis and colleagues (28).

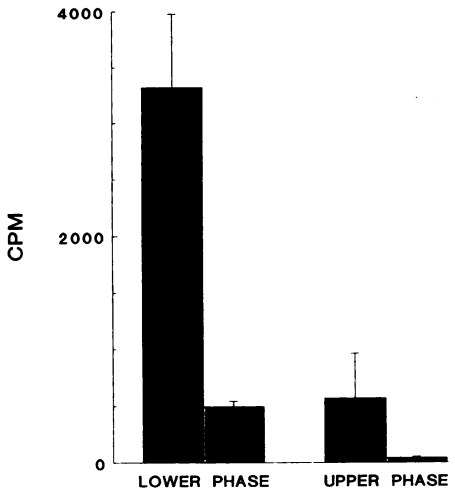

FIG. 1. Binding of ^{3}H-labeled (hatched bars) and ^{35}S-labeled (solid bars) *P. aeruginosa* to rabbit corneal epithelial glycosphingolipids extracted in the upper and lower phases. Data are expressed as means ± standard errors of the mean of six determinations. The counts per minute of the background spots (197 ± 12 and 1,367 ± 204 cpm for ^{3}H- and ^{35}S-labeled bacteria, respectively) were deducted from the binding values for the upper and lower phases.

RESULTS

Binding of the radiolabeled *P. aeruginosa* to the glycolipids of the rabbit corneal epithelium. (i) Dot-blot assay. A dot-blot assay was used to determine whether *P. aeruginosa* bound to corneal epithelial lipids extracted in the Folch lower phase (NGSL and cholesterol), the upper phase (gangliosides), or both. Compared with the upper phase, *P. aeruginosa* binding to the lower phase was 5.8 times greater when ^{35}S-labeled *P. aeruginosa* was used and 11.5 times greater when ^{3}H-labeled *P. aeruginosa* was used (Fig. 1). Thus, regardless of whether ^{3}H- or ^{35}S-labeled *P. aeruginosa* was used, more bacteria were found to bind to the lower phase than to the upper phase. Binding of ^{3}H- and ^{35}S-labeled *P. aeruginosa* to the lower phase was 3.5 times above the background, whereas binding to the upper phase was only 1.2 times above the background.

(ii) Thin-layer chromatogram overlay assay. A thin-layer chromatogram overlay procedure was used to determine whether *P. aeruginosa* binding to the lower phase observed by the dot-blot assay was caused by binding to one or more of the NGSL components extracted in the lower phase. For this study, we analyzed the *P. aeruginosa*-binding patterns

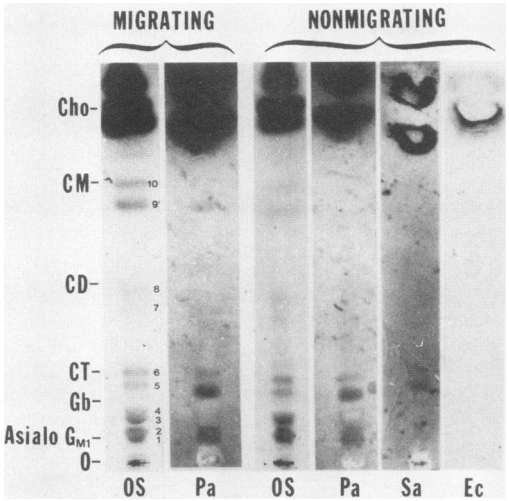

FIG. 2. Binding of ^{35}S-labeled bacteria to NGSLs of nonmigrating and migrating rabbit corneal epithelia in culture. Abbreviations: OS, orcinol staining; Pa, *P. aeruginosa*; Sa, *S. aureus*; Ec, *E. coli*; O, origin; Gb, globoside; CT, ceramidetrihexoside, CD, ceramidedihexoside; CM, ceramidemonohexoside; Cho, cholesterol. The OS lane of migrating epithelium was reprinted from *Investigative Ophthalmology and Visual Science* (Panjwani et al., in press) with permission of the publisher.

of thin-layer chromatograms of two preparations of migrating cell cultures and six preparations of nonmigrating cell cultures and compared them with the respective patterns obtained by orcinol staining. To ensure the reproducibility of the data, materials from different preparations were not pooled but were analyzed individually. Of the 10 orcinol-positive NGSL components detected in the thin-layer chromatogram of the lower phase of the migrating cultures, *P. aeruginosa* was found to bind only to components 1, 2, 5, 6, and 9 (Fig. 2). Of the five *P. aeruginosa*-reactive NGSL components detected in migrating epithelia, four components, 1, 2, 5, and 6, were also present in nonmigrating epithelia (Fig. 2). In all six chromatograms of nonmigrating epithelia and both chromatograms of migrating epithelia, orcinol spray reagent stained components 5 and 6 with approximately equal intensities, but radiolabeled *P. aeruginosa* stained component 5 with a much greater intensity than it did component 6 (Fig. 2). On a thin-layer chromatogram, *P. aeruginosa*-reactive NGSL components 6 and 9 migrated with chromatographic mobilities similar to those of the standards CT and CM, respectively; NGSL components 1 and 2 migrated slightly ahead of asialo G_{M1}; and NGSL component 5 migrated faster than globoside but slower than CT. *P. aeruginosa* also stained several nonpolar lipid components which migrated in the cholesterol area on the TLC plate (Fig. 2).

To determine whether the bacterial binding pattern of rabbit corneal NGSLs observed in this study was specific to *P. aeruginosa*, we also analyzed the *S. aureus*-binding pattern of five preparations and the *E. coli*-binding pattern of one preparation of nonmigrating epithelia. Unlike *P. aeruginosa*, which stained NGSL components 1, 2, 5, and 6, *S. aureus* stained only NGSL components 2 and 5 (Fig. 2) and *E. coli* did not stain any of the 10 orcinol-positive NGSL components that were present in the lower phase of the nonmigrating epithelia (Fig. 2). As was observed with *P. aeruginosa*, *S. aureus* and *E. coli* also stained the components that migrated in the cholesterol area, but mainly at the margins (Fig. 2). The *S. aureus*- and *E. coli*-binding patterns of migrating epithelia were not analyzed because of the limited availability of the starting material.

TABLE 1. Binding of *P. aeruginosa* to various glycosphingolipid standards and cholesterol

Standard	Amt (μg)	*P. aeruginosa* binding[a]
CM	2.0	−
CD	2.0	+
CT	2.0	+
Globoside	2.0	−[b]
G_{M3}	2.0	−
Asialo G_{M1}	0.5	+++
G_{M1}	2.0	−
G_{D1A}	2.0	−
Cholesterol	2.0	−
Cholesterol	15.0	±
Cholesterol	40.0	+++

[a] Detected by a thin-layer chromatogram overlay procedure. Symbols: +++, intense; ++, moderate; +, weak; ±, trace.
[b] The result was − in 4 of 6 experiments and ± in 2 of 6 experiments.

Estimation of the approximate amounts of various corneal NGSLs. Approximate amounts of various corneal NGSLs were determined from the thin-layer chromatograms of the lower-phase samples by using appropriate standard curves. Microgram amounts of components 1 to 4 were estimated from the asialo G_{M1} standard curve. Similarly, CT, CD, and CM standard curves were used to determine the amounts of NGSL components 5 and 6, 7 and 8, and 9 and 10, respectively. By this method, in a fraction of the lower phase representing 2.0 mg of cell protein of nonmigrating epithelia, the combined amounts of NGSL components 1 and 2 and components 3 and 4 were 1.72 and 1.89 μg, respectively; and the amounts of components 5, 6, 7, 8, 9, and 10 were 1.15, 1.10, 0.51, 0.50, 0.79, and 0.76 μg, respectively. We were unable to calculate the amounts of components 1 to 4 individually since they were poorly resolved on the thin-layer chromatogram (Fig. 2). A fraction of the lower phase derived from 2.0 mg of cell protein contained 6.4 μg of total carbohydrate, as determined by the orcinol method of Balazs and colleagues (1). In an equivalent fraction of the lower phase, there was 58 μg of cholesterol.

Binding of radiolabeled *P. aeruginosa* to various glycosphingolipid standards and cholesterol. In a thin-layer chromatogram overlay assay, *P. aeruginosa* was found to bind to asialo G_{M1} and, to a lesser extent, to CT and CD (Table 1). *P. aeruginosa* did not bind to G_{M1}, G_{D1A}, G_{M3}, or CM (Table 1). These results were observed in all six chromatograms analyzed for G_{D1A}, G_{M1}, asialo G_{M1}, CT, CD, and CM and in all four chromatograms analyzed for G_{M3}. *P. aeruginosa* did not bind to globoside in four of the six chromatograms analyzed. On the other hand, in two of the six experiments, *P. aeruginosa* bound to globoside, but to a lesser degree than it did to CD and CT (Table 1). *P. aeruginosa* was also found to bind to cholesterol, although we were unable to detect binding to cholesterol in amounts below a concentration of 15 μg.

DISCUSSION

In the present study we demonstrated that *P. aeruginosa* binds to NGSLs extracted from rabbit corneal epithelial cells grown in culture. Specifically, *P. aeruginosa* bound to 5 of the 10 orcinol-positive NGSL components of migrating rabbit corneal epithelia. On the basis of the comparison of the chromatographic mobilities of the five *P. aeruginosa*-

reactive components with those of the standards, it appears that components 1 and 2 contain four sugar residues, whereas NGSL component 6 contains three sugar residues and NGSL component 9 contains one sugar residue. NGSL component 5 may contain three or four sugar residues, because it migrated faster than globoside but slower than CT. It remains to be determined whether one or more of the five *P. aeruginosa*-reactive NGSL components identified in this study play a role in the pathogenesis of corneal ulceration. Nevertheless, we have previously shown, using both cell and organ culture techniques, that component 9, one of the five *P. aeruginosa*-reactive NGSL components identified in this study, is synthesized and accumulates in greater amounts in migrating than in nonmigrating epithelia (N. Panjwani, G. Michalopoulos, J. Song, B. S. Tanweer, S. Zaidi, G. Yogeeswaran, J. Baum, Invest. Ophthalmol. Vis. Sci., in press). This may be of biological importance since it is generally accepted that traumatized (migrating) epithelia are more susceptible to infection than normal (nonmigrating) epithelia are (14, 24).

Of the five *P. aeruginosa*-reactive NGSL components detected in migrating epithelia, four were also present in nonmigrating epithelia. It should be noted, however, that the confluent corneal epithelial cell cultures designated in this study as nonmigrating epithelia were equivalent to the basal cells of corneal epithelia in vivo. These basal cells lie several cell layers beneath the corneal surface, unless the cornea has been traumatized. A recent study (18) has shown that even a subtle corneal injury that removes only one or two layers of the corneal epithelium can facilitate the adherence of *P. aeruginosa*. It is therefore plausible that any of the four *P. aeruginosa*-reactive NGSL components identified in this study in the nonmigrating cultures may play a role in the pathogenesis of corneal ulceration, especially when the injury has been mild, as in patients who wear extended-wear contact lenses.

Most known glycosphingolipids, including those which migrated on TLC plates with chromatographic mobilities similar to those of the *P. aeruginosa*-reactive corneal NGSLs identified in this study, such as asialo G_{M1}, asialo G_{M2} (19, 20), globoside, and CT, contain an internal glucose residue linked to a ceramide moiety. To this end, Spurr-Michaud et al. (27) have shown that binding of *P. aeruginosa* to injured rat corneas could be blocked by concanavalin A. However, in that study, *P. aeruginosa* was found to bind mainly to the denuded basal lamina in front of the leading edge; it is unlikely that NGSLs are present in the basal lamina. Hazlett et al. (12, 13) have shown that binding of *P. aeruginosa* to scarified adult and uninjured newborn mice corneas could be blocked by *N*-acetylmannosamine (13) and sialic acid residues (12), respectively. Neither *N*-acetylmannosamine nor sialic acid residues are likely to have been present in our neutral glycosphingolipid fraction.

Consistent with the studies of Krivan and associates (19), who demonstrated that *P. aeruginosa* derived from sputum samples of patients with cystic fibrosis bind to asialo G_{M1}, our *P. aeruginosa* strain, which was isolated from a clinical bacterial corneal ulcer, was also found to bind to asialo G_{M1}. On the other hand, unlike the findings of Krivan et al. (19, 20), in our study *P. aeruginosa* was found to bind to CT and CD, although to a lesser degree than it bound to asialo G_{M1}. In our study, we did not establish the identity of the various *P. aeruginosa*-reactive NGSL components, but component 5 may be asialo G_{M2}, because it migrated on the TLC plate in the area where asialo G_{M2} is expected to migrate, i.e., between globoside and asialo G_{M1} (19, 20), and it is known that *P. aeruginosa* derived from sputum samples binds to asialo G_{M2} (19, 20). However, in the present study we did not determine whether *P. aeruginosa* derived from a bacterial corneal ulcer also bound to the standard asialo G_{M2}.

We recognize the importance of determining how many of the five *P. aeruginosa*-reactive corneal NGSL components identified in this study are located on the cell surface. However, since about 80% of cellular glycolipids usually are located on the cell surface, the majority of *P. aeruginosa*-reactive NGSL components identified in this study are likely to be associated with the cell surface.

In the present study we also demonstrated that two of the four *P. aeruginosa*-reactive NGSL components of nonmigrating epithelia also recognized *S. aureus*. On the other hand, none of the 10 corneal NGSL components was found to recognize *E. coli*. These observations are consistent with our earlier observations (Panjwani et al., in press) that, compared with *S. aureus* (30 ± 1.5 bacteria per epithelial cell), a much greater number of *P. aeruginosa* (186 ± 11 bacteria per epithelial cell) bind to corneal epithelial cells in culture. In contrast, we found that *E. coli* did not bind to corneal epithelial cells in culture.

We have shown (Panjwani et al., in press) that NGSLs synthesized by rabbit corneal epithelia in organ and tissue cultures are remarkably similar; the NGSLs of corneal epithelia have not been analyzed thus far in an in vivo system. It is well known that studies performed in vitro may not mirror events in vivo. Furthermore, mechanisms of bacterial adherence to corneas may vary among animal species, especially since it is known that the lectin-binding patterns of corneal epithelia vary among species (N. Panjwani and J. Baum, Invest. Ophthalmol. Vis. Sci. Suppl. **27**:64, 1986). In the present study, a rabbit animal model was chosen because we have shown previously that the lectin-binding patterns of the corneal epithelia of rabbits and dogs are most similar to that of human corneal epithelium (Panjwani and Baum, Invest. Ophthalmol. Vis. Sci. Suppl. **27**:64, 1986). Moreover, we have recently established that NGSLs synthesized by human and rabbit corneal epithelial cells in culture are remarkably similar (N. Panjwani, G. Michalopoulos, and J. Baum, unpublished data).

In summary, in the present study we demonstrated that *P. aeruginosa* binds to rabbit corneal NGSLs. We hope that future studies will indicate which, if any, of the five *P. aeruginosa*-reactive corneal NGSL components identified in this study plays a role in the development of corneal infection.

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