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Characterization of *Pseudomonas aeruginosa* Adherence to Mouse Corneas in Organ Culture

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The present study was designed to obtain further information on the nature of the corneal macromolecule(s) to which *Pseudomonas aeruginosa* adheres and how adherence might be prevented. Scarified adult mouse corneas in organ culture were treated with trypsin or lipase to determine whether the receptor molecule(s) was protein or lipid in nature. Trypsin (20 μg/ml) treatment of the cornea for 5 min had no significant effect on bacterial adherence, and longer periods of enzyme exposure resulted in extensive surface cell lysis. In contrast, lipase treatment (50,000 U/ml) for 1 h caused little visible cell lysis and significantly reduced bacterial adherence. To test further the lipid nature of the receptor, a highly purified monosialoganglioside (G_{M1}) preparation (500 μg/ml) was used to preincubate (1 h) the cornea prior to bacterial application, and this also inhibited bacterial adherence. Similar corneal treatment with gangliotetraosylceramide (asialo G_{M1}) (500 μg/ml) had little effect on ocular bacterial binding. Premixing of the bacterial inoculum with G_{M1} prior to corneal application had no significant effect on inhibiting bacterial binding, but similarly premixing the bacterial inoculum with asialo G_{M1} transiently decreased adherence. Lastly, premixing of the bacterial inoculum or preincubation of corneas with fibronectin (500 μg/ml for 1 h) both decreased bacterial adherence. These findings provide evidence that the receptor-adhesin interactions of *P. aeruginosa* at the ocular surface in organ culture are complex, involve a glycolipid moiety, and may be blocked by a ganglioside containing at least one sialosyl residue or by fibronectin, which may bind to membrane-associated gangliosides.

Pseudomonas aeruginosa, an opportunistic bacterial pathogen, produces a fulminating, highly destructive corneal infection in humans which may result in decreased visual acuity or blindness (16). In human and experimental cases of disease, corneal injury or other host compromise is usually requisite to produce infection by the organism (4, 6, 11, 12, 16, 25). An in vivo study (9) with immature mice compromised by age (12) provided evidence that sialic acid inhibits bacterial adherence to the unwounded cornea, while another study (10) provided evidence that *N*-acetylmannosamine, its C_6 precursor, functions to inhibit *P. aeruginosa* binding to the scarified adult mouse cornea. Other saccharide moieties also have been implicated in bacterial binding to the corneal surface. For example, an in vitro inhibition study (13) using corneal epithelial cells in culture and a lectin study (23) employing concanavalin A treatment of trephined rat cornea in organ culture suggested that mannose is involved in bacterial adherence. In all of these models, the precise nature of the binding site on the corneal surface remains uncharacterized. Thus, the present study was designed (i) to further examine, in organ culture, the corneal epithelial cell receptor-adhesin interactions of *P. aeruginosa* at the wounded ocular surface of the adult mouse in a model in which the epithelial basal lamina is breached and (ii) to determine how to best prevent adherence of the bacterium to ocular surface cells. The results suggest that the ocular receptor facilitating binding of *P. aeruginosa* to the cornea involves a glycolipid molecule and that adherence is blocked significantly if the glycolipid is sialylated.

MATERIALS AND METHODS

Mice. Female Swiss Webster (ICR) mice purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.) were used. Mice 37 to 60 days old, weighing 20 to 30 g, were housed in clear plastic cages and fed laboratory rodent chow (Ralston-Purina Co., St. Louis, Mo.) and acidified water ad libitum. Prior to use of the animals, each eye was observed for corneal clarity with a stereoscopic microscope at 40×. Only those mice whose corneas were clear and normal in appearance were used for the experiments described below.

Bacterial cell cultures. Stock cultures of *P. aeruginosa* ATCC 19660 stored at 25°C on tryptose agar slants (Difco Laboratories, Detroit, Mich.) were used for inoculation of 60 ml of broth medium containing 5% peptone and 0.25% Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). The culture was hemolytic and proteolytic and produced lecithinase and exotoxin A. Cultures were grown on a rotary shaker at 37°C for 18 h, centrifuged at 27,000 × *g* for 20 min at 4°C, and diluted with saline (Travenol Laboratories, Inc., Deerfield, Ill.) to a concentration of 2.0×10^{10} CFU/ml by using a standard curve relating viable counts to optical density at 440 nm (1,2). An inoculum of 5 μl containing 5.0×10^7 organisms was used in each of the experiments.

Culture wells. Cell culture dishes (GIBCO Laboratories, Grand Island, N.Y.) 35 by 10 mm and rubber tubing (Norton Plastics, Akron, Ohio) with diameters of 3/16 in. and 2/16 in. were obtained. The 3/16-in. tube was cut to 0.5 cm, and the 2/16-in. tubing was inserted inside up to a 0.25-cm depth mark. Two centered diagonal and vertical cuts were then made on the 3/16-in. tubing down to the 0.25-cm mark, and horizontal cuts along this plane were made in order to remove four cut sections of tubing. This was done to facilitate resting of the posterior part of the eye on the 2/16-in. tubing. Locating the optic nerve down inside the 2/16-in. tubing provided greater stability and a superior orientation of the cornea within the 3/16-in. tubing (Fig. 1). The entire tubing apparatus was superglued to the bottom of

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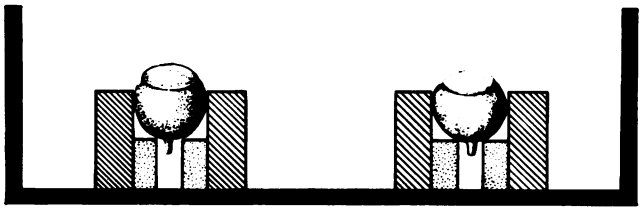

FIG. 1. Diagrammatic cross-sectional representation of the culture apparatus.

a cell culture dish and placed under UV light for sterilization prior to use.

Organ culture and infection. Mice were anesthetized with ether and sacrificed immediately via cervical dislocation. Following this, the animals were placed beneath a 40× stereoscopic microscope and three 1-mm incisions were made to the center of both the right and the left corneas with a sterile 26-gauge needle. The deepest wounds penetrated the epithelial cell basal lamina and into the superficial corneal stroma. The depth of the wound was determined by light (1-μm plastic sections) or scanning electron microscopic (SEM) examination of randomly selected corneas (data not shown). Following scarification, eyes were enucleated with a sterile scissors and placed into sterilized prepared culture wells containing 4.5 ml of Eagle minimal essential medium with Earle salts and L-glutamine (GIBCO) at room temperature (pH 7.2 to 7.4, 260 to 320 mosM). The corneal surface, oriented superiorly in the wells, was covered by a thin layer of minimal essential medium.

Adherence assay. A 5-μl bacterial cell suspension containing 5.0×10^7 CFU of *P. aeruginosa* ATCC 19660 was delivered onto the surface of each eye in culture by using a calibrated micropipette (Oxford Laboratories, Foster City, Calif.) with a sterile, disposable tip. All eyes were immediately placed in a water-jacketed CO_2 incubator (American Scientific Products, McGaw Park, Ill.) at 37°C and 5% CO_2 and cultured for 15, 30, or 60 min. The eyes were then rinsed vigorously in phosphate-buffered saline (PBS), fixed, dehydrated, and critical point dried in preparation for SEM observation. Prior to adherence testing, the integrity of this eye model was examined by SEM. Eyes were wounded as described above and cultured for up to 24 h. Such eyes cultured without antimycotics or antibiotics were free of exogenous bacterial growth for 8 h. At 12 h a few bacteria were seen, and at 24 h the eyes were covered with organisms of unidentified type (data not shown). Thus, the eyes were free from exogenous bacterial contamination for eight times the incubation period used for adherence testing.

Quantitation of adherence. Adherent bacteria were quantitated at 15, 30, and 60 min after ocular application by SEM procedures described previously (9, 10). In brief, the number of organisms in a microscopic field of a fixed size was observed. The field size was fixed by utilizing a consistent working distance of 25 mm, fixing the angle (30°) from which the sample was viewed, and using a uniform magnification of 3,000×. For each test, five randomly selected fields were photographed on each of three eyes and the negatives were enlarged to a total magnification of 6,000×. Counts were done with a square cut to measure 80 mm^2 at a magnification of 6,000×, and the data are expressed as the mean numbers of bacteria per square micrometer of scarred surface. This method has previously been shown to yield results which agree well with those obtained from parallel experiments using microbiological culture of infected eyes. (10).

Specimen preparation for SEM. Following incubation of the organ-cultured eyes with the bacteria, the eyes were vigorously rinsed with PBS (pH 7.5) to dislodge nonadherent organisms and immediately fixed at 4°C in a fixative containing 2.0% osmium tetroxide, 2.5% glutaraldehyde, and 0.1 M phosphate buffer (1:1:1; pH 7.5) for a total of 3 h, with a change to freshly prepared fixative after 1.5 h. The eyes were then dehydrated through graded ethanols to 100% and critical point dried in an AutoSamdri critical point dryer (Tousimis Research Laboratories, Rockville, Md.) with liquid CO_2 as the transition fluid. Eyes, with corneas superiorly oriented, were mounted on brass specimen stubs with colloidal silver paint suspended in methanol (Tousimis). A thin layer of gold (approximately 15 to 20 nm) was evaporated onto the surface of each specimen by using a Hummer II (Technics, Alexandria, Va.) sputter coater. The eyes were viewed with a JEOL JSM-840A SEM (JEOL Ltd., Tokyo, Japan) at the settings described above.

Enzyme treatment. Trypsin type III from bovine pancreas was purchased (Sigma Chemical Co., St. Louis, Mo.) and dissolved in PBS at a concentration of 20 μg/ml (1 μg = 10 U). Scarified eyes were exposed to trypsin for 5 and 10 min at 37°C and then subjected to bacterial adherence testing as described above. Control eyes were exposed to PBS for the same time and at the same temperature and similarly tested for bacterial adherence.

Lipase type XI from *Rhizopus arrhizus* was purchased (Sigma) and diluted with PBS to concentrations of 50,000 and 100,000 U/ml. The lipase used in this experiment was tested for contaminating protease activity with a Bio-Rad Laboratories (Richmond, Calif.) protease detection kit. In this system, diffusible proteases cause clear zones in the gel whose diameters are directly related to protease concentration (3). Protease from *Streptomyces griseus* (Sigma type XIV, 4 U/mg) and bovine pancreatic trypsin (Sigma type III, 10,000 U/mg) were used as standards for this assay. The gel was photographed after 1 h of incubation at room temperature. Slight contaminating protease activity was seen at a concentration of 100,000 U/ml and less at a concentration of 50,000 U/ml (Fig. 2). The scarified eyes in organ culture were incubated with either PBS (control) or each concentration of the lipase solution for 1 h at 37°C and were then rinsed thoroughly with PBS prior to topical application of the bacteria for adherence testing.

G_{M1} and asialo G_{M1}. A highly purified monosialoganglioside (G_{M1}) from bovine brain (Calbiochem-Behring, La Jolla, Calif; lot 801615) was used in adherence inhibition studies with the bacteria. The ganglioside was reported by Calbiochem to be greater than 98% pure by thin-layer chromatographic analysis. The preparation was mixed with PBS at concentrations of 500 μg/ml and 1 mg/ml. The bacteria were then either mixed directly with the ganglioside suspension for a brief period (5 min) prior to ocular application or were applied to scarified eyes that had been preincubated with the ganglioside for 1 h as described above for the lipase study. Eyes from each of these two groups were then incubated for 15, 30, and 60 min for bacterial adherence testing. Control eyes were incubated with bacteria mixed with PBS instead of the ganglioside or were preincubated with PBS for 1 h followed by topical application of the organisms in PBS. A similar series of experiments were performed with asialo G_{M1} purified from bovine brain (Sigma, lot 27F4011). The purity of this compound also was tested by the vendor and was reported to be 98% pure by thin-layer chromatography.

FN. Fibronectin (FN) from human plasma was purchased from Calbiochem (lot 801589). The purity of the compound

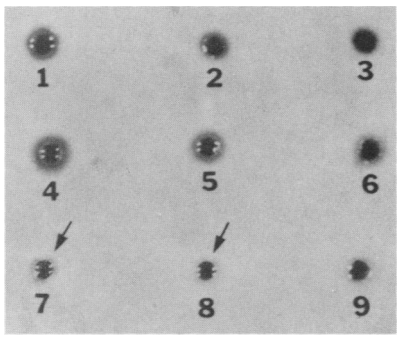

FIG. 2. Agar gel containing casein used for the detection of proteases. Wells 1 to 3 were filled with 9 μl of bovine pancreatic trypsin (Sigma) (6.25, 3.12, and 1.56 μg/ml, respectively) in PBS. Wells 4 to 6 contained 9 μl of protease from *S. griseus* (Sigma) (75, 37.5, and 18.7 μg/ml, respectively) in PBS. Wells 7 and 8 contained 9 μl of lipase (100,000 and 50,000 U/ml, respectively) in PBS. Well 9 was filled with 9 μl of PBS (pH 7.5; buffer control). The gel was photographed after 1 h of incubation at room temperature. Slight contaminating protease activity is seen for lipase at 100,000 U/ml (well 7, arrow) and less at 50,000 U/ml (well 8, arrow).

was tested by the vendor, and the preparation was reported to contain a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The glycoprotein also was described as having no contaminants as determined by immunoelectrophoresis with antifibronectin and anti-human sera. FN was similarly tested in the adherence assay model to determine whether it would significantly alter or inhibit bacterial adherence. FN (500 μg/ml) was diluted in PBS and preincubated for 1 h with the eyes in organ culture as described above for the lipase studies or was briefly mixed (5 min) with the bacterial inoculum prior to ocular application. Control eyes were preincubated in vitro with PBS for 1 h prior to adherence testing or received organisms directly mixed for 5 min with PBS prior to ocular application.

Statistical analysis. A paired Student's *t* test using separate estimates for the variance was used to test for significance of the data.

RESULTS

Enzyme treatment. Scarified mouse corneas in organ culture were treated with trypsin or lipase to determine whether the receptor molecule was a protein or a lipid. Scarified corneas were exposed to trypsin at a concentration of 20 μg/ml for 5 and 10 min at 37°C prior to testing for in vitro adherence of *P. aeruginosa*. No significant difference in adherence was noted between eyes incubated for 5 min with trypsin and control, PBS-treated scarified corneas. The longer time period (10 min) of trypsin exposure produced extensive cell lysis (data not shown). The adherence assay data obtained following 5 min of trypsin treatment of the scarified corneas are presented in Table 1.

Scarified corneas were preincubated with either PBS (Fig. 3A) or lipase (Fig. 3B) for 1 h at a concentration of 50,000 or 100,000 U/ml at 37°C prior to bacterial application for adherence testing. This treatment significantly reduced adherence at all time periods examined. The quantitative data from the lipase experiments are presented in Table 2.

G_{M1} and asialo G_{M1} treatment. Concentrations of 500 μg/ml and 1 mg/ml of purified G_{M1} were used to preincubate the scarified corneas for 1 h prior to adherence testing with the bacteria. This treatment significantly reduced the number of adherent bacteria at all time periods examined compared with corresponding control, PBS-treated scarified corneas (Table 3 and Fig. 3C). When the bacteria were premixed briefly for 5 min with 500 μg or 1 mg of G_{M1} per ml and were immediately applied to the scarified corneas, no significant difference in the numbers of adherent organisms was noted between G_{M1}-treated and control, PBS-treated scarified corneas for all time periods tested (Table 4).

Asialo G_{M1} (500 μg/ml) pretreatment of the scarified corneas for 1 h also had no significant effect on bacterial adherence. On the other hand, when the bacteria were premixed with asialo G_{M1} (500 μg/ml) for 5 min prior to incubation with the scarified corneas, bacterial adherence was reduced significantly at the 15-min interval compared with the corresponding PBS (control) mean. The data from the asialo G_{M1} experiments are presented in Table 5.

FN treatment. Preincubation of scarified corneas for 1 h with FN (500 μg/ml) resulted in a significant reduction of bacterial adherence at all time periods examined (Fig. 3D). When FN was mixed with the bacterial inoculum for 5 min prior to adherence testing, significantly decreased bacterial adherence was seen at 15 and 30 but not at 60 min. The adherence assay data for FN treatment of scarified corneas are presented in Table 6.

TABLE 1. Preincubation of scarified corneas with trypsin

Inoculum containing *P. aeruginosa*[a]	Bacteria per μm² at time (min)[b]:		
	15	30	60
PBS	7.53 ± 0.7	6.33 ± 0.4	4.62 ± 0.2
Trypsin	6.39 ± 0.5	7.69 ± 1.0	3.56 ± 0.2

[a] The 5-μl inoculum consisted of 5.0×10^7 CFU of bacteria. PBS or trypsin (20 μg/ml) was preincubated with the scarified eyes for 5 min before adherence testing.

[b] Randomly chosen SEM photomicrographs were used to count adherent organisms. Counts were done with a square cut to measure 80 mm² at 6,000×. Fifteen areas were observed for each treatment. Data are expressed as mean numbers of bacteria per square micrometer of scarred surface ± the standard error of the mean. A paired Student's *t* test using separate estimates for the variances showed that all the trypsin means were not significant compared with the PBS means.

DISCUSSION

Previous in vivo data have shown that binding of *P. aeruginosa* to the unwounded, mouse pup ocular epithelium is prevented and pup survival is significantly enhanced by sialic acid and/or neuraminidase treatment of the bacterial inoculum prior to its ocular delivery (9). Other work, with rat corneas in organ culture which were wounded with trephine with the basal lamina left intact (23) or rabbit corneal epithelial cells grown in culture (13), suggested that mannose is involved in initial binding of *P. aeruginosa* to corneas. In contrast, in vivo studies using adult mice whose corneas were scarified to a depth just below the basal lamina provided evidence that *N*-acetylmannosamine, the C_6 precursor of sialic acid, significantly decreases initial bacterial adherence (10). These data all provided information regarding sugar sequences which appeared efficacious in blocking bacteria binding but did not provide information about the molecular species to which the sugars attach. The present study extended our earlier in vivo work by employing an organ-cultured-cornea model to determine whether the ocular receptor molecule involved in *P. aeruginosa* adherence is a lipid or a protein. In this regard, scarified corneas incubated with trypsin and subjected to adherence assay testing

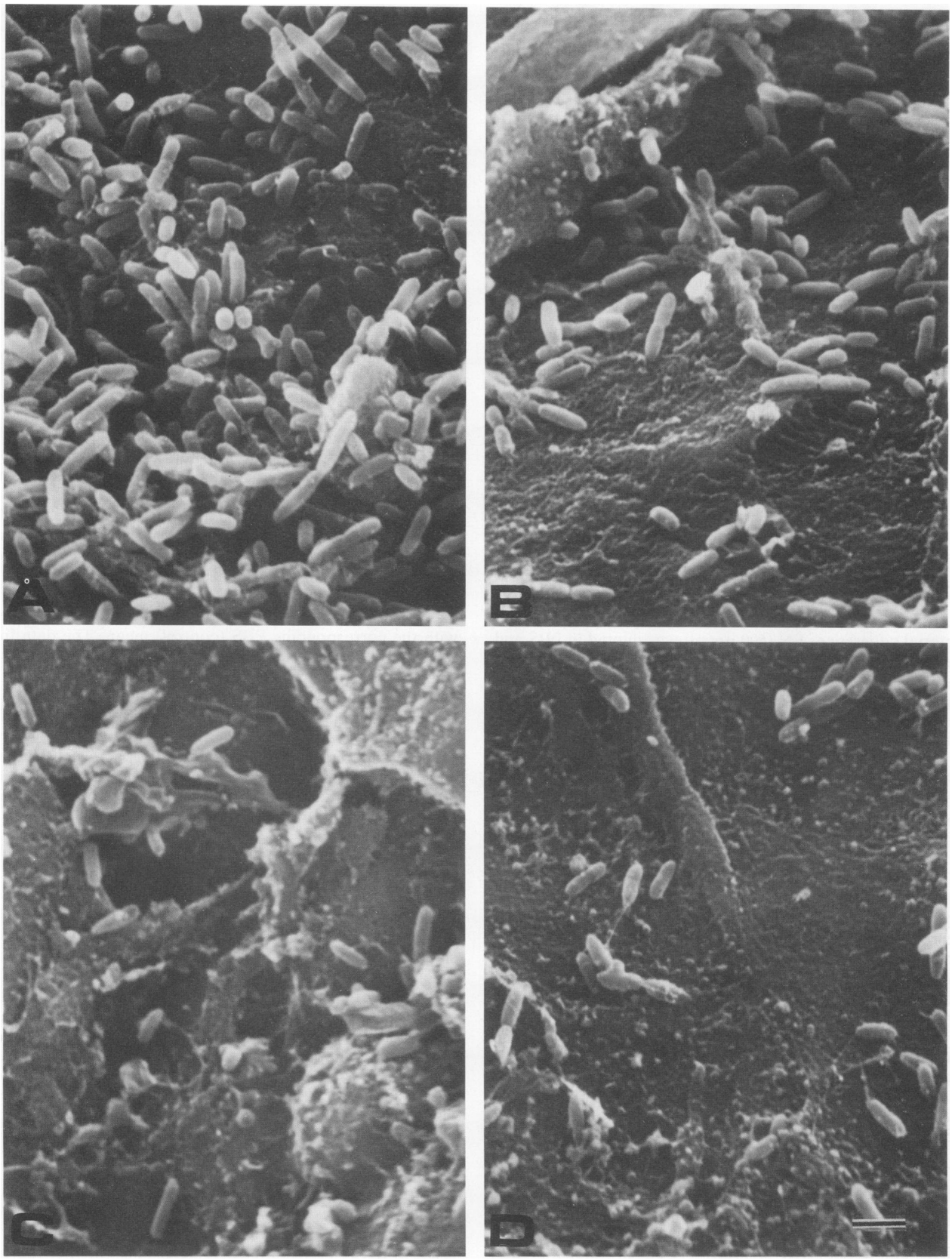

FIG. 3. SEM micrographs showing typical adherence of *P. aeruginosa* to the scarified corneal epithelial surface at 60 min after topical bacterial application. The eyes were preincubated with PBS (A), 50,000 U of lipase per ml (B), 500 μg of G_{M1} per ml (C), or 500 μg of FN per ml (D) for 1 h prior to bacterial application. Bar = 1.0 μm.

TABLE 2. Preincubation of scarified corneas with lipase

Inoculum containing *P. aeruginosa*[a]	Bacteria per μm^2 at time (min)[b]:		
	15	30	60
PBS	5.82 ± 0.3	5.20 ± 0.4	8.62 ± 0.4
Lipase (50,000 U/ml)	3.23 ± 0.3	3.47 ± 0.2	5.56 ± 0.5
PBS	4.44 ± 0.4	5.22 ± 0.4	6.27 ± 0.4
Lipase (100,000 U/ml)	1.15 ± 0.1	1.34 ± 0.1	1.69 ± 0.2

[a] The 5-μl inoculum consisted of 5.0×10^7 CFU of bacteria. The PBS or lipase (50,000 or 100,000 U/ml) was preincubated with the scarified eyes for 1 h before adherence testing.

[b] Randomly chosen SEM photomicrographs were used to count adherent organisms. Counts were done with a square cut to measure 80 mm^2 at 6,000×. Fifteen areas were observed for each treatment. Data are expressed as mean numbers of bacteria per square micrometer of scarred surface ± the standard error of the mean. A paired Student's *t* test using separate estimates for the variances showed that the lipase means all were significant ($P \leq 0.001$) compared with the corresponding PBS means.

showed no significant differences in bacterial binding compared with PBS control eyes at all time periods. However, exposure of the scarified eyes to lipase significantly decreased bacterial binding, providing evidence that a lipid, rather than a protein, molecule functions as the ocular receptor for binding of *P. aeruginosa* to the scarified cornea. Because of the slight contaminating protease activity in our lipase preparation, it is also remotely possible that the effect observed with lipase could be modulated by the presence of a contaminating protease capable of acting on a protein(s) which is not trypsin sensitive. Nonetheless, these results are in agreement with those of similar past work which used an in vitro acid-injured trachea model to examine *Pseudomonas* adherence; in that study a lipid was suggested as involved in bacterial binding (21). Unfortunately, in the latter study, the lipase used was not tested for contaminating proteolytic activity prior to use.

A more recent study with *P. aeruginosa* and *Pseudomonas cepacia* isolated from cystic fibrosis patients has demonstrated a preferred binding of both isolates to nonsialyated separated glycolipid standards (14). In that (14) and other work (15) employing thin-layer chromatographic procedures, both asialo G_{M1} and gangliotriaosylceramide (asialo G_{M2}) (14), as well as fucosylasialo G_{M1} (15), bound the bacteria equally well, whereas other glycolipids did not. Because the isolates did not bind to lactosylceramide, it was concluded that the β-*N*-acetylgalactosamine residues positioned internally in asialo G_{M1} and fucosylasialo G_{M1} and

TABLE 3. Preincubation of scarified corneas with purified G_{M1}

Inoculum containing *P. aeruginosa*[a]	Bacteria per μm^2 at time (min)[b]:		
	15	30	60
PBS	5.88 ± 0.4	8.58 ± 0.9	10.09 ± 0.9
G_{M1} (500 μg/ml)	2.52 ± 0.5	4.00 ± 0.5	3.13 ± 0.4
PBS	4.43 ± 0.5	6.44 ± 0.7	6.42 ± 0.8
G_{M1} (1 mg/ml)	1.41 ± 0.3	1.78 ± 0.4	1.56 ± 0.5

[a] The 5-μl inoculum consisted of 5.0×10^7 CFU of bacteria. PBS or G_{M1} (500 μg and 1 mg/ml) was preincubated with the scarified eyes for 1 h before adherence testing.

[b] Randomly chosen SEM photomicrographs were used to count adherent organisms. Counts were done with a square cut to measure 80 mm^2 at 6,000×. Fifteen areas were observed for each treatment. Data are expressed as mean numbers of bacteria per square micrometer of scarred surface ± the standard error of the mean. A paired Student's *t* test using separate estimates for the variances showed that the purified-G_{M1} means all were significant ($P \leq 0.001$) compared with the corresponding PBS means.

TABLE 4. Effect of purified G_{M1} on bacterial adherence to scarified corneas

Inoculum containing *P. aeruginosa*[a]	Bacteria per μm^2 at time (min)[b]:		
	15	30	60
PBS	6.72 ± 0.6	5.21 ± 0.5	3.82 ± 0.4
G_{M1} (500 μg/ml)	5.59 ± 0.8	3.77 ± 0.6	2.64 ± 0.2
PBS	1.96 ± 0.3	4.03 ± 0.5	7.44 ± 0.7
G_{M1} (1 mg/ml)	2.05 ± 0.2	5.08 ± 0.5	9.79 ± 0.7

[a] The 5-μl inoculum consisted of 5.0×10^7 CFU of bacteria. The PBS or G_{M1} (500 μg or 1 mg/ml) was combined with a bacterial suspension at room temperature (5 min) before adherence testing.

[b] Randomly chosen SEM photomicrographs were used to count adherent organisms. Counts were done with a square cut to measure 80 mm^2 at 6,000×. Fifteen areas were observed for each treatment. Data are expressed as mean numbers of bacteria per square micrometer of scarred surface ± the standard error of the mean. A paired Student's *t* test using separate estimates for the variances showed that none of the purified-G_{M1} means were significant compared with the corresponding PBS means.

terminally in asialo G_{M2} probably were minimal requirements for binding. Also, both isolates failed to bind to sialylated ganglioside G_{M1}, G_{M2}, or G_{D1a} fractions of separated glycolipid standards, suggesting that substitution of the glycolipids with sialosyl residues might prevent binding. These data agree with earlier results from our laboratory showing sialic acid prevention of bacterial binding to nonscarified, immature mouse corneas (9). Unfortunately, similar sialic acid treatment did not prevent binding in adult scarified eyes (10). Recent work from another laboratory also suggests binding of *P. aeruginosa* to nonsialylated neutral glycolipids of rabbit corneal epithelial cells grown in culture but not to the fraction containing the gangliosides (20). Glycosphingolipids also have been reported to be cell surface receptors for other pathogenic bacteria. For example, uropathogenic *Escherichia coli* isolates bind to Galα1-4Gal sequences in globoseries glycosphingolipids that are found in epithelial cells lining the urinary tract (17, 18).

The biological relevance of these data was tested in the current study by using an organ culture system similar to that described for the rat (23), which preserves the normal apical-to-basal stratified state of the corneal epithelium and the relationship between the epithelium and the basal lam-

TABLE 5. Effect of asialo G_{M1} on bacterial adherence to scarified corneas

Expt[a]	Inoculum containing *P. aeruginosa*[b]	Bacteria per μm^2 at time (min)[c]:		
		15	30	60
1	PBS	5.23 ± 0.5	9.39 ± 0.5	11.5 ± 0.9
	Asialo G_{M1} (500 μg/ml)	4.47 ± 1.0	10.27 ± 0.2	9.20 ± 0.5
2	PBS	7.3 ± 0.5	6.8 ± 0.8	6.21 ± 0.7
	Asialo G_{M1} (500 μg/ml)	1.78 ± 0.3	3.0 ± 0.5	4.56 ± 0.4

[a] In experiment 1, scarified eyes were preincubated with PBS or asialo G_{M1} (500 μg/ml) for 1 h before adherence testing. In experiment 2, the PBS or asialo G_{M1} (500 μg/ml) was mixed with a bacterial suspension before ocular application.

[b] The 5-μl inoculum consisted of 5.0×10^7 CFU of bacteria.

[c] Randomly chosen SEM photomicrographs were used to count adherent organisms. Counts were done with a square cut to measure 80 mm^2 at 6,000×. Fifteen areas were observed for each treatment. Data are expressed as mean numbers of bacteria per square micrometer of scarred surface ± the standard error of the mean. A paired Student's *t* test using separate estimates for the variances showed that only the 15-min mean for experiment 2 was significant ($P \leq 0.001$). All other means were not significant.

TABLE 6. Effect of FN on bacterial adherence to scarified corneas

Expt[a]	Inoculum containing *P. aeruginosa*[b]	Bacteria per μm^2 at time (min)[c]: 15	30	60
1	PBS	6.49 ± 0.3	7.14 ± 0.5	14.97 ± 0.6
	FN (500 μg/ml)	2.37 ± 0.3	2.65 ± 0.2	2.51 ± 0.2
2	PBS	3.12 ± 0.3	3.86 ± 0.4	2.41 ± 0.2
	FN (500 μg/ml)	1.26 ± 0.1	1.59 ± 0.2	1.96 ± 0.2

[a] In experiment 1, scarified eyes were preincubated with PBS or FN (500 μg/ml) for 1 h before ocular application. In experiment 2, the PBS or FN (500 μg/ml) was mixed with a bacterial suspension before application.

[b] The 5-μl inoculum consisted of 5.0×10^7 CFU of bacteria.

[c] Randomly chosen SEM photomicrographs were used to count adherent organisms. Counts were done with a square cut to measure 80 mm^2 at 6,000×. Fifteen areas were observed for each treatment. Data are expressed as mean numbers of bacteria per square micrometer of scarred surface ± the standard error of the mean. A paired Student's *t* test using separate estimates for the variances showed that all means for FN (experiment 1) were significant ($P \leq 0.001$) compared with PBS means. For experiment 2, only 15- and 30-min FN means were significant ($P \leq 0.001$); the 60-min mean was not significant.

ina. Such preservation of spatial relationships is not possible with cells in culture. In this system, when scarified corneas were incubated with G_{M1}, bacterial adherence was significantly reduced for 60 min. The molecular mechanism by which this occurs was not shown in the present study but may involve G_{M1} competition with or blocking of the ocular receptor required for bacterial recognition and binding. In support of this hypothesis, when G_{M1} was premixed with the bacterial inoculum prior to adherence testing, there was no significant effect on binding.

Preincubation of scarified adult mouse corneas with asialo G_{M1} also produced no significant change in bacterial adherence compared with PBS controls. These data suggest that tissue treatment with soluble asialo G_{M1}, a putative receptor molecule for *Pseudomonas* binding (14, 15), does not provide increased binding sites at the ocular surface, even at a high (1 mg/ml) concentration. In contrast, when this nonsialylated glycolipid was premixed briefly with the bacterial inoculum prior to incubation with the scarified corneas, binding was significantly blocked after 15 min of incubation. Although the inoculum was not rinsed prior to its application to the scarified cornea, it is not feasible to suggest that transfer of asialo G_{M1} influenced adherence, since preincubation of the tissue with the nonsialylated glycolipid failed to significantly alter bacterial binding. More than likely, when the bacteria were premixed with asialo G_{M1}, ligands at the bacterial surface (pili, outer membranes, or flagella?) recognized and bound its receptor, thereby decreasing available bacterial binding sites and thus reducing ocular binding. At later times of incubation, it is likely that turnover of the ligands on the organism occurred, providing for an increase in unblocked ligands on the bacterial surface capable of recognizing the native ocular surface receptor(s). These hypotheses are made more complex by reports that *P. aeruginosa* contains a cell surface lectin not in pili that binds to and agglutinates various red blood cells (7) as well as two other hemagglutinating lectins intracellularly (8).

Lastly, FN, a high-molecular-weight glycoprotein which binds to gangliosides (27) and has a binding site for gram-positive but not gram-negative organisms (5, 19, 22, 24, 28, 29), significantly reduced bacterial adherence following preincubation of scarified corneas. However, premixing of the bacterial inoculum with FN prior to incubation with the scarified corneas inhibited bacterial adherence only at 15 and 30 min. Since the bacterial inoculum was not rinsed following premixing with FN prior to its application to the ocular surface, it is possible that sufficient FN was transferred to the corneas in organ culture, providing transient blockage of the ocular receptor. Furthermore, since all sialic acid in human plasma FN is α2,6 linked to galactose (26), it is possible that the sialylated residues associated with this high-molecular-weight protein molecule are involved in inhibiting bacterial binding. Whether sialic acid is α2,6 or α2,3 linked does not appear to be of significance, however, since G_{M1}, which also blocks binding, contains sialic acid-galactose in an α2,3 linkage (15). Whatever the mechanism, these data also support a previous study (21) which demonstrated that FN, perhaps by binding to G_{M1}, blocks the binding of *P. aeruginosa* to the acid-injured tracheal epithelium in vitro. In another model system (30), adherence of *P. aeruginosa* to buccal epithelial cells in vitro was shown to coincide with the loss of FN from the cell surface, and further, the loss of the glycoprotein appeared to be induced by proteases. This model differs somewhat from the cornea model described in our study, since corneal epithelial preparations obtained from organ-cultured normal or wounded mouse corneas, electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, and passively transferred to nitrocellulose paper for immunoblot analysis failed to reveal the presence of FN either before or after wounding (L. D. Hazlett, unpublished data).

Based on these data, it is clear that the interactions of *P. aeruginosa* at the corneal surface are complex and that sialic acid-containing glycolipids or the presence of the sialylated glycoprotein FN significantly decrease *Pseudomonas* binding to the scarified corneal epithelium. The in vivo biological significance of these data will require further evaluation and study in order to determine a possible therapeutic relevancy.

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