In the Immature Mouse, *Pseudomonas aeruginosa* Pili Bind a 57-kd (α2–6) Sialylated Corneal Epithelial Cell Surface Protein: A First Step in Infection

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**Purpose.** To test the hypothesis that in the unscarified immature eye, *Pseudomonas aeruginosa* pili bind glycoprotein receptors, one or more of which are surface associated.

**Methods.** Several methods—including radioiodination of bacterial pili and surface-associated corneal epithelial proteins (CEPs), solid-phase binding assays, carbohydrate detection, and immunoblotting techniques in which periodate oxidation and preincubation of blots with purified pili, neuraminidase, sialic acid, other sugars, and SNA and MAA lectins—were used to identify and characterize host proteins. Some of these proteins in the immature mouse corneal epithelium interacted with bacterial pili.

**Results.** Seven proteins, with molecular weights from 14 to 66 kd were identified that strongly bound PAK/PR1 pili. To determine if any protein(s) was cell surface localized, corneal epithelial surface membrane proteins were radioiodinated and examined using a pilus overlay assay and lectin analysis. Only one protein of 57 kd was cell surface labeled and bound pili in an overlay assay. This protein was α(2–6) sialylated, as shown by SNA binding. Furthermore, SNA lectin was able to block pilus binding to CEPs. Labeling of pilus and a solid-phase binding assay confirmed that pili bind to CEPs and, further, that binding could be competitively inhibited by excess unlabeled pilus and that the receptors appeared saturable. Carbohydrate reagents were used to show that the epithelial proteins of the postnatal day 5 (P5) mouse cornea were glycosylated. Removal of carbohydrates by preincubation of blots with periodate, neuraminidase, sialic acid, or preincubation of blots with sialic acid, eliminated pili binding. Pretreatment of blots with either neuraminidase (N'ase) to decrease and/or remove sialic acid residues, or pretreatment with SNA lectin with specificity for α(2-6) linked sialic acid to galactose, also diminished pili binding to CEPs. Other sugars or MAA lectin, specific for sialic acid α(2–3) linked to galactose, had no inhibitory effect.

**Conclusions.** These data show that a 57-kd surface membrane protein bound pili in the immature cornea and that for both this protein and the other nonsurface proteins, sialic acid α(2–6) linked to galactose was important in receptor recognition by the pilus adhesin. The 57-kd protein is putatively important in the initial interaction of pili with the unwounded ocular epithelium and may be the initial pathogenic event in this model. Invest Ophthalmol Vis Sci. 1995;36:634-643.

*Pseudomonas aeruginosa,* the most common cause of corneal bacterial ulcers, binds to adult scarified and immature unwounded corneal epithelium. The importance of this interaction as a prerequisite for colonization and infection is well established for various tissues, but the host receptors that interact with pili are only recently characterized. Because much is known about the pilus adhesin of *P. aeruginosa,* including data that have suggested its binding domain resides in the C-terminal region of the pilin subunit, the objective of the current study was to identify and characterize the host receptors for pilus adhesion at the unwounded corneal epithelial surface. This model was chosen to investigate because it provides an experimental system to decipher the events that may occur in microbial keratitis in infancy and early childhood, particularly in those patients in whom an immunocompromised state was identified as contributing to the onset of corneal disease.
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article, corneal epithelial proteins (CEPs) were identified that bound pili in an overlay assay using a pilus-specific monoclonal antibody (mAb) XLR-3. Only one protein was unequivocally cell surface associated and contained α(2-6) linkages of sialic acid to galactose. Furthermore, preincubation of blots with SNA lectin, which detects such a linkage, decreased pilus binding and was concentration dependent. Results from solid-phase binding assays confirmed that corneal epithelial proteins bind pilus and that binding sites are competed for by the addition of excess unlabeled pili, and they suggested that the receptors were saturable. Carbohydrates were determined to be important for pilus receptor activity because periodate oxidation of blots, preincubation of pili with sialic acid, and N’ase treatment of blots all decreased binding.

MATERIALS AND METHODS

Pili Purification

P. aeruginosa strain PAK/PR1 were cultured as described, and pili were purified according to the method of Frost and Paranchych, also as described. The purity of the pilus preparations was determined by transmission electron microscopy and 13% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Pili-associated endotoxin was decreased to an acceptable level (=1 endotoxin U/mg) with a Detoxi-Gel affinity column (Pierce Chemical, Rockford, IL) as described. The method to produce mAb XLR-3 for this study also has been described in detail. The mAb XLR-3 was isotypes as an IgG2b antibody, and its specificity was determined by Western blot analysis.

Preparation of P5 Mouse Eyes

Midterm pregnant female Swiss (ICR) mice were purchased from Harlan Sprague–Dawley (Indianapolis, IN), housed individually in clear plastic cages, and fed laboratory enriched rodent chow (Ralston–Purina, St. Louis, MO). The pregnant females were observed twice each day to record the exact day of delivery. Mice were classed as postnatal day 1 (P1) on the day of birth. On the day after delivery, litters were standardized to no more than 11 pups each to ensure animal health and homogeneity of size among litters. At P5, each mouse pup was rapidly and humanely killed by decapitation. Subsequently, the skin of the fused eyelids was excised to expose the eye, and right and left eyes were enucleated, placed into phosphate-buffered saline (PBS) containing 20 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2 to 7.4, and incubated for 3 hours at 37°C to remove the corneal epithelium. Corneal epithelial sheets were teased from the underlying stroma with forceps and solubilized in Laemmli sample buffer (0.0625 M Tris-HCl, pH 6.8, 2.0% SDS, and 10.0% glycerol) without 2-mercaptoethanol as described. All animals were cared for in accordance with the guidelines established by the National Institutes of Health (DHEW publication number 85-23, 1985), and we complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Gel Electrophoresis and Immunoblotting

The discontinuous SDS–PAGE system described by Laemmli was used with 10% acrylamide gels. Before electrophoresis, 2-mercaptoethanol (5.0% in final concentration; Bio-Rad, Richmond, CA) was added to samples, and 6.65 µg of protein was loaded onto each lane on the gel. Gel electrophoresis was conducted at 20 mA/gel at constant voltage using the Bio-Rad Mini Protein II Dual Slab Cell (Bio-Rad). The electrophoretically separated material was transferred to nitrocellulose paper (pore diameter 0.2 µm; Schleicher & Schuell, Keene, NH) at 100 V at constant voltage for 1 hour at 4°C using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad).

After transfer, blots were washed with 50 mM Tris-buffered saline (TBS), pH 7.5, with 0.1% Tween 20 (TTBS) for 3 hours, changing to fresh TTBS every hour. Nitrocellulose-blotted CEPs were stained with 20 µl of AuroDye Forte solution (Amersham International, Amersham, UK) for 2 hours to allow visualization of the transferred proteins. The blots were then rinsed with distilled water and allowed to dry. Immunoblotting was performed as described previously. In brief, blots were blocked for nonspecific binding with 5% bovine serum albumin (BSA, essentially fatty acid and globulin free; Sigma, St. Louis, MO) in 50 mM TBS, pH 7.5, placed in an Orbital water bath shaker (Lab-Line Instruments, Melrose Park, IL) at 37°C and at 100 rpm overnight. All subsequent steps were performed at 37°C and at 100 rpm. Blots were washed three times with PBS for 10 minutes each, incubated with purified PAK/PR1 pili (100 µg/ml) or PBS (control) for 2 hours, and washed three times with TTBS. Next, the blots were incubated with mAb XLR-3 (25 µg/ml) in 1% normal rabbit serum in TTBS for 1 hour, followed by three washes with TTBS. A goat anti-mouse IgG (heavy and light chain specific) — alkaline phosphatase conjugate (Cappel, Organon Teknika, Durham, NC) 1:3000 dilution in TTBS with 1% normal goat serum — was added and incubated for 1 hour, and blots were washed three times with TTBS and once with PBS. Color development was initiated using 0.3 mg of p-nitro blue tetrazolium chloride per milliliter, (0.15 mg of 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt per milliliter [Bio-Rad]) in 0.1 M sodium bicarbonate buffer, 1.0 mM magnesium chloride [Sigma], pH 9.8); develop-
ment was stopped by rinsing the blots in distilled water.

Iodination of Corneal Epithelial Surface Membrane Proteins and Pili

Radiiodination of surface membrane proteins was performed using an Enzymobead Radiiodination reagent (Bio-Rad). Enzymobeads were rehydrated with 0.5 ml distilled water at least 1 hour before use. Afterward, 135 µl of the iodination mixture (50 µl Enzymobeads, 25 µl of 1% β-D-glucose [Sigma], 10 µl of 1.0 mCi Na[125]I [ICN Biomedicals, Irvine, CA], and 50 µl of 0.2 M phosphate buffer, pH 7.2) was added to five enucleated P5 eyes per microcentrifuge tube. Iodination was allowed to proceed at room temperature for 30 minutes with agitation every 5 minutes. Eyes were then washed three times with PBS, 7.5, and placed in PBS–EDTA buffer to incubate for 3 hours to allow removal of corneal epithelium from each eye. Corneal epithelial sheets were homogenized in 30 µl of Laemmli sample buffer with 2-mercaptoethanol, and the sample was loaded onto a 10% SDS–PAGE gel for electrophoresis as described above. Parallel control eyes were similarly treated but were incubated in radioiodination mixture lacking Enzymobeads. The resultant gel was electrophoretically transferred onto nitrocellulose, and the transferred proteins were autoradiographed using Kodak Diagnosis Film X-Omat (Eastman Kodak, Rochester, NY) at room temperature overnight. The same transferred proteins were used for overlay assay with pili as described, as well as for binding and inhibition assays with lectins as described in Lectin Analysis.

For solid-phase binding assays, pili were labeled with [125]I using the solid-phase reagent tetrachlorodipurified pili protein in 400 µl intactness of the Iodogen film. Approximately 500 µg were added to 0.5 ml distilled water at least 1 hour before use. Afterward, 135 µl of the iodination mixture (50 µl Enzymobeads, 25 µl of 1% β-D-glucose [Sigma], 10 µl of 1.0 mCi Na[125]I [ICN Biomedicals, Irvine, CA], and 50 µl of 0.2 M phosphate buffer, pH 7.2) was added to five enucleated P5 eyes per microcentrifuge tube. Iodination was allowed to proceed at room temperature for 30 minutes with agitation every 5 minutes. Eyes were then washed three times with PBS, 7.5, and placed in PBS–EDTA buffer to incubate for 3 hours to allow removal of corneal epithelium from each eye. Corneal epithelial sheets were homogenized in 30 µl of Laemmli sample buffer with 2-mercaptoethanol, and the sample was loaded onto a 10% SDS–PAGE gel for electrophoresis as described above. Parallel control eyes were similarly treated but were incubated in radioiodination mixture lacking Enzymobeads. The resultant gel was electrophoretically transferred onto nitrocellulose, and the transferred proteins were autoradiographed using Kodak Diagnosis Film X-Omat (Eastman Kodak, Rochester, NY) at room temperature overnight. The same transferred proteins were used for overlay assay with pili as described, as well as for binding and inhibition assays with lectins as described in Lectin Analysis.

To examine the ability of labeled pili protein to bind to epithelial glycoproteins, a solid-phase binding assay was used. For these assays, 50 µl of P5 mouse CEP sample was added to each experimental well (10 µg/well) of a 96-well plate (Falcon 3912-micro test III, Becton Dickinson, Oxnard, CA). Control wells were similarly coated but with an irrelevant protein, BSA (10 µg/ml). The plate was covered with parafilm and incubated overnight (4°C), and the next day excess buffer was removed by rapidly inverting the plate. Nonspecific binding sites were blocked by adding TBS-BSA buffer (0.05 M Tris-HCl, pH 7.8, containing 0.15 M sodium chloride and 1% BSA) and incubating for 2 to 3 hours at room temperature. Afterward, the buffer was removed. For competition and determination only of saturation experiments (the latter described in the next paragraph), wells were treated with a 10-fold excess of unlabeled pili protein (10 µg volume) for 30 minutes before the addition of [125]I-labeled protein (30 µg volume; activity of 1.0 × 10⁶ counts per minute). The plate was covered and incubated overnight at room temperature. The next day, the radioactive solution was removed, and the wells were washed five times with cold PBS. Individual wells were cut out, and radioactivity was counted in a gamma counter (CliniGamma 1272; LKB-Wallac, Turku, Finland).

To determine if the glycoprotein receptors could be saturated, 96-well plates were treated essentially as described in the preceding paragraph, including overnight incubation at 4°C and blocking of nonspecific binding sites with TBS-BSA. However, instead of using only one concentration of [125]I-labeled pili protein, the epithelial samples were incubated with several concentrations of radiolabeled pili. Nonspecific binding was determined in the presence of an excess amount of unlabeled pili protein as described in the preceding paragraph. After overnight incubation with labeled pili, the radioactivity was removed, wells were washed with cold PBS, and incorporated radioactivity was measured as described above.

Carbohydrate Detection

Carbohydrates associated with epithelial proteins were detected using a GlycoTrack Carbohydrate Detection kit from Oxford GlycoSystems (Rosedale, NY). Blots were washed with PBS for 10 minutes and incubated in 0.1% sodium periodate in sodium acetate–EDTA buffer for 20 minutes in the dark at room temperature. The blots were washed three times with PBS (10 minutes per wash), incubated with biotin-hydrazide solution (2 µl biotin–hydrazide to 10 ml sodium acetate–EDTA) for 60 minutes, washed three times with TBS, and blocked with 0.5% nonfat dry milk for 30 minutes. After washing three times with TBS, blots were incubated with streptavidin–AP conjugate (S-AP, 636 Investigative Ophthalmology & Visual Science, March 1995, Vol. 36, No. 3
were incubated with N'ase for 20 minutes at room temperature, followed by incubation in the same buffer for 1 hour. Experimental blots were incubated with various concentrations of periodate (0 to 30 mM) in 50 mM sodium acetate buffer, pH 4.5, for 1 hour in the dark, at room temperature. Control blots were similarly incubated, but in sodium acetate buffer, pH 4.5 alone. All blots were rinsed with sodium acetate buffer and incubated with 50 mM sodium borohydride (Sigma) in PBS for 30 minutes in the dark at room temperature, rinsed six times with PBS, and assessed by immunoblot for pili binding as described.

**Effect of Sugars on Pili Binding**

N-acetylglucosamine, N-acetylgalactosamine, methyl A-D-mannopyranoside, L-fucose (40 mg/ml, all from Sigma) and sialic acid (250 µg/ml; 1, 10, and 40 mg/ml, Sigma) were individually preincubated with pili (100 µg/ml) at 37°C for 1 hour before incubation with the protein blots. When combined with PBS, all the sugars except sialic acid were in a neutral pH range from 6.35 to 7.37; sialic acid was adjusted to neutrality, pH 7.5, with 2 N NaOH. Control blots were preincubated with PBS at 37°C for 1 hour. As an additional control for sialic acid adjusted to neutral pH with 2 N NaOH, pili were incubated with PBS that had first been acidified with HCl, pH 2.0, and adjusted to neutrality at pH 7.5 with NaOH. This was done to ensure that the effects observed with sialic acid were not due to adjusting this sugar from acid to neutral pH. Immunoblots were prepared and color development was performed as described.

**Neuraminidase (N'ase) Treatment of Blots**

N'ase, type V, from Clostridium perfringens was purchased from Sigma (lot 39F-8015) and diluted in PBS to a concentration of 2.2 U/ml. Before its experimental use, it had been tested and found free of contaminating protease activity using a Bio-Rad Laboratories protease determination kit (data not shown). Blots were incubated with N'ase for 20 minutes at room temperature, washed three times with PBS, and used for immunoblot or lectin blot analysis.

**Lectin Analysis**

For identification of sugar linkages in CEPs, blots were incubated with 3% BSA (Sigma) for 2 hours. After washing twice with PBS, blots were incubated with either digoxigenin-labeled Maackia amurensis (MAA, 5 µg/ml, specific for sialic acid linked α[2–3] to galactose; Boehringer Mannheim Biochemica, Indianapolis, IN) or digoxigenin-labeled Sambucus nigra (SNA, 1 µg/ml, specific for sialic acid linked α[2–6] to galactose; Boehringer Mannheim Biochemica) for 1 hour each according to the dilution recommended by the manufacturer. Blots were washed three times with TBS and then incubated with anti-digoxigenin-AP (0.75 U/ml; Boehringer Mannheim Biochemica) for 1 hour. AP color development was carried out as described.

For examination of the effects of lectins on pili binding, blots were blocked with 3% BSA (Sigma) overnight at 37°C and with shaking at 100 rpm. They were washed twice with TTBS and once with lectin buffer (0.15 M sodium chloride, 0.01 M manganous chloride, 0.05 M Tris-HCl, 0.01 M calcium chloride, and 0.05% Tween 20, pH 7.0, Sigma). Blots were incubated with lectin buffer (controls) or SNA or MAA lectin (200 or 500 µg/ml in lectin buffer) at 37°C for 1 hour with shaking at 100 rpm. The blots were washed twice with lectin buffer and once with PBS and were then assessed for pilin binding as described.

**RESULTS**

**Binding of Pili to CEP Blots**

An Aurodye stain was used to visualize electrophoretically transferred proteins after loading of 6.65 µg protein/lane and electrophoresis on 10% SDS gels. Numerous epithelial proteins were stained intensely using this procedure (Fig. 1, lane 2). Using similarly blotted CEPs and a pilus overlay assay, strong binding was seen for protein bands with approximate molecular weights of 14, 31, 33, 38, 45, 57, and 66 kd after immunoblotting with mAb XLR-3 specific for PAK/PR1 pili (Fig. 1, lane 3). Minor protein bands (e.g., 32 and 97 kd) also interacted with pili using this procedure (Fig. 1, lane 4). The staining intensity for these proteins was less than for proteins at either 33 or 66 kd, for example. No protein bands were identified by mAb XLR-3 in the PBS control blot in which PBS rather than an overlay of pili protein was used in the first incubation step (Fig. 1, lane 4).

**Iodination of CEPs and Pili**

SDS gel (10%) electrophoresis of CEPs also was performed after incubation of the whole eye with an Enzymobead and ¹²⁵I radioiodination mixture to label surface plasma membrane-associated proteins of the corneal epithelium. After removal of the epithelium, SDS gel electrophoresis and transfer of the proteins to nitrocellulose, the blotted proteins were analyzed by autoradiography. As shown in Figure 2, lane 2, little to no cell surface labeling was observed when Enzymobeads were omitted from the reaction mixture. Although
FIGURE 1. Identification of epithelial proteins that bind *Pseudomonas aeruginosa* pili by immunoblotting. Lane 1 = Ponceau S-stained blot of low molecular weight (LMW) markers (Bio-Rad) in kd; lane 2 = Aurodye-stained nitrocellulose transfer of P5 CEPs; lane 3 = nitrocellulose transfer of P5 CEPs overlayed with PAK/PR1 pili and incubated with anti-PAK/PR1 mAb XLR-3 as described in Materials and Methods. Arrowheads indicate major reactive proteins; 4, control for overlay binding shown in lane 3 in which PAK/PR1 pili were omitted from the overlay procedure.

Several cell surface proteins were identified with the complete reaction mixture (Fig. 2, lane 3), a single surface membrane associated CEP of 57 kd also bound pili in a subsequent pili overlay assay (Fig. 2, lane 4). A duplicate blot was reacted with the lectin SNA and demonstrated that the 57-kd protein contained sialic acid α(2–6) linked to galactose (Fig. 2, lane 6).IODination of the 57-kd CEP did not interfere with pilus binding (compare Fig. 2, lanes 4 and 5). When preincubated with a duplicate blot before pilus overlay, SNA lectin at a concentration of 500 μg/ml (Fig. 2, lane 7) but not at 200 μg/ml (data not shown) blocked pilus binding. MAA lectin, which distinguishes sialic acid α(2–3) linked to galactose, was similarly used and had no effect on pilus binding at either concentration (data not shown).

Pili labeled with 125I were used in a solid-phase binding assay27 to test further receptor–ligand interactions between pili and host proteins. Labeled pili bound to CEP-coated wells significantly above control, BSA-coated well levels, and binding sites were effectively competed for by the addition of a 10-fold excess of unlabeled (cold) pili (Fig. 3). Use of the same assay system suggested that the corneal protein receptors were saturable (Fig. 4).

Glycoprotein Staining, Periodate Oxidation, Monosaccharides, and N′ase

GlycoTrack reagents were used to determine if the proteins of the corneal epithelium routinely displayed by gel electrophoresis and blotting to nitrocellulose were glycoproteins. This technique showed that many proteins of the P5 corneal epithelium were glycosylated (Fig. 5). To determine if carbohydrates participated in the receptor–adhesin interactions between bacterial pili and host proteins, blots were oxidized by mild periodate treatment followed by borohydride reduction. Pilus binding activity was significantly decreased when blots were treated with 10 mM periodate.
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FIGURE 4. Saturation curve of $^{125}$I labeled PAK/PR1 pili to P5 glycoprotein receptors. Increasing concentrations of $^{125}$I labeled pili were added to wells coated with P5 CEPs (10 μg/well). Nonspecific binding was determined in cell wells incubated with labeled pili in the presence of 10-fold excess of unlabeled pili. The experiment was repeated three times, and data from a typical assay are shown.

and was eliminated entirely at 30 mM (Fig. 6, lanes 3 and 4, respectively). Aurodye staining of a periodate-treated CEP blot showed that the proteins remained after carbohydrate removal (Fig. 6, lane 5).

Because periodate treatment completely inhibited pili binding, several sugars were tested individually for their ability to block pili binding to CEPs. Sialic acid alone inhibited pili binding to CEP blots, and the inhibitory effect was concentration dependent. Preincubation of blots with 1 mg/ml sialic acid significantly inhibited pili binding (Fig. 6, lane 6), whereas incubation of blots with 250 μg/ml sialic acid (data not shown) had no effect. At a concentration of 10 (Fig. 6, lane 7) or 40 (data not shown) mg/ml, sialic acid eliminated pili binding to CEP blots. Other sugars tested, including N-acetylgalactosamine, N-acetylglucosamine, methyl α-D-mannopyranoside, and L-fucose, all at a concentration of 40 mg/ml, had no inhibitory effect on pili binding (data not shown).

Adjustment of the pH of PBS to acidic with 1 N HCl and then to basic with 2 N NaOH did not affect pili binding, and blots treated in this manner appeared the same as routine PBS control blots (data not shown).

Pretreatment of the CEP blots with 2.2 U/ml N’ase, which removes and/or decreases sialic acid residues, also decreased the ability of pili to bind to CEPs (Fig. 7, lane 3). Because sialic acid was the only sugar capable of completely inhibiting pili binding to CEPs and because N’ase treatment also reduced binding, two lectins, MAA and SNA, that specifically recognize sialic acid residues linked α(2-3) or α(2-6) to galactose, respectively, were used to analyze binding of these lectins by CEPs and to establish that binding could be significantly decreased with the removal or decrease of sialic acid by N’ase. MAA lectin bound to only a few proteins of molecular weights greater than 66 kd on nitrocellulose blots of 10% SDS gels (Fig. 8, lane 2). After N’ase treatment of the blots, no binding of MAA was detected (Fig. 8, lane 3). SNA lectin bound a number of proteins of molecular weights ranging from 14 to greater than 97 kd (Fig. 8, lane 4), and N’ase treatment of the blots also significantly decreased binding for SNA (Fig. 8, lane 5).

FIGURE 6. Analysis of pili binding to CEPs after periodate oxidation or incubation of pili with sialic acid before pili overlay assay. Lane 1 = Ponceau S-stained blot of LMW markers in kd; lane 2 = pili binding to control immunoblot (no periodate treatment); lane 3 = pili binding to CEP blot treated with 10 mM periodate; lane 4 = pili binding to CEP blot treated with 30 mM periodate; lane 5 = 10 mM periodate-treated blot stained with Aurodye to show that the proteins are not degraded by periodate treatment; lanes 6 and 7 = pili preincubated with 1 mg/ml (6) or 10 mg/ml (7) of sialic acid before pili overlay assay. Arrowheads indicate pili-binding proteins.

FIGURE 5. Detection of epithelial glycoproteins. Lane 1 = Ponceau S-stained blot of LMW markers in kd; lane 2 = nitrocellulose blot of P5 CEPs reacted with GlycoTrack reagent for the detection of carbohydrate residues.

FIGURE 7. Detection of sialic acid residues after treatment with 2.2 U/ml N’ase. Lane 1 = control blot; lane 2 = N’ase-treated blot.
FIGURE 7. Analysis of pilus binding to CEP blots after N'ase treatment or preincubation of the blots with 500 /ig/ml SNA lectin before pilus overlay assay. Lane 1 = Ponceau S-stained nitrocellulose blot of LMW markers in kd; lane 2 = pilus binding to control immunoblot (no N'ase treatment); lane 3 = pilus binding to blot treated with 2.2 U/ml N'ase; lane 4 = blot preincubated with 500 /ig/ml SNA lectin before pilus overlay assay. Arrowheads indicate pilus-binding proteins.

MAA and SNA lectins also were used in a binding inhibition assay to determine if these sialic acid-specific lectins could compete with and inhibit pilus binding to epithelial proteins. Preincubation of blots with SNA lectin (at 500 /ig/ml but not at 200 /ig/ml concentration) decreased pilus binding (Fig. 7, lane 4), whereas similar use of MAA lectin did not (data not shown).

DISCUSSION

P. aeruginosa adheres to the unwounded cornea of the immature mouse,2-5 but in the adult, bacterial adherence requires prior corneal scarification.2-4 Hypothetically, as in microbial keratitis in childhood versus in adults,20 different mechanisms operate to ensure these different bacterial adhesin-host receptor interactions. In this regard, results from this laboratory6 have suggested that sialic acid may be an ocular receptor for P. aeruginosa adherence in vivo in the immature mouse cornea. Other studies have shown that the corneal receptor(s) for P. aeruginosa in the immature mouse are enhanced by trypsin treatment and that the receptors exposed by trypsin are not lipase sensitive, indicating that host proteins are important for bacterial binding to immature mouse cornea.10 In the adult animal, bacterial binding to the scarified cornea also is enhanced by trypsin treatment, but, in contrast to the immature eye, exposed receptors are lipase sensitive.31 Recent evidence suggests that asialo GM1, representative of a class of neutral glycolipids, is a lipase-sensitive receptor.32 In addition, it has been shown recently that several corneal epithelial glycoproteins are host-receptor components for pilus-mediated bacterial binding to adult mouse scarified cornea.21 Results using that model, as well as bovine corneal epithelium from which a sufficient quantity of neutral glycolipids for analysis could be extracted, provided further evidence that the pilus adhesin and endotoxin, but not flagella, bind to asialo GM1 in the corneal epithelium.33

In the current study, we have determined that there are proteins in the unwounded immature mouse cornea to which pili bind. Results show that seven proteins (approximate molecular weights: 14, 31, 33, 38, 45, 57, and 66 kd) from the P5 mouse cornea bind purified pili, as revealed by pilus overlay assay and immunostaining with mAb XLR-3. Because the P5 mouse2 and human infant cornea require no wounding to establish an experimental or clinical infection, respectively, it was critical to determine whether the pilus adhesin bound to CEPs, which were surface plasma membrane associated. Radioiodination of surface membrane proteins of corneal epithelium using an Enzymobead and 125I radioiodination mixture showed that only one protein was surface plasma membrane associated and that this same protein also bound pilus in an overlay assay. Furthermore, this same 57-kd protein was glycosylated; it bound the lectin SNA, which recognizes sialic acid α(2→6) linked to galactose. The importance of this linkage in adherence to this same protein also was established because incubation of blots with SNA lectin prevented pilus binding in a concentration dependent manner to the 57-kd protein. Other nonsurface-associated proteins also bound pilus. Events that follow the initial binding of pilus to the 57-kd surface protein and that allow this to occur have not been established experimentally. However, in vivo results of a scanning electron microscopic study7 have shown previously that P. aeruginosa bind to the P5 epithelial proteins.

FIGURE 8. Binding of lectins MAA and SNA to P5 epithelial proteins. Lane 1 = Ponceau S-stained blot of LMW markers in kd; lane 2 = MAA binding to P5 CEPs; lane 3 = MAA binding after treatment of blot with 2.2 U/ml N'ase; lane 4 = SNA binding to P5 CEPs; lane 5 = SNA binding after treatment of blot with 2.2 U/ml N'ase.
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corneal surface within 5 minutes of inoculation beneath the fused eyelid with no cytopathology visible until at least 10 minutes later. Hypothetically, during that time bacterial and/or host proteases cytopathic to host cells are elaborated, resulting in exposure of intracellular proteins and allowing additional binding interactions to occur, as shown in the current study.

Several carbohydrates, including sialic acid,
N-acetylmannosamine, N-acetylgalactosamine, and L-fucose, have been implicated as receptor components for *P. aeruginosa*. Recent examination of the glycosylation profiles of pilus-binding proteins in the adult mouse corneal epithelium also showed that carbohydrates may be required for receptor activity. As a logical extension of the latter studies, and to test the hypothesis that carbohydrates also are of importance in pilus binding in the unwounded immature mouse eye, periodate oxidation was performed to determine if carbohydrate removal decreased pilus binding. Inhibition of pilus binding was dependent on the concentration of periodate used, and complete loss of pilus binding was seen at a periodate concentration of 30 mM, indicating strongly that the carbohydrate portions of the P5 CEPs are required for receptor activity. These data agree well with other studies, showing that similar periodate oxidation treatment of adult mouse CEP blots or human buccal epithelial blots eliminates subsequent pseudomonal pilus binding. However, in competitive inhibition experiments using several different sugars, only preincubation of pili with sialic acid, but not the other sugars, completely inhibited pilus binding to CEP blots and was concentration dependent. These results are consistent with the documented presence of sialic acid as an important constituent of surface plasma membrane glycoproteins and subcellular membrane fractions. It is present in all fractions of cultured cells and tissue extracts. In addition, these findings substantiate earlier work from this laboratory showing that sialic acid inhibited *P. aeruginosa* binding to the P5 cornea in vivo and in vitro. One of these and other such studies, sugars also have been shown to lessen or prevent infection. For example, Aronson et al showed that methyl-$\alpha$-D-mannopyranoside reduced the extent of infection of the urinary tract of the mouse by *Escherichia coli*. Similarly, others found that globotetraose (containing Gal-$\alpha$1-4 Gal residues) could prevent the colonization of a P-fimbriated bearing strain of *E. coli* onto mouse urinary tract tissue. Hazlett et al showed that in the P5 mouse eye, saturation of binding sites on the surface of *P. aeruginosa* with sialic acid not only prevented attachment to the corneal epithelium, but a significant number of animals infected with sialic acid-treated organisms also were protected from septicemia and death.

N$\alpha$ase treatment of CEPs also was assessed, and it was found that such treatment decreased but did not entirely eliminate pilus binding. These data may be explained if one considers that the sialidase from *C. perfringens* used in this study, like many other bacterial sialidases, hydrolyzes $\alpha$-(2-3) linkages of sialic acid faster (2 to 5 times) than $\alpha$-(2-6) linkages. Further, sialic acid bound to internal galactose residues to which N-acetylgalactosamine is linked by its glycosidic groups (as in some glycoproteins and gangliosides) is almost or completely resistant to the action of most sialidases. Use of specific sialidases to hydrolyze various linkages of sialic acid selectively or use of a sialidase that would increase the hydrolysis rate of $\alpha$-(2-6) bonds would test the validity of this hypothesis.

Two sialic acid-specific lectins, MAA and SNA, were used to determine, respectively, $\alpha$-(2-3) and $\alpha$-(2-6) linkage patterns of sialic acid of the P5 CEPs. SNA lectin identified many low molecular weight proteins of the corneal epithelium. In contrast, MAA lectin bound to mainly higher molecular weight proteins, indicating that many low molecular weight proteins of the P5 epithelium exhibited $\alpha$-(2-6)- but not $\alpha$-(2-3)-linked sialic acid residues. Both lectins were tested in binding inhibition assays to attempt to compete with and inhibit pili and host protein interactions. SNA lectin, but not MAA lectin subsequently put to similar use, decreased the binding of pseudomonal pili to P5 CEPs. SNA lectin did not completely inhibit pili binding to CEPs, suggesting that the binding site(s) on the pili adhesin interact differently with sialylated epithelial glycoproteins than the lectin. In addition, it is possible that hydrophobic interactions between pili and epithelial proteins differ from those interactions of the lectin with its carbohydrate.

In summary, the results show that *P. aeruginosa* pili bind to proteins in the P5 mouse eye; that one protein is surface membrane associated with a molecular weight of 57 kd; that this protein is $\alpha$-(2-6) sialylated; and that the $\alpha$-(2-6) sialic acid-specific lectin SNA decreases pilus binding to the 57-kd protein. In addition, intracellular membrane proteins also bind pseudomonal pilus. For both interactions, carbohydrates are of importance and sialic acid $\alpha$-(2-6) linked to galactose is an important constituent. Practically, these data may provide valuable insight into the mechanism of pathogenesis and thereby improve treatment for childhood cases of *P. aeruginosa* keratitis, where immunosuppression or underlying systemic disease leads to keratitis without perturbation of the ocular surface.

**Key Words**

pseudomonas, pili, cornea, epithelium, adhesion

**References**


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Pili Initially Bind a Single Epithelial Surface Glycoprotein


