Corneal Epithelial Glycoproteins Exhibit *Pseudomonas aeruginosa* Pilus Binding Activity

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Adherence of *Pseudomonas aeruginosa* to the cornea is a requisite step in the pathogenesis of bacteria-induced corneal disease. *P. aeruginosa* is capable of attaching to host epithelial cells by its pili, but there is little information regarding the epithelial receptors of this adhesin in the cornea. Using nitrocellulose blotting of polyacrylamide gels of solubilized adult mouse corneal epithelium, four major proteins (molecular weights: 38, 42, 57, and 66 kD) and several minor proteins were identified that bound purified pili from strain PAK and its hyperpiliated mutant PAK/PR1. These proteins were identified by immunoblotting either with pilus-specific monoclonal antibodies, XLR-3 and PK 3B, or using peptide PAK 128-144 (OX). The glycosylated nature of the proteins was determined using similar gel electrophoresis of corneal epithelial proteins, blotting onto nitrocellulose, and staining the blots with lectins conjugated to either horseradish peroxidase or alkaline phosphatase. All four major pilus-binding proteins were stained with concanavalin A lectin (mannose and glucose) and either wheat germ agglutinin lectin (WGA, specific for sialic acid and N-acetylglucosamine) or succinylated WGA lectin (only N-acetylglucosamine). Staining for peanut agglutinin lectin (galactose β(1-3) N-acetylgalactosamine) was seen for the 42-, 57-, and 66-kD proteins. The importance of the carbohydrate portions of these corneal proteins in pili binding was confirmed by preincubation of corneal epithelial blots with periodate or pili with sialic acid, both of which abolished the pilus binding. These studies indicate that corneal epithelial pilus-binding proteins are glycoproteins in nature and that sialic acid may be a constituent of these pilus-specific receptors in the adult mouse corneal epithelium. Invest Ophthalmol Vis Sci 33:2185-2193, 1992

*Pseudomonas aeruginosa*, an opportunistic bacterial pathogen, is a common cause of severe corneal infection. The infection is difficult to treat because it progresses rapidly and may result in decreased visual acuity or blindness. The ability of *P. aeruginosa* to adhere to the epithelium of a host is thought to be important in the initiation of an infection. *P. aeruginosa* can bind to various epithelial cells through its pili in several nonocular systems, and the pilus-binding domain may be located in the carboxy-terminal region of the pilin subunit. Several glycoproteins with pilus-binding ability have been identified in human buccal epithelial cells. However, there is little information regarding the epithelial receptors of this adhesin in the cornea. Several carbohydrates, including sialic acid, N-acetylmannosamine, mannose, and galactose have been suggested as bacterial-receptor components for corneal epithelium, but these studies did not distinguish between receptors specific for pili versus other potential adhesins. We used nitrocellulose blotting of polyacrylamide gels of solubilized adult mouse corneal epithelial proteins, immunostaining with pilus-specific antibodies, reactivity with a peptide conjugate PAK 128-144(OX), and lectin staining to determine pilus-specific receptor proteins in the cornea. We report that several corneal epithelial proteins bind purified pili or peptide PAK 128-144(OX) and that the proteins are glycoproteins because they stained with four different lectins, including wheat germ agglutinin (WGA), succinylated WGA (sWGA), peanut agglutinin (PNA), and concanavalin A (Con A). Periodate oxidation of the blots eliminated pilus binding, indicating that the carbohydrate portions of the glycoproteins appear to be necessary for receptor activity.

Materials and Methods

Pili Purification

Pili were purified essentially according to an earlier method. PAK/PR1 bacteria, a hyperpiliated mutant

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of the PAK strain, were grown on solid medium in stainless-steel pans, harvested, suspended in 15% sucrose in standard saline citrate (SSC) buffer (pH 7.0), and stirred overnight at 4°C. The bacterial cell suspension was blended for 2 min at 2000 rpm in a Sorvall Omnimixer (RMC, Tucson, AZ). The cells were removed by centrifugation at 10,000 × g for 15 min at 4°C, after which the supernatant solution was dialyzed for 72 hr against tap water at 4°C. Ammonium sulfate [(NH₄)₂SO₄] then was added to the dialysate to 50% saturation, and the solution was stirred overnight at 4°C. Both flagella and pili were precipitated and removed by centrifugation at 27,000 × g for 1 hr at 4°C. The pellet was resuspended in 20% w/v (NH₄)₂SO₄–SSC buffer, stirred for 4 hr at 4°C, and centrifuged at 27,000 × g for 1 hr to remove the flagella. This ammonium sulfate precipitation step was repeated two or three times. The pellet was resuspended in SSC buffer and centrifuged at 3000 × g for 15 min. The final pellet, enriched for pili, was redissolved in SSC buffer. The pili preparation was dialyzed against distilled water overnight and then negatively stained with 2% uranyl acetate and examined by transmission electron microscopy to ensure the purity of the preparation. In addition, samples of each preparation underwent electrophoresis on 13% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) to characterize sample purity further.

**Protein Determination**

The total protein concentration for each preparation was determined by the bichinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). In this assay, 0.1 ml of each standard protein sample and samples to be tested were pipetted into 2.0 ml of BCA working reagent and incubated at 37°C for 30 min. After incubation, the samples were cooled to room temperature, and the absorbance was measured spectrophotometrically at 562 nm. A standard curve was prepared, and the sample concentration was determined from this standard curve. All samples were lyophilized and stored at −70°C until used.

**Immunization**

Six-week-old female BALB/cByJ mice (Jackson, Bar Harbor, ME) were immunized with 100 µg/mouse of purified PAK/PR1 pili in a 0.1-ml emulsion of a 1:1 mixture of Freund's complete adjuvant (Difco, Detroit, MI) and phosphate-buffered saline (PBS) in the foot pads of their hind feet (0.05 ml per foot). A second injection with the same amount of pili in Freund's incomplete adjuvant (Difco) was administered similarly 2 weeks later. Two weeks after the second injection, the mice were bled, and their sera were analyzed using an enzyme-linked immunosorbent assay (ELISA) for the presence of antibody specific to pili. A final intravenous injection of antigen was given 1 week later, consisting of 10 µg pili in 0.1 ml PBS. Then, 0.1 ml of lipopolysaccharide (200 µg/ml; Difco) was injected intravenously into each mouse 3 hr later. All experiments in this study involving animals were done humanely and according to the ARVO Resolution on the Use of Animals in Research.

**Production and Selection of Hybridomas**

Cell fusion and cloning of the antibody-secreting hybridomas of interest were accomplished by a modification of procedures described previously. Three days after the final intravenous injection of pili, the mice were killed by cervical dislocation, and their spleens were removed. Spleen cells were fused with NS1 myeloma cells at a ratio of 5:1 using 50% saturated polyethylene glycol 4000 (Sigma, St. Louis, MO). Then, 50 µl of the resulting cell suspension (3 × 10⁶ cells/ml in Dulbecco's modified Eagle's medium [DMEM]) with 15% horse serum (GIBCO, Grand Island, NY) was pipetted into each well of a 96-well microtiter culture plate. Twenty-four hours later, 50 µl of DMEM, supplemented with 15% horse serum, 0.1 mmol/l hypoxanthine, 4 × 10⁻⁴ mmol/l aminopterin, and 1.6 × 10⁻³ mmol/l thymidine (Sigma), was added to each well. After allowing the cells to grow for 10–14 d, the hybridomas were tested by ELISA for specific antibodies against pili. The cells of positive wells were cloned by limiting dilution, and the supernatant was tested for pili specificity by western blotting. The cells of positive wells were cloned again. The resulting monoclonal cell lines of interest were used to produce ascitic fluid in pristane-primed (0.1 ml; Aldrich, Milwaukee, WI) female BALB/cByJ mice. Each mouse was injected with 1 × 10⁶ hybridoma cells 2 weeks after pristane priming. Ascitic fluid was tapped from each mouse only once and pooled for purification and subtyping.

**Purification of Monoclonal Antibody (mAb) and Subtyping**

Ascitic fluid was centrifuged at 2000 × g to remove hybridoma cells. The mAb, designated XLR-3, was purified by ammonium sulfate precipitation (40% saturation) and dialyzed against distilled water. The mAb then was subtyped using a mouse immunoglobulin subtyping kit (Zymed, San Francisco, CA). It was aliquoted, lyophilized, and stored at −70°C until needed.
Preparation of Mouse Corneal Epithelia

Adult female Swiss-Webster (ICR) mice (Harlan Sprague Dawley, Indianapolis, IN) were used to prepare corneal epithelial sheets. The mice were killed by cervical dislocation, and their eyes were enucleated. The eyes were placed into PBS containing 20 mmol/l ethylenediaminetetraacetic acid (EDTA), pH 7.2-7.4, and incubated for 3 hr at 37°C. After incubation, the corneal epithelial sheet was teased from the underlying stroma with forceps, and all conjunctival epithelium was removed under a dissecting microscope. These sheets were processed as follows. Approximately 15-20 corneal epithelial sheets were pooled and homogenized using a 1-ml glass tissue homogenizer in 200 μl of Laemmli sample buffer (0.0625 mol/l Tris base, 2.0% SDS, and 10.0% glycerol, without 2-mercaptoethanol, pH 6.8, Bio-Rad, Richmond, CA) and placed into a boiling-water bath for 30 sec. Protein concentration of the sample was determined by BCA protein assay as described earlier. Corneal epithelial sheet was removed under a dissecting microscope. These sheets were processed as follows. Approximately 15-20 corneal epithelial sheets were pooled and homogenized using a 1-ml glass tissue homogenizer in 200 μl of Laemmli sample buffer (0.0625 mol/l Tris base, 2.0% SDS, and 10.0% glycerol, without 2-mercaptoethanol, pH 6.8, Bio-Rad, Richmond, CA) and placed into a boiling-water bath for 30 sec. Protein concentration of the sample was determined by BCA protein assay as described earlier. Corneal epithelial protein samples were stored at —20°C until needed.

PAGE

The discontinuous SDS–PAGE system described previously was used with 10% acrylamide gels. Before electrophoresis, an appropriate amount of 2-mercaptoethanol (5.0% in final concentration; Bio-Rad) was added to the corneal epithelial protein samples. To each lane on the gel, 6.5 μg of corneal epithelial protein sample was added. The SDS–PAGE was conducted using 20 mA per gel under constant current (Mini Protean II Dual Slab Cell; Bio-Rad). The electrophoretically separated material was transferred to nitrocellulose paper (Bio-Rad) at 100 V under constant voltage for 1 hr on ice (Mini Trans-Blot Electrophoretic Transfer Cell; Bio-Rad).

Immunoblotting

After electrophoretic transfer of corneal epithelial proteins, nitrocellulose blots were blocked for nonspecific binding activity with 3% bovine serum albumin (BSA) (essentially fatty acid and globulin free), 0.05% Nonidet P-40, 5 mmol/l EDTA, and 150 mmol/l sodium chloride (Sigma) and 0.25% gelatin in 50 mmol/l Tris buffer (Bio-Rad), pH 7.5, at 37°C with shaking at 100 rpm overnight. The blots were washed twice with 0.1% Tween 20 in 50 mmol/l Tris-buffered saline (TTBS), pH 7.5 for 10 min each at 37°C with shaking at 100 rpm. They then were incubated with purified PAK/PR1 pili (100 μg/ml), PAK pili (100 μg/ml), or PBS (control) at 37°C at 100 rpm. After 2 hr, the blots were washed three times with TTBS (10 min per wash). The mAb XLR-3 (immunoglobulin [Ig]G 2b, 25 μg/ml) or mAb PK 3B (IgG isotype, specific for the PAK pilin subunit, dilution in 1% normal rabbit serum in TTBS were incubated with the blot at 37°C for 1 hr at 100 rpm. The blots were washed three times with TTBS. A goat anti-mouse IgG (heavy and light chain specific)-alkaline phosphatase conjugate (Cappel, West Chester, PA) at 1:3000 dilution in TTBS with 1% normal goat serum was added and incubated at 37°C for 1 hr at 100 rpm. The blots were washed three times with TTBS and once with TBS. The color development solution used consisted of 0.3 mg of p-nitro blue tetrazolium chloride per ml, 0.15 mg of 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt per ml (Bio-Rad), 0.1 mol/l sodium bicarbonate, 1.0 mmol/l magnesium chloride (Sigma), pH 9.8. Color development was stopped by rinsing the blots in distilled water.

An alkaline phosphatase-PAK 128-144(OX) peptide conjugate produced by one of us (RTI) was also used to identify pilus binding proteins. The conjugate is a synthetic peptide with an amino acid sequence identical to the carboxy-terminal region of PAK pilin (residues 128-144), with a formed disulfide bridge between the amino acid residues Cys-129 and Cys-142, which recognizes the pilus binding domain. Nitrocellulose blots of corneal epithelial proteins were blocked for nonspecific binding activity as described above. After washing with TTBS, blots were incubated with peptide PAK 128-144(OX) conjugated to alkaline phosphatase in a ratio of 8:1 (19 μmol alkaline phosphatase, 1:100 dilution) at 37°C for 2 hr at 100 rpm. After the incubation, blots were washed three times with TTBS and once with TBS. Color was developed as described previously.

Lectin Blotting

Nitrocellulose blots of corneal epithelial proteins were washed with lectin buffer consisting of 0.15 mol/l sodium chloride, 0.05 mol/l Tris HCl, 0.01 mol/l calcium chloride, 0.01 mol/l manganese chloride, and 0.05% Tween 20, pH 7.0, for 1 hr. All lectins (sWGA, WGA, PNA, and Con A; 0.2 mg/ml; EY, San Mateo, CA) were conjugated to horseradish peroxidase (HRP) and used to stain individual blots for 1 hr. Because Con A conjugated to HRP was not able to detect lectin-specific reactivity, Con A (0.2 mg/ml; EY) conjugated to alkaline phosphatase was used. To determine that the lectins bound specifically to the respective corneal epithelial proteins, the appropriate inhibitory sugar for each was added to the lectin solution as a control. This was done 1 hr before the lectins
were incubated with the corneal epithelial protein blots. N'N' diacetylchitobiose (0.02 mol/l; EY), the dimer of N-acetylglucosamine, was used as the inhibitory sugar for WGA and sWGA. Galactose and methyl α-D-mannopyranoside (Sigma), at a concentration of 0.2 mol/l for each, were inhibitory sugars for PNA and Con A, respectively. The blots subsequently were washed three times with lectin buffer and once with TBS. A substrate solution of HRP color development solution consisting of 0.6 mg/ml 4-chloro-1-naphthol (Bio-Rad), 20% ice-cold methanol, and 0.3% hydrogen peroxide in TBS was added to the blots. The substrate solution for alkaline phosphatase color development has been described. Color development was stopped by rinsing the blots with distilled water.

Effect of Amino Sugars on Pilus Binding to Blotted Corneal Epithelial Proteins

Amino sugars, sialic acid, N-acetylglucosamine, N-acetylgalactosamine, methyl α-D-mannopyranoside, L-fucose (Sigma), and N'N' diacetylchitobiose (40 mg/ml), were preincubated individually with pili (100 μg/ml) at 37°C for 1 hr before incubation with the corneal epithelial blots. Immunoblots were prepared and color development was done as described.

Periodate Oxidation of Corneal Epithelial Protein Blots

We used a method described earlier. Briefly, BSA-blocked blots were rinsed with 50 mmol/l sodium acetate buffer (pH 4.5); after this, control blots were incubated in the same buffer for 1 hr. Experimental blots were incubated with varying concentrations of periodate (0–30 mmol/l; Sigma) in 50 mmol/l sodium acetate buffer, pH 4.5, for 1 hr in the dark at room temperature. Both control and experimental blots then were rinsed with sodium acetate buffer and incubated with 50 mmol/l sodium borohydride (Sigma) in PBS for 30 min in the dark at room temperature. The blots were rinsed six times with PBS and were assessed for pili binding as described.

Results

Characterization of PAK/PR1 Pili

Examination of the purified pili of P. aeruginosa PAK/PR1 by transmission electron microscopy (Fig. 1) revealed that the biochemically purified sample was highly enriched for pilus. These structures were approximately 2.5 μm in length and 5.2 nm in diameter. During SDS-PAGE of PAK/PR1 pili (Fig. 2), the band containing the pili migrated at about 18,000 Daltons.

Reactivity and Specificity of mAb for Pili

The mAb XLR-3 was isotyped as an IgG 2b antibody. The mAb PK 3B (IgG) specific for PAK pili previously had been characterized and used to identify pilus-receptor molecules on human buccal epithelial cells. The specificity of both of these mAb was tested by ELISA and western blot analysis. During ELISA, XLR-3 and PK 3B reacted with both PAK and PAK/PR1 pili (data not shown). During immunoblot analysis, both mAb reacted specifically with the PAK/PR1 pili band of 18 kD molecular weight (Fig. 2).

Fig. 1. Transmission EM of negatively stained, biochemically purified pili from PAK/PR1. Pili have a central diameter of 5–6 nm. Magnification ×75,000; Bar = 100 nm.
Binding of Pili and Peptide PAK 128-144(OX) to Corneal Epithelial Protein Blots

Both PAK/PR1 and PAK pili bound to several corneal epithelial proteins by immunoblotting with pilus-specific mAb, XLR-3 and PK 3B, respectively (Figs. 3, 4, Table 1). Strong staining intensity with mAb XLR-3 was seen for four epithelial proteins of approximate molecular weights 38, 42, 57, and 66 kD after incubation with PAK/PR1 pili. Two other proteins of molecular weights 33 and 97 kD reacted weakly with XLR-3. Strong staining intensity with mAb PK 3B was seen for seven corneal epithelial proteins of molecular weights 38, 42, 45, 57, 62, 66, and 97 kD. Peptide PAK 128-144(OX) bound strongly to four epithelial proteins of molecular weights 38, 42, 45, and 66 kD and less strongly to two other proteins of molecular weights 57 and 97 kD (Fig. 5, Table 1). Thus, pili from PAK and PAK/PR1 and the peptide conjugate PAK 128-144(OX) bound to corneal epithelial proteins similarly. Four proteins (molecular weights, 38, 42, 57, and 66 kD) in common exhibited strong immunostaining by XLR-3 and PK 3B and reactivity with peptide PAK 128-144(OX) conjugated to alkaline phosphatase (strongly reactive, except for 57-kD protein). No protein bands were identified by either pilus-specific mAb in the control blots.

Fig. 2. SDS-PAGE gels stained with Coomassie brilliant blue G-250 and immunoblot. Lanes: 1, low molecular weight markers (Bio-Rad) in kD; 2, purified pili (PAK/PR1, 40 µg protein loaded); 3, immunoblot of PAK/PR1 pili reacted with mAb XLR-3.

Fig. 3. Immunoblots of mouse corneal epithelial proteins reacted with pili and mAb XLR-3. Lanes: 1, Ponceau S-stained nitrocellulose blot of low molecular weight markers (Bio-Rad) in kD; 2, PAK/PR1 pili and mAb XLR-3, arrowheads indicate reactive proteins; 3, control, mAb XLR-3, no pili.

Fig. 4. Immunoblots of mouse corneal epithelial proteins reacted with pili and mAb PK 3B. Lanes: 1, Ponceau S-stained nitrocellulose blot of low molecular weight markers (Bio-Rad) in kD; 2, PAK pili and mAb PK 3B, arrowheads indicate reactive proteins; 3, control, mAb PK 3B, no pili.
Glycosylated Nature of Pilus-Binding Epithelial Proteins

All pilus-binding proteins were found to react reproducibly with the lectins used (Fig. 6, Table 1). Staining of the four major proteins identified in common by the two mAb and the peptide was as follows: the 38-kD protein stained with WGA, sWGA, and Con A; the 42-kD protein stained with sWGA, PNA, and Con A; and the 57- and 66-kD proteins stained with WGA, sWGA, PNA, and Con A. No lectin staining was observed in any of the sugar-blocked control blots.

Effect of Amino Sugars on Pilus Binding to Corneal Epithelial Proteins

Only sialic acid was found to inhibit the binding of both PAK and PAK/PR1 pili to all epithelial proteins of interest (Fig. 7). All other haptenic sugars (N-acetylgalactosamine, N'N' diacetylchitobiose, N-acetylgalactosamine, methyl a-D-mannopyranoside, and L-fucose; Fig. 7) had no effect on inhibiting pilus binding.

Effect of Periodate Oxidation on Pilus Binding to Corneal Epithelial Proteins

Exposure of blotted corneal epithelial proteins to periodate decreased the ability of pili to bind to corneal epithelial proteins; this decrease in activity was proportional to an increase in the concentration of periodate. Complete loss of pilus-binding activity occurred at a periodate concentration of 30 mmol/l (Fig. 8).

Discussion

Successful colonization of host tissue by P. aeruginosa depends on the ability of the bacterium to bind to the epithelial cell surface of the host.19,20 Adherence to the corneal epithelium is a prerequisite for colonization and infection.21 The bacteria adhere only to injured tissue areas,7-11,21-24 to exposed corneal stroma,21,23 or to the unwounded immature cornea.25-27 Bacterial adhesion to epithelial cells occurs by specific interactions between adhesins of the bacteria and complementary receptors on the epithelial cell surface. Numerous studies show that pili play an important role in bacterial adherence to mucosal epithelium in nonocular systems.2-4,28 Using an in vitro ocular model, it was found that pili mediated adherence of P. fluorescens to human cornea.29 However, little is known about the corneal epithelial receptors for P. aeruginosa. Previous studies suggest that N-acetyl-
mannosamine,7 mannose,8,9 and galactose9 may serve as ocular receptors for *P. aeruginosa* in adult wounded cornea with sialic acid6 as a receptor in the immature unwounded cornea. However, neither the actual corneal proteins to which these carbohydrates attach, nor the nature of the bacterial adhesin has been clarified.

Recently, some progress was made in identifying the host receptor molecules for *P. aeruginosa* pili on human buccal epithelial cells.5 Five major glycoproteins that bound purified PAK pili were identified by immunoblotting with mAb PK 3B, a mAb that does not affect pilus binding to buccal epithelial cells. We reported the use of two mAb (PK 3B and XLR-3) specific for pseudomonal pilin and peptide PAK 128-144(OX) conjugated to alkaline phosphatase to identify host receptor molecules for pili in adult mouse corneal epithelium. Peptide PAK 128-144(OX) is a synthetic peptide with an amino acid sequence identical to the carboxy-terminal region of PAK pilin (residues, 128-144), which contains only one hydrophobic region.28 This peptide functions as an epithelial cell-binding domain and binds to human respiratory and buccal epithelial cells. The peptide also adheres to the same locations on human tracheal and buccal epithelial cells that bind native PAK pili.17,28 Therefore, peptide PAK 128-144(OX) directly conjugated to alkaline phosphatase provided an alternate method to identify pilus-binding corneal proteins without using antibodies. Our results provide evidence that four major corneal epithelial proteins (molecular weights, 38, 42, 57, and 66 kD) can be identified by their strong immunostaining with two monoclonal antibodies prepared against pili from two different PAK strains and by peptide PAK 128-144(OX) (strongly reactive with all but 57 kD) with an identical carboxy-terminal region to one of the strains (PAK). The rea-
Fig. 8. Immunoblots of PAK/PR1 pili binding to periodate oxidized blots of mouse corneal epithelial proteins. Lanes: 1, Ponceau S-stained nitrocellulose blot of low molecular weight markers (Bio-Rad) in kD; 2, control immunoblot (no periodate treatment); 3, 10 mmol/l periodate; 4, 30 mmol/l periodate.

son for the weak reactivity of the peptide with the 57-kD protein is hypothetic, but it may involve hydrophobic and/or conformational changes in the peptide that might decrease its corneal protein-binding ability.

Previous in vivo studies 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 neuraminidase treatment of the bacterial inoculum before its ocular delivery. In an in vivo adult mouse model, N-acetylmannosamine, the C6 precursor of sialic acid significantly decreased initial bacterial adherence to scarified corneas. Other work, with trephine-wounded rat corneas in organ culture or rabbit corneal epithelial cells grown in culture, suggested that mannose and galactose were involved in initial binding of _P. aeruginosa_ to the cornea. In nonocular systems, sialic acid and N-acetylglucosamine are important constituents of the binding sites for _P. aeruginosa_ on human tracheobronchial mucin and injured tracheal cells. Therefore, four lectins, including WGA (specific for sialic acid and N-acetylglucosamine), sWGA (only N-acetylglucosamine), PNA (galactose β(1-3) N-acetylgalactosamine), and Con A (mannose and glucose), were used for lectin staining of the blots. We found that all pilus-binding proteins stained with Con A (only Con A conjugated to alkaline phosphatase) and either WGA or sWGA. Staining for PNA was seen for the 42-, 57-, and 66-kD proteins. No lectin-specific reactivity was detected by Con A conjugated to HRP, related either to its low sensitivity or the high amounts of mannose on HRP interfering with lectin reactivity (information from the vendor). Our results indicate that these pilus-binding proteins are glycoproteins that contain sialic acid, N-acetylglucosamine, galactose β(1,3) N-acetylgalactosamine, and mannose moieties. The results also suggest that, because some proteins stained with several lectins, pilus-binding proteins contain a complex oligosaccharide structure.

Preliminary characterization of these pilus-receptor proteins also was done using periodate oxidation. At acid pH, periodate oxidation can cleave carbohydrate vicinal hydroxyl groups on sugars without altering the structure of the polypeptide chain. Periodic acid (30 mmol/l concentration) incubation of corneal epithelial protein blots before immunoblotting eliminated pilus binding, indicating that the carbohydrate portions of the various protein molecules are necessary for receptor activity. Another recent report also showed that periodate oxidation of buccal epithelial cell blots eliminated pilus binding.

In competitive inhibition experiments, preincubation of pili with several amino sugars, including sialic acid, N-acetylglucosamine, N' N' diacetylchitobiose, N-acetylgalactosamine, methyl α-D-mannopyranose, and L-fucose, provided evidence that only sialic acid inhibited pilus binding to corneal epithelial protein blots. These findings correlated with earlier work from this laboratory that suggested that sialic acid was an effective blocking sugar to inhibit _P. aeruginosa_ binding in vivo to the 5-postnatal day mouse cornea. In addition, others showed that sialic acid inhibited pilus binding to buccal epithelial cells. In these studies, a series of monosaccharides were preincubated with pili before their incubation with buccal epithelial cells. They found that, not only sialic acid, but also L-fucose had an inhibitory effect on pilus binding. D-fucose, D-mannose, N-acetyl-D-mannosamine, D-galactose, N-acetyl-D-galactosamine, D-glucose, N-acetyl-D-glucosamine, and D-arabinose had no effect on binding. Sialic acid alone was capable of inhibiting pilus binding to corneal epithelial proteins; therefore, it appears that this amino sugar may be a constituent of the pilus receptor in the cornea. Because sialic acid is the only negatively charged amino sugar we studied, it also is possible to hypothesize that its charge may contribute to its inhibition of pilus binding.

In summary, we identified several glycoproteins that act as receptors for _P. aeruginosa_ pili in the adult mouse cornea. Identification of specific bacterial adhesin receptor proteins should provide information necessary for unraveling complex bacterial and host
interactions and may facilitate future development of a vaccine to prevent this organism from adhering to the cornea. In this regard, preliminary studies were done using human corneas. These showed the existence of similar molecular weight epithelial proteins to those identified in the mouse cornea and epithelial proteins of other molecular weights that similarly interact with PAK/PR1 pilus (Rudner, unpublished data).

Key words: adhesion, pili, corneal receptor, monoclonal antibody, lectins

References
