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# Role of Pili in Adhesion of *Pseudomonas aeruginosa* to Human Respiratory Epithelial Cells

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**The ability of pili from *Pseudomonas aeruginosa* K (PAK) to act as an adhesin to human respiratory epithelial cells was examined using an in vitro adhesion assay. Equilibrium analysis of PAK binding to human buccal epithelial cells (BECs) and tracheal epithelial cells (TECs) by means of a Langmuir adsorption isotherm revealed that the maximum numbers of binding sites per epithelial cell ($N$) were 255 for BECs and 236 for TECs, with apparent association constants ($K_a$) of $2.8 \times 10^{-9}$ and $5.8 \times 10^{-9}$ ml/CFU, respectively. Trypsinization of the BECs before the binding assay increased $N$ to 605 and decreased the $K_a$ to $1.7 \times 10^{-9}$ ml/CFU. Addition of homologous pili to the binding assay with BECs or TECs or the addition of anti-pilus Fab fragments inhibited PAK adherence. Binding of purified pili to BECs was shown to reach saturation. Purified pili and PAK competed for the same receptor on the BEC surface. Further, by using peptide fragments of PAK pilin (derived from the native pili or produced synthetically) in the binding assay for PAK to BECs, we have presumptively identified the pilus binding domain in the C-terminal region of the pilin and shown that the C-terminal disulfide bridge is important in maintaining the functionality of the binding domain.**

The binding of *Pseudomonas aeruginosa* to an epithelial surface is thought to be the first step in the establishment of a respiratory tract infection by this bacterium in a susceptible patient (20). Therefore, the binding of this bacterium to respiratory epithelial cells has been examined to elucidate the adhesion mechanisms used (12, 17–19, 25, 26). Two adhesins, pili and the alginic acid capsule, have been shown to be involved in the binding of *P. aeruginosa* to human buccal epithelial cells (BECs) (6, 12, 26), acid-injured mouse trachea (17, 19), and hamster trachea (11).

The pili of *P. aeruginosa*, which have been shown to be polarly located (2), serve as an attachment site for various *Pseudomonas*-specific bacteriophages (3) and are responsible for twitching motility (4) but are not involved in bacterial conjugation (3). The pili purified from the strain used in this study, *P. aeruginosa* K (PAK), have been extensively characterized. The pilin has been purified to homogeneity and sequenced (22), and this amino acid sequence agrees with that deduced from the DNA sequence of the structural gene for pilin (16). The PAK pilin appears to be representative of the pilin produced by a number of *P. aeruginosa* strains (unpublished data). However, pili exhibit considerable antigenic variation between strains (24). Controversy has arisen over the effectiveness of preventing adhesion mediated by pili. Pili from a heterologous strain inhibit adhesion of *P. aeruginosa* to injured mouse trachea; however, only antibody against the homologous pili, not the heterologous pili, was found to prevent adhesion (19). These data indicate that the binding domain of pili may be somewhat conserved, yet may not be antigenically active. This would make effective anti-adhesion-based treatment strategies of patients with *P. aeruginosa* potentially difficult. The identification of the pilus binding domain would allow the resolution of this problem.

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## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** PAK has been previously described (3). This strain was maintained on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) slants at −70°C and was routinely cultured on BHI agar. A single colony was used to inoculate 10 ml of M-9 medium (1), which was incubated at 37°C for 18 h with shaking at 150 rpm in a New Brunswick gyroshaker. This culture was the source of a 10% (vol/vol) inoculum for 10 ml of M-9, which was cultured, as described above, for 3 h. The culture was then supplemented with [$^{35}$S]L-methionine (10 μCi/ml) having a typical specific activity of 1,100 mCi/mmol (New England Nuclear Corp., Boston, Mass.) and incubated for another 3 h. The $^{35}$S-labeled *P. aeruginosa* organisms were then harvested by centrifugation (12,000 × *g* for 10 min at 4°C) and washed three times with 0.01 M sodium phosphate-buffered saline (PBS) at pH 7.2.

**BECs.** Human BECs were collected with wooden applicator sticks from healthy, nonsmoking, male volunteers ($n$ = 10). BECs were removed from the applicator sticks by agitation in PBS. The BECs were washed three times (2,000 × *g* for 10 min at 4°C) with PBS. BECs were either used at this stage or first trypsinized by the addition of 50 μg of trypsin per ml for 30 min at 37°C. Trypsinized BECs were washed an additional three times to remove the trypsin. The cell concentration for the BECs was determined with a hemacytometer, and the BEC concentration was adjusted to $2.0 \times 10^5$ cells per ml before use in further assays.

**TECs.** Human tracheal epithelial cells (TECs) were obtained by bronchoscopic brushing of the tracheal bronchial mucosa. All procedures were approved by the Toronto General Hospital Ethics Committee, and informed consent was obtained from the relatives of the patient.

TECs were obtained on two separate occasions (replicate binding isotherm experiments were performed) from an intubated female patient without a tracheostomy (age 64) in the intensive care unit. The patient had been admitted for

surgical treatment of an oropharyngeal fistula and experienced respiratory failure during the procedure. Subsequently, she developed pneumonia, with both mucoid and nonmucoid *P. aeruginosa* and *Klebsiella pneumoniae* being isolated from the sputum, and was treated with antibiotics. At the time of the bronchoscopy, the patient was exhibiting clearing of chest infiltrates, as determined by chest X-ray. However, *P. aeruginosa* was isolated from the tracheal cell sample on both occasions.

As this patient was, for the most part, conscious, the procedure was preceded by intravenous administration of diazepam (5 to 10 mg) and the injection of 5 ml of 2% (wt/vol) Xylocaine down the airway. Bronchoscopy was performed with a flexible Olympus type 2 BF bronchoscope inserted through an endotracheal tube. A cytology brush was passed through the suction channel and was used to abrade the tracheal-bronchial mucosa. The bronchoscope and brush were removed following each brushing to avoid loss of epithelial cells by withdrawing the brush through the suction channel itself. The cells were eluted from the bronchial brush by agitation in high-glucose Dulbecco modified Eagle medium (DME) containing 1% (wt/vol) sodium citrate and kept at 4°C. A total of 10 brushings per sampling were performed. TECs were isolated from contaminating blood cells and mucous as described by Franklin et al. (8). Contaminating bacteria in the sample were removed during this procedures. Since the bound bacteria are in a reversible equilibrium which is shifted to favor free bacteria during the fractionation procedure, the Percoll gradient centrifugation used during the TEC isolation protocol caused the bacteria to migrate to a separate and distinct density. Upon completion of the TEC isolation protocol, isolated TECs appeared relatively free of bacteria.

**Bacterial binding assay.** The bacterial binding assay described by McEachran and Irvin (12) was performed. BECs (1.0 ml at $2.0 \times 10^5$ cells per ml) or TECs (1.0 ml at $10^5$ cells per ml) were mixed with an equal volume of $^{35}S$-labeled bacteria suspended in PBS in a 15-ml polystyrene test tube and incubated for 2 h. The number of bacteria added to each tube varied from a ratio of bacteria to epithelial cells of 100:1 to a ratio of 10,000:1. Epithelial cells with bound bacteria were then collected by filtration on 12.0-μm-pore-size polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.) that had been pretreated with 2% (wt/vol) bovine serum albumin in PBS to reduce nonspecific binding, washed with 15 ml of PBS, and then dried in scintillation vials. Aquasol (5 ml) was added to each vial, and the amount of radioactivity was determined by scintillation counting in a Beckman LS-150 liquid scintillation counter. All binding assays were performed in triplicate. Binding of bacteria to epithelial cells was corrected for nonspecific binding of bacteria to the 12.0-μm-pore-size filter (nonspecific binding was generally less than 15% of the experimental value). Epithelial cell concentration was determined at the end of the assay to correct for cells lost during incubation. Experiments were replicated independently at least three times using BECs. The TEC binding data were generated by performing the binding assay on TECs collected from a single patient. Two identical binding assays were performed on TECs collected 10 days apart, and the data were pooled for subsequent analysis. A time course experiment was performed by using PAK binding to BECs to demonstrate that maximal binding had occurred by the end of 2 h. It was also demonstrated that labeled PAK could be displaced from the BECs by unlabeled PAK. This established that the binding assay was run under conditions of reversible equilibrium. TECs and BECs were observed microscopically at the end of each assay to assure that bacterial binding was still reversible and to observe that the epithelial cells were still intact.

**Pilus inhibition studies.** Pilus inhibition assays were performed by either a preincubation method or a direct competition method. In the case of preincubation, pili and BECs ($2.2 \times 10^5$ BECs per ml) were mixed together and incubated at 37°C with shaking at 300 rpm. After 1 h, the BECs were collected by centrifugation (2,000 × *g* for 10 min at 4°C), followed by a single wash with PBS. The BECs were resuspended in PBS, and the concentration was adjusted to $2.0 \times 10^5$ BECs per ml. The pretreated BECs were then used immediately in the bacterial binding assay. Direct competition of pili and bacteria was achieved by the simultaneous addition of pili, bacteria, and BECs or TECs at the commencement of the bacterial binding assay.

**Effect of anti-pilus Fab fragments on PAK binding.** Rabbit anti-pilus Fab fragments were prepared from the immunoglobulin G fraction of PAK pilus-specific antisera, as described by Sastry et al. (22).

Labeled PAK (0.5 ml of approximately $2 \times 10^9$ CFU/ml) and the Fab fragments (0.5 ml of 0 to 3.5 mg/ml) suspended in PBS were mixed together in 15-ml polystyrene test tubes and incubated together with shaking at 300 rpm for 30 min at 37°C. At this time, 1 ml of BECs ($2.0 \times 10^5$ BECs per ml) was added to each tube; for nonspecific binding controls, 1 ml of PBS was added to each tube. This mixture was incubated for 2 h at 37°C with shaking at 300 rpm. The number of bacteria bound per BEC was then determined as described above.

**Preparation of natural and synthetic pilin fragments.** Natural pilin fragments cT-II, cT-III, and cT-IV were prepared as described previously (24).

Peptides were synthesized using the Merrifield solid-phase procedure on a Beckman model 990 peptide synthesizer. The carboxy-terminal lysine was coupled to a 1% cross-linked Merrifield resin (Pierce Chemical Co., Rockford, Ill.; 0.9 meq of Cl/g of substitution) via the cesium salt of *t*-butyloxycarbonyl lysine 2-chlorobenzoxycarbonyl (9). The addition of subsequent protected amino acids, removal of protecting groups from the peptide, and cleavage of the peptides from the resin using hydrogen fluoride were performed as previously described (15). The peptides were purified by reversed-phase high-performance liquid chromatography on a Synchropak C18 column (Synchrom, Linden, Indiana; 250 by 10 mm; flow, 2.0 ml/min; solvent A, 1.0% trifluoroacetic acid (TFA)-$H_2O$; solvent B, 1.0% TFA-$CH_3CN$; gradient, 1.0% solvent B per min). A diagrammatic summary of the fragments used is given in Fig. 1.

**Assessment of the effect of pilin fragments on binding.** The effect of pilin fragments on binding was assessed by adding labeled bacteria, fragments, and BECs ($10^5$ cells per ml) simultaneously and incubating for 2 h at 37°C with shaking at 150 rpm. The number of bacteria bound per BEC was determined by the filter assay described above.

**Pilus binding to BECs.** An immunoassay was performed to assess the binding of pili from PAK to BECs. BECs (1 ml at $2.0 \times 10^5$ BECs per ml) were added to an equal volume of PAK pili (1.0 to 0 mg/ml in PBS) and incubated at 37°C with shaking at 300 rpm in a New Brunswick gyroshaker. After 1 h, BECs were collected by centrifugation (12,000 × *g* for 10 min at 4°C) and washed twice with PBS. Rabbit anti-PAK pilus antibody (prepared as described in reference 22) was added to the BEC pellet (1 ml of a $10^{-4}$ dilution) and incubated as described above for 1 h. The BECs were then collected by centrifugation and washed twice with PBS.

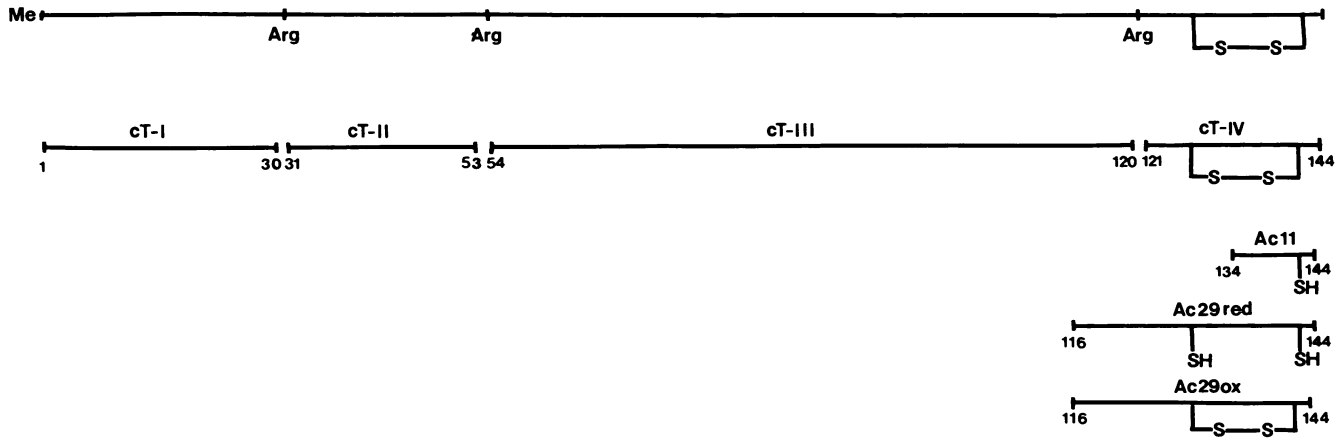


FIG. 1. Schematic representation of PAK pilin and pilin peptide fragments. The respective positions of the peptide fragments produced by arginine-specific cleavage and synthetic peptides (Ac11 and Ac29) are shown. The two cysteine residues of synthetic peptide Ac29 were either in the oxidized (i.e., the intrachain disulfide bridge was formed) or reduced state. Amino acid residue numbers at the ends of the fragment or peptide are shown.

Goat anti-rabbit immunoglobulin G (heavy and light chains) peroxidase-conjugated antibodies (Jackson Laboratory, Bar Harbor, Maine) were added to the BEC pellet (1 ml diluted per instructions for use), and the mixture was incubated as described above for 1 h. The BECs were collected by centrifugation, transferred to a clean test tube, and washed twice with PBS. The pellet was suspended in 1 ml of 1 mM 2,2'-azino-di-(3-ethylbenzothiazoline sulfonic acid) (ABTS) in 10 mM citrate buffer (pH 4.2) supplemented with 0.03% (wt/vol) hydrogen peroxide. The reaction was stopped by the addition of 1 ml of 4 mM $NaN_3$, and the optical density at 405 nm was determined after removal of the BECs by centrifugation. The BEC concentration in each tube was determined with a hemacytometer at the end of the assay prior to the removal of BECs by centrifugation.

## RESULTS

**Binding of PAK to BECs and TECs.** PAK bound to both BECs and TECs in a concentration-dependent manner (Fig. 2). The binding approaches saturation over the range of bacterial concentrations used. Saturation of bacterial binding to both BECs and TECs, although not attained, would occur at a level of >220 bacteria per BEC. Since the binding was found to be in equilibrium, further analysis of the binding data was carried out with the Langmuir adsorption isotherm: $(U/B) = (U/N) + 1/(K_a N)$, where $U$ is the unbound concentration of bacteria, $B$ is the bound bacteria per BEC, $N$ is the number of binding sites per BEC, and $K_a$ is the apparent association constant. Plotting $U/B$ versus $U$ for the BEC data produced a single straight line (correlation coefficient = 0.93) (Fig. 2), indicative of a single class of receptors on the BEC surface, and allowed for the determination of $N$ from the slope ($N = 255 \pm 12$ CFU/BEC) and the association constant, calculated from the $y$ intercept ($K_a = 2.8 \times 10^{-9} \pm 0.2 \times 10^{-9}$ ml/CFU). A single straight line was also produced with the TEC data (correlation coefficient = 0.84), with $N$ calculated to be $236 \pm 1$ CFU/TEC and $K_a$ calculated to be $5.7 \times 10^{-9} \pm 1.6 \times 10^{-9}$ ml/CFU (Fig. 2). Binding of PAK to trypsinized BECs approached saturation at a level of >450 bacteria per cell (Fig. 3). Analysis of these binding data using the Langmuir adsorption isotherm produced a single straight line (correlation coefficient = 0.93) with an $N$ value of $605 \pm 12$ CFU/BEC and a $K_a$ of $1.7 \times 10^{-9} \pm 0.4 \times 10^{-9}$ ml/CFU (Fig. 3).

**Effect of pili on binding.** Either preincubating BECs with pili before the addition of the bacteria or adding pili, BECs, and bacteria simultaneously (direct competition) resulted in the inhibition of bacterial binding to virtually identical levels (Fig. 4). Pili also inhibited the binding of bacteria to TECs by a direct competition assay (Fig. 4). Maximum inhibition occurred in all cases when greater than 0.2 mg of pili per ml was added. Inhibition of binding of PAK by pili was shown to be competitive by generating a Lineweaver-Burk plot in which various concentrations of bacteria were added to a fixed concentration of pili and BECs (Fig. 5).

**Effect of anti-pilus Fab fragments on PAK binding.** Fab framents caused a concentration-dependent decrease in binding of PAK to BECs when compared with control levels (Table 1).

**Pilus binding to BECs.** Increasing the amount of pili initially added to the BECs resulted in an increased level of pilus binding and reached saturation over the range exam-

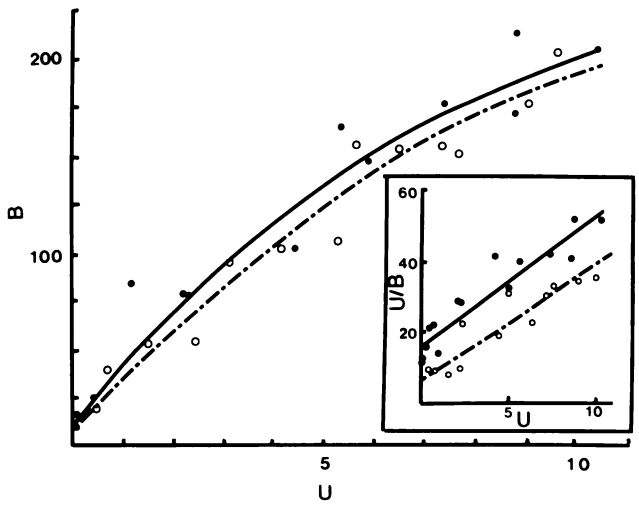


FIG. 2. Binding isotherm for PAK adhering to human BECs (●) and TECs (○). Inset, Langmuir adsorption isotherm for binding data ($B$ = bound bacteria per epithelial cell; $U$ = unbound bacteria per ml $\times 10^8$; and $U/B$ = [unbound bacteria per ml/bound bacteria per epithelial cell] $\times 10^5$).

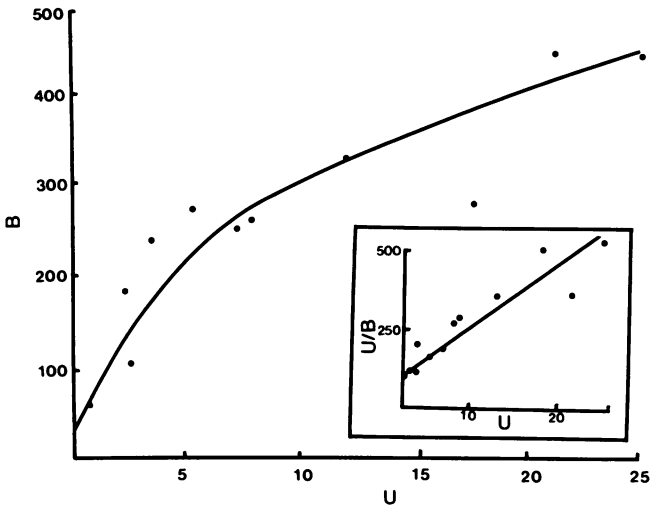


FIG. 3. Binding isotherm for PAK adhering to trypsinized BECs. Inset, Langmuir adsorption isotherm for binding data (*B*, *U*, and *U*/*B* as defined in the legend to Fig. 2).

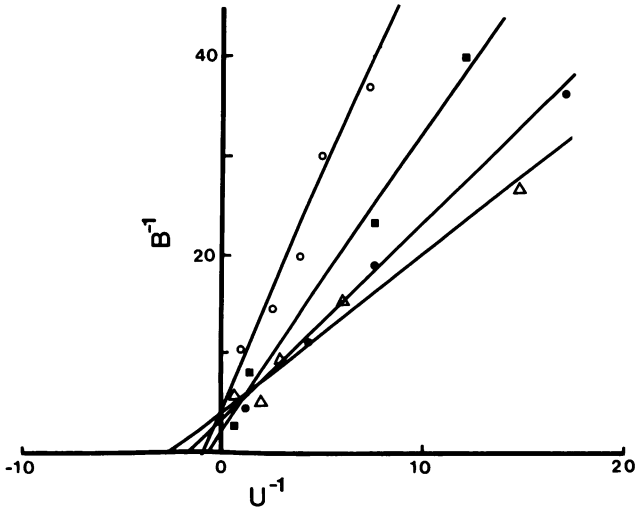


FIG. 5. Lineweaver-Burk plot for the determination of the type of inhibition due to pili of the binding of PAK to human BECs. Isotherm generated in the presence of 0 (control) (△), 1.0 (○), 0.5 (■), and 0.25 (●) mg of pili per ml.

ined (Fig. 6). Saturation occurred at concentrations of pili greater than 0.125 mg/ml.

**Effect of pilus fragments on binding.** Natural fragments of cT-II and cT-III had no effect on the binding of PAK to BECs. The C-terminal fragment, cT-IV, caused a significant increase in the level of binding. Since the C-terminal region was the only fragment to cause an effect on binding, a further examination of this region was made. A synthetic peptide consisting of the last 11 C-terminal amino acids (Ac11) had no effect on binding, while a synthetic peptide consisting of the last 29 amino acids of the C terminus of PAK pilin, but with a reduced disulfide bridge (Ac29red), caused a slight increase in PAK binding to BECs. Oxidation of the reduced Ac29 to the oxidized state before use in the adhesion assay increased PAK binding to BECs more than 10-fold. The results are summarized in Table 2.

## DISCUSSION

*P. aeruginosa* adherence to human respiratory epithelial cell surfaces is thought to be important in the initiation of a respiratory tract infection in a susceptible patient (20). Thus, a more complete understanding of the adhesion mechanisms and adhesins of this bacterium is pivotal in the elucidation of its pathogenic mechanism.

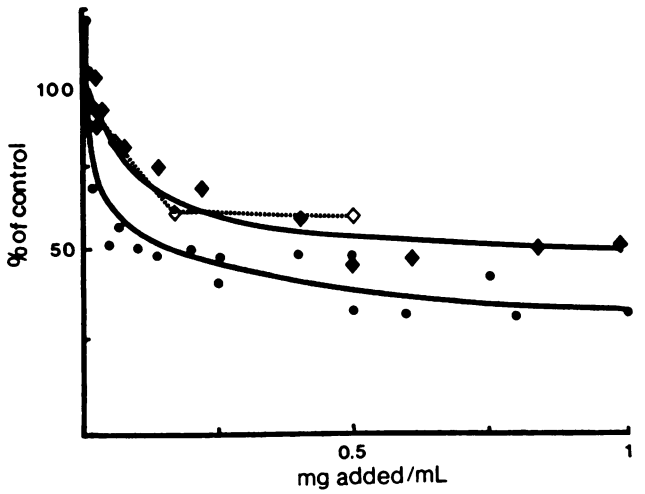


FIG. 4. Effect of pili on the binding of PAK to human TECs (◇) and to human BECs by a preincubation method (●) and direct competition (◆).

The use of binding isotherms and the Langmuir adsorption isotherm in the study of bacterial adherence has allowed the comparison of the binding of different strains of bacteria to different types of epithelial cells (7, 12, 13). The data from these isotherms indicated that PAK can bind to a single class of receptor sites on BECs. PAK also bound to TECs by a single class of receptor sites. The maximum number of binding sites per epithelial cell and the association constants for PAK binding to BECs and TECs are similar, suggesting that similar binding mechanisms or receptors are used for both types of epithelial cells. The differences observed between the maximum number of receptors and the $K_a$s for BECs and TECs may reflect the fact that BECs were collected from a group of individuals, hence the equilibrium parameters obtained are an average, whereas TECs were obtained from a single patient. Microscopic examination of the binding of PAK to these epithelial cell types revealed that PAK bound over the entire BEC surface, while it bound only to the cilia of TECs (data not shown). This may be due either to PAK using different binding mechanisms for the different epithelial cell types or to the pilus-specific class of receptors on TECs being primarily localized on the cilia.

Trypsinization of BECs presumably modifies the receptor sites on untrypsinized BECs or similar surface components

TABLE 1. Effect of anti-pilus Fab fragments on the binding of PAK to human BECs

| Fab fragment concn[a] (mg/ml) | % of control |
|---|---|
| 0.35 | 47.3 |
| 0.035 | 57.7 |
| 0.0035 | 58.1 |
| 0.00035 | 75.7 |
| 0.000035 | 95.6 |

[a] Values shown are from a representative experiment. The initial concentration of PAK was $2.3 \times 10^9$ CFU/ml, and the ratio of PAK to BEC was 6,750:1. Inhibition of PAK binding to BECs by anti-pilus Fab could be demonstrated when lower ratios of PAK to BEC were used.

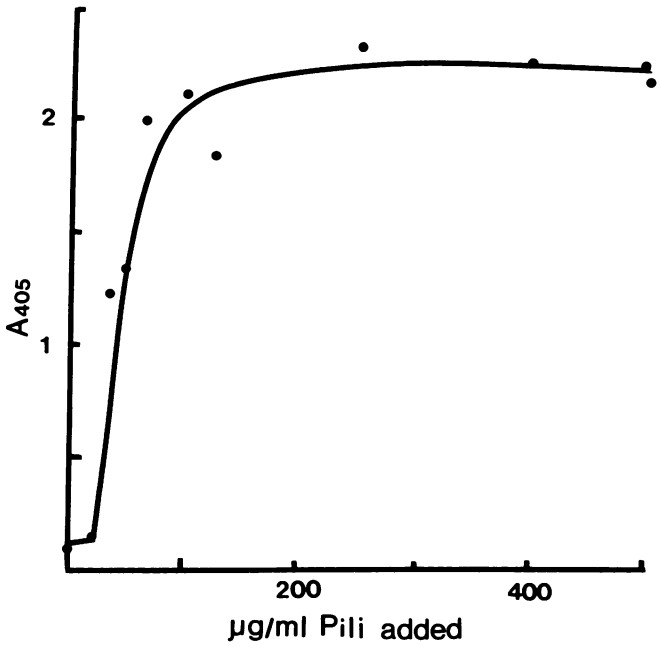


FIG. 6. Binding of PAK pili to BECs ($A_{405}$ versus micrograms of pili added per milliliter). Pilus binding was assessed by an indirect enzyme-linked immunoassay using saturating conditions of both rabbit anti-pilus immunoglobulin G (primary antibody) and goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated antibodies (second antibody).

to create an increased number of receptors to which PAK binds less effectively. It is unlikely that trypsinization removed only cell components which sterically blocked preexisting receptor sites. If the sites were identical to those already exposed, then the association constant of newly exposed sites would not be expected to be lower. If the newly exposed sites were different and had a lower association constant, this would be resolved as two classes of receptor sites by the Langmuir analysis. The PAK receptor sites are presumably partly proteinaceous and thus are different from the pilus receptor sites recognized by strain 492c, as the copy number of those receptor sites did not change with trypsinization (12).

A direct demonstration that pili are a *P. aeruginosa* adhesin was accomplished by showing that purified pili bound in a concentration-dependent manner to BECs, that pili inhibited bacterial binding, and that anti-pilus Fab fragments inhibited bacterial binding. This inhibition was competitive (demonstrated by the Lineweaver-Burk plot [Fig. 5]) and, thus, was not due to steric blocking of an adjacent site. The ability of pili to inhibit the binding of PAK to TECs indicates that the adhesin-receptor interaction for the trachea, and possibly for the lower respiratory tract, is similar to that observed for BECs.

TABLE 2. Effect of natural and synthetic peptide fragments of PAK pilin on the adhesion of PAK to BECs

| Fragment | Concn (nmol/ml) | Adhesion index (% of control) |
|---|---|---|
| cT-IV | 25 | 379[a] |
| cT-III | 25 | 102[a] |
| cT-II | 25 | 110[a] |
| Ac11 | 25 | 104[a] |
| Ac29red | 15[b] | 287[c] |
| Ac29ox | 15[b] | 1,060[c] |

[a] Determined at a ratio of 3,061:1 (bacteria/BECs) and by a direct competition assay.

[b] The concentrations of the Ac29red and Ac29ox fragments were normalized to an $A_{280}$ value of 0.183 (~15 nmol/ml, final concentration).

[c] Determined at a ratio of 2,240:1 (bacteria/BECs) and by a direct competition assay.

The peptide fragments derived from PAK pilin were used to examine the pilin binding domain. The binding domain of pilin, as well as domains important in pilin-pilin interaction, would be expected to exhibit more sequence conservation than regions that are not involved in these functions (10). The areas not involved in pilin function could be important in the immunological variation of pili between strains (10). The central region of the pilin, represented by pilin fragments cT-II and cT-III, is considerably more variable, with cT-III accounting for a considerable proportion of the immunological response to the pilus (22, 24). Neither of these fragments had any effect on the binding of PAK to BECs and, therefore, are not likely involved in the pilin binding domain. Examination of the sequence of *P. aeruginosa* pilin (21) showed that conservation appears to be most stringent in the N and C termini (fragments cT-I and cT-IV), implicating these regions in pilin function. The N-terminal fragment, cT-I, is conserved between a number of genera, including *Bacteroides*, *Moraxella*, and *Neisseria* (5), and appears to function in the pilin-pilin assembly process (23). Because of its hydrophobic nature, it could not be used in the investigations reported here. The cT-IV region of the pilin did affect the binding of PAK to BECs. The increase in PAK binding could be explained by the presence of two functional domains: one involved in pilus-BEC interaction and the other involved in pilin-pilin interaction. The cT-IV fragment does have two hydrophilic domains capable of such functions (23). Previous work (14) had reported that the cT-IV fragment inhibited binding. This is opposite to what was observed here. This difference may be explained by the low ratio of bacteria to BECs and a relatively higher concentration of the fragment in studies by these researchers. We used a higher ratio of bacteria to BECs for greater accuracy in our determination of the number of bacteria bound per BEC. However, the higher relative concentration of cT-IV used previously was impossible to achieve here as a result of limitations on the amount of cT-IV available. It is possible that in the previous work (14), the higher concentration of the fragment did not allow resolution of the pilus-fragment interaction thought to be observed here. To further investigate these domains, synthetic peptides were used. When a synthetic peptide consisting of the 11 C-terminal amino acids (Ac11) was used, no effect was observed, whereas a synthetic peptide consisting of the C-terminal 29 amino acids did increase binding. However, the state of the disulfide bond in this fragment had profound effects on binding. If the bond was in the oxidized state, then the fragment increased binding to a higher level than cT-IV. This was not surprising, since this fragment is larger than cT-IV and completely covers the two hydrophilic domains of the C-terminal region. It is likely, therefore, that the C-terminal region of the pilin is involved in the pilus binding domain and that the C-terminal disulfide bridge is important in maintaining the pilin binding domain conformation.

## ACKNOWLEDGMENTS

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