INFECTION AND IMMUNITY, Jan. 1990, p. 124–130
0019-9567/90/010124-07$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 58, No. 1

Inhibition of Pilus-Mediated Adhesion of *Pseudomonas aeruginosa* to Human Buccal Epithelial Cells by Monoclonal Antibodies Directed against Pili

PETER DOIG,[1] PARIMI A. SASTRY,[2] ROBERT S. HODGES,[2] K. K. LEE,[2] WILLIAM PARANCHYCH,[2] AND RANDALL T. IRVIN[1,3]†*

Departments of Botany and Microbiology, Erindale College, University of Toronto, Mississauga, Ontario L5L 1C6,[1] Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7,[2] and Department of Microbiology, Toronto General Hospital, Toronto, Ontario M5G 2C4,[3] Canada

Received 8 June 1989/Accepted 22 September 1989

The *Pseudomonas aeruginosa* PAK pilus is capable of mediating the binding of this strain to human respiratory epithelial cells. We have produced monoclonal antibodies (MAbs) to the PAK pilus in order to elucidate the location of the binding domain of the pilus for human buccal epithelial cells (BECs). Four MAbs are described. MAbs PK41C and PK34C were found to react with *P. aeruginosa* pilins produced by a large number of strains. The epitope recognized by PK41C was determined to lie within the N-terminal region of the pilin and is likely constituted by amino acid residues 22 through 33. The epitope for PK34C was located in the C-terminal region of the pilin and was partially dependent on an intact intrachain disulfide bridge between cysteine residues 129 and 142. PK99H and PK3B were found to react specifically with PAK pilin. The epitope for PK99H was also localized in the C-terminal region of the pilin protein and appears to reside between amino acid residues 130 and 138. The epitope for PK3B was not localized by using the methods of this study, but it is likely dependent on the three-dimensional structure of the pilin. Fab fragments of PK99H inhibited adhesion of strains PAK and 492c to BECs, but the adherence of five other strains was not affected. Fab fragments of PK34C inhibited adhesion of all piliated strains examined. Fab fragments from both of these antibodies inhibited PAK pilus binding to BECs. Fab fragments of PK41C and PK3B had no effect on *P. aeruginosa* binding to BECs. These results confirm that the C-terminal region of the pilin has adhesin qualities and that a conserved epitope lies within this region.

Pseudomonas aeruginosa is a major respiratory pathogen of immunocompromised and immunosuppressed patients (20). Successful colonization of the respiratory system of a patient by this bacterium is thought to be dependent upon the ability of the bacterium to bind to the epithelial cell surface of the host (19, 20, 26). Adherence of *P. aeruginosa* to the respiratory epithelium is thus an important first step of the infection process.

One method by which *P. aeruginosa* mediates its attachment to an epithelial cell surface is via pili (5, 19, 26). Pili are proteinaceous appendages composed of a single monomer termed pilin (21). The primary amino acid sequence of the pilin produced by a number of strains has been determined (21). The strain-to-strain variation in the pilin amino acid sequence is substantial; however, certain regions of the pilin sequence are conserved (21). It is uncertain whether the epithelial cell-binding domain sequence of pilin is conserved or whether a common antigenic epitope may be associated with the epithelial cell-binding domain of pilin. Pili from a heterologous strain have been demonstrated to inhibit the binding of *P. aeruginosa* to an epithelial surface (19). Homologous antipilus antiserum has been demonstrated to inhibit adherence, but heterologous antipilus antiserum did not inhibit adherence (19). This suggests that the binding domain of the pilus is either poorly conserved or poorly antigenic.

* Corresponding author.
† Present address: Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Alberta T6G 2H7, Canada.

We have recently demonstrated that *P. aeruginosa* PAK uses pili to bind to human buccal epithelial cells (BECs) and tracheal epithelial cells (5) and that the binding domain of the pilus may involve the C-terminal region of the pilin subunit (5, 16). We have produced monoclonal antibodies (MAbs) directed against PAK pili. These antibodies have been used to map the putative epithelial cell-binding domain of *P. aeruginosa* pilin and to investigate antigenic variation of pilin.

MATERIALS AND METHODS

Strains and culture conditions. *P. aeruginosa* PAK (3) and *P. aeruginosa* PAO (2), 492c (8), 492a (8), P1 (18), and K122-4 (18) have been previously described. PAK/3 is a nonpiliated variant of strain PAK. PAO 579 is a leucine-requiring, mucoid variant of PAO. Strain HD1 is a clinical isolate from the blood of a cancer patient undergoing chemotherapy and was a gift from M. Joffee, University of Alberta, Edmonton, Alberta, Canada. These strains were stored at −70°C and were routinely subcultured on brain heart infusion agar at 37°C. For adhesion assays, the strain was grown in M9 medium supplemented with 0.4% (wt/vol) glucose (1) and grown overnight at 37°C in a gyroshaker (New Brunswick Scientific Co., Inc.) shaking at 150 rpm. This culture served as a 10% (vol/vol) inoculum for fresh M9. After 3 h of incubation as described above, 10 μCi of L-[^{35}S]methionine per ml was added and the culture was incubated a further 3 h. At this time the bacteria were harvested by centrifugation (12,000 × *g*, 10 min, 4°C) and were washed three times with 0.01 M sodium phosphate-buffered saline (PBS), pH 7.2.

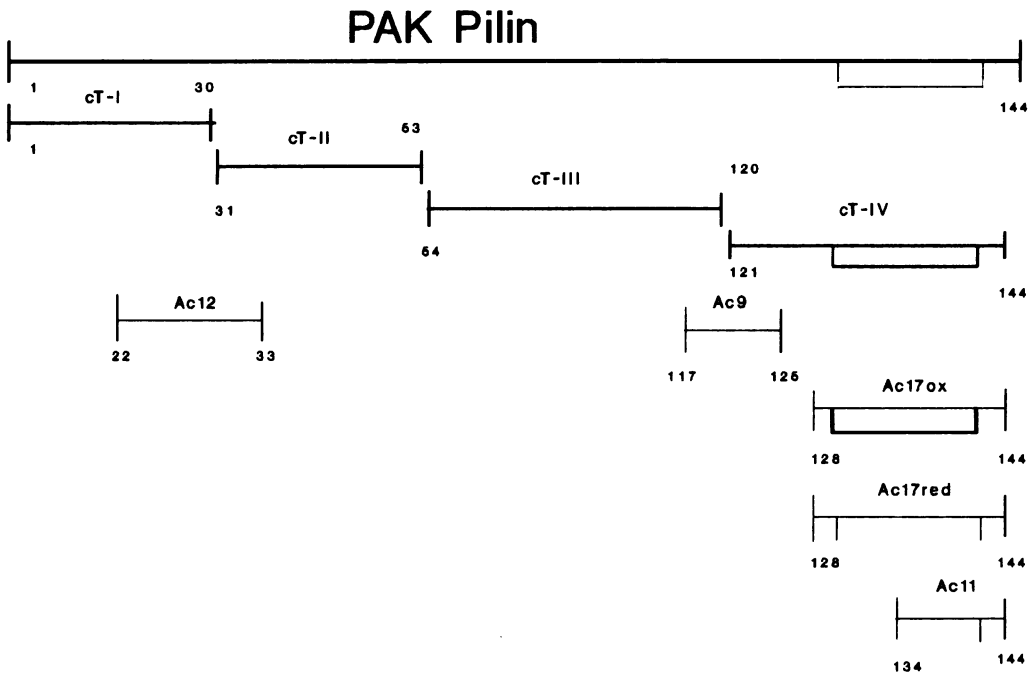

FIG. 1. Schematic representation of the map location of the pilin peptides used in this study. Fragments labeled cT were produced by tryptic digest of PAK pilin, while those labeled Ac were synthetically produced.

Pilus and peptide preparations. PAK and PAO pili were purified as previously described (21, 25). Pilin fragments were prepared as described by Sastry et al. (22). Synthetic peptides Ac17red (residues 128 to 144), Ac17ox, Ac11 (residues 134 to 144), Ac12 (residues 22 to 33), and Ac9 (residues 117 to 125) were prepared as previously described (5). A summary of the peptides used is given in Fig. 1. The cysteine residues of the Ac17red peptide cleaved from the Merrifield chloromethylated resins with hydrogen fluoride were maintained in the reduced state due to the presence of reducing agents during the cleavage process. The oxidation of these cysteine residues to form an intrachain disulfide bridge (Ac17ox) was carried out in a 0.1-mg/ml solution of peptide dissolved in an aqueous buffer (0.05 M NaH_2PO_4 [pH 7.5], containing 0.5 M NaCl and 0.02 M $CuCl_2$) and stirred overnight at room temperature (6). The oxidized peptide was desalted and purified by reversed-phase high-pressure liquid chromatography. The air oxidation was verified by circular dichroism (13). The oxidized peptide showed a distinct negative shift in its relative ellipticity between 250 and 300 nm. Reduction of the air-oxidized peptide with 1.0 mM dithiothreitol gave rise to a circular dichroism spectrum similar to its reduced conformer.

Immunization. BALB/c female mice (Charles River Breeding Laboratories, Inc.) were immunized by weekly intraperitoneal injections of 100 μg of PAK pili in 0.5 ml of PBS supplemented with 1% (wt/vol) $Al(OH)_3$. These injections were performed for 4 weeks. The mice were not immunized for the following 4 weeks. The mice were then further intraperitoneally immunized with 100 μg of PAK pili per mouse in 0.5 ml of PBS weekly for 4 weeks. A final intravenous injection (5 μg per mouse) of PAK pili in PBS was given 4 days prior to the fusion experiment.

Cell line and culture conditions. The mouse myeloma cell line NS1 was cultured in high-glucose Dulbecco modified Eagle medium supplemented with 2 mM L-glutamine and 10% (vol/vol) fetal calf serum (GIBCO Laboratories) at 37°C in the presence of 5% CO_2. Cells were passed every 48 h at a split ratio of 1:5 or 1:4.

Cell fusion and hybridoma selection. Fusion of the NS1 myeloma cells with spleen cells from immunized mice was performed as described by Irvin and Ceri (7), except that the medium was not supplemented with β-mercaptoethanol.

Clones were tested for the ability to synthesize antibody directed against PAK pili by enzyme-linked immunosorbent assay (ELISA). Positive clones were scaled up into 5-ml Dulbecco modified Eagle medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, hypoxanthine, aminopterin, and thymidine. Clones were frozen and subsequently subcloned in semisolid agarose (9, 10).

Ascites tumors were produced by injecting 10^6 hybridoma cells into pristine primed BALB/c male mice (11). Ascites fluid was recovered daily with a 25-gauge needle after the development of an ascites tumor. Typically, 15 ml of ascites fluid was collected over a period of 7 to 10 days.

Purification of antibody and subtyping. Ascites fluid was centrifuged at 12,000 × *g* to remove hybridoma cells. The antibody was partially purified by ammonium sulfate fractionation. The partially purified antibody was dialyzed against PBS, pH 7.4. Antibodies were then subtyped by using the Zymed mouse immunoglobulin subtyping kit. Immunoglobulin G (IgG) antibodies were further purified by applying the partially purified antibody to a protein A-agarose column equilibrated with PBS, pH 7.4. The antibody was eluted from the column with 10 mM citrate buffer, pH 2.75. Affinity-purified antibody was dialyzed against PBS, aliquoted, and frozen at −20°C until further use.

ELISA. Antigens were coated on enzyme immunoassay polystyrene cuvettes (Gilford Instrument Laboratories, Inc.). Antigen (10 to 50 μg/ml in 0.01 M carbonate buffer, pH 9.5) was added to each cuvette (100 μl per cuvette) for 6 h at room temperature. Cuvettes were then washed three times with 250 μl of PBS supplemented with 0.02% (wt/vol) bovine serum albumin (BSA) (buffer A). Cuvettes were blocked

with 5% (wt/vol) BSA in PBS overnight at 4°C. Cuvettes were used immediately or were washed twice with buffer A and frozen at −20°C until future use. To each cuvette, 100 μl of primary antibody was added for 2 h. Each cuvette was then washed three times with 250 μl of buffer A by using aspiration. A goat anti-mouse IgG (heavy plus light chains [H+L]) immunoglobulin-horseradish peroxidase conjugate (Jackson Laboratory) in buffer A (100 μl per cuvette) was added, and the cuvettes were incubated for 2 h at room temperature. The cuvettes were washed three times with buffer A, and 250 μl per cuvette of substrate solution [1 mM 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid), 0.03% (vol/vol) hydrogen peroxide in 10 mM sodium citrate buffer, pH 4.2] was added. The reaction was stopped by the addition of 250 μl per cuvette of 4 mM sodium azide, and A_{405} nm was determined by using an enzyme immunoassay manual reader (Gilford).

Competitive ELISA. Competitor and antibody were mixed together in buffer A for 1 h at room temperature. This mixture was then added to cuvettes (100 μl per cuvette) coated with PAK pili and blocked with BSA as described above. The ELISA was then performed as described above.

Preparation of Fab fragments. Fab fragments were prepared by using immobilized papain (Pierce Chemical Co.). Briefly, antibody was dialyzed against 20 mM cysteine · HCl–10 mM EDTA in 20 mM sodium phosphate buffer, pH 6.2. Antibody (1 ml containing approximately 2 mg of antibody) was added to 0.5 ml of immobilized papain and incubated at 37°C for 20 h (PK99H, PK34C, and PK41C) or for 5 h (PK3B) with shaking at 150 rpm. The immobilized papain was removed by centrifugation, and the supernatant containing the Fab fragments was diluted with 3 ml of 10 mM Tris buffer, pH 8.0. This was then applied to a protein A-agarose column and eluted with 10 mM Tris buffer, pH 8.0. Fab fragments were collected in the flowthrough, while Fc fragments were eluted from the column with 10 mM citrate buffer, pH 2.75. Fab fragments were concentrated by placing the Fab effluent in dialysis tubing (molecular weight cutoff of < 8,000) and extracting liquid from the dialysis sack with polyethylene glycol (molecular weight of 15,000 to 20,000). The fragments were then dialyzed against PBS.

Protein assay. Protein concentrations were determined by the BCA protein assay (Pierce), with BSA as a standard.

Adhesion assays. The effect of Fab fragments on binding was studied as follows. Fab fragments (0.5 ml of 0 to 3,200 μg/ml in PBS) were added to 0.1 ml of bacteria (0.7×10^8 to 2×10^8 CFU/ml) in PBS and incubated for 30 min at room temperature. To this, 0.4 ml of PBS and either 1.0 ml of human BECs (2×10^5 cells/ml) or 1.0 ml of PBS (to assess nonspecific binding of the bacteria to the filters) were added. The mixture was then incubated at 37°C with shaking at 300 rpm for 2 h. The remainder of the filter-based adhesion assay was performed as described by McEachran and Irvin (14), as modified by Doig et al. (5).

Effect of Fab fragments on PAK pilus binding to BECs. An immunoassay was performed to assess the effect of Fab fragments from PK99H and PK34C on the binding of PAK pili to BECs. Fab fragments (0.1 ml of 400 μg/ml or 800 μg/ml) were added to an equal volume of PAK pili (20 μg/ml) in PBS, and they were incubated for 30 min at room temperature. BECs (0.2 ml at 2.0×10^5 BECs per ml) were then added, and the mixture was incubated at 37°C with shaking at 300 rpm in a gyroshaker (New Brunswick Scientific). After 2 h, BECs were collected by centrifugation ($12,000 \times g$ for 2 min) and washed three times with PBS. Antipilus MAb PK3B was added to the BEC pellet (0.2 ml of a 10^{-4} dilution), and the mixture was incubated as described above for 1 h. The BECs were then collected by centrifugation and washed three times with PBS. Goat anti-mouse IgG (H+L) conjugated to peroxidase (Jackson Laboratory) was added to the BEC pellet (0.2 ml diluted 1/5,000), and the mixture was incubated as described above for 30 min. The BECs were collected by centrifugation and washed as described above. The pellet was suspended in 0.2 ml of a solution containing 1 mM 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) and 0.03% (vol/vol) hydrogen peroxide in 10 mM citrate buffer, pH 4.2, and transferred to a clean test tube. The reaction was stopped by the addition of 0.2 ml of 4 mM NaN_3, and the optical density at 405 nm was determined after removal of the BECs by centrifugation. Control values were between A_{405} 0.7 to 1.0. 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.

Polyacrylamide gel electrophoresis and immunoblotting. The discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis described by Laemmli and Favre (12) was used. To each lane, 0.1 μg of PAK or PAO pili heated at 100°C for 15 min in 2% (wt/vol) sodium dodecyl sulfate–5% (vol/vol) β-mercaptoethanol–10% (vol/vol) glycerol in 0.625 mM Tris buffer, pH 6.8, was added. Soluble whole-cell extracts of the various bacterial strains were prepared by adding an equal volume of an overnight culture grown in M-9 medium as described above to the solubilization buffer and heating at 100°C for 20 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was conducted at constant current. The electrophoretically separated material was transferred to nitrocellulose (Schleicher & Schuell, Inc.) by electrophoretic transfer as described by Towbin et al. (24). After transfer, nitrocellulose blots were blocked with 3% (wt/vol) BSA–0.25% (wt/vol) gelatin–0.1% (vol/vol) normal rabbit serum–0.05% (vol/vol) Nonidet P-40–5 mM EDTA–150 mM sodium chloride in 50 mM Tris buffer, pH 7.5, at 37°C for at least 3 h.

Before use, the blot was washed with 0.1% (vol/vol) Tween 20 in 50 mM Tris-buffered saline, pH 7.5 (TTBS) for 10 min at 37°C with shaking at 100 rpm. Primary antibody (10^{-2} dilution of antibody with an endpoint titer of 10^6, determined by ELISA) in TTBS was added, and the mixture was incubated for 2 h as described above. The blot was washed three times with TTBS (10 min per wash). A goat anti-mouse IgG (H+L)-alkaline phosphatase conjugate (Jackson Laboratory) in TTBS was added, and the mixture was incubated for 1 h as described above. The blot was washed three times with TTBS and once with Tris-buffered saline. A substrate solution (NBT/BCIP) consisting of 0.33 mg of Nitro Blue Tetrazolium chloride per ml, 0.165 mg of 5-bromo-4-chloro-3-indolyl phosphate per ml, 100 mM sodium chloride, and 5 mM magnesium chloride in 100 mM Tris buffer, pH 9.5, was added, and color development was stopped by rinsing the blot in distilled water.

Dot blotting. Dot blots were performed by using a Bio-Rad dot blotting manifold. Hydrophobic antigens cT-I and Ac12 (5 μg per well in TTBS) or Bio-Rad molecular weight standards (control for nonspecific binding to coated nitrocellulose) (5 μg per well) were applied to a TTBS-prewetted nitrocellulose membrane. After 2 h, any remaining fluid was drawn through the membrane with a vacuum. The wells were washed four times with TTBS (200 μl per well) and blocked with 100 μl of 3% (wt/vol) BSA in TTBS for 1 h. The wells were then washed four times with TTBS, and the antibody in TTBS was added (100 μl per well) for 1 h. The

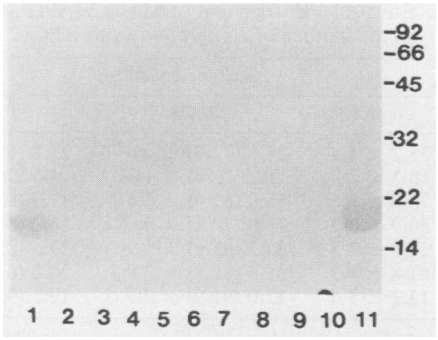

FIG. 2. Western immunoblot of whole-cell extracts of various strains of *P. aeruginosa* and purified pili probed with MAb PK99H (molecular mass markers [kilodaltons] are listed on the right). Lanes: 1, PAK; 2, PAO; 3, PAO 579; 4, HD1; 5, K122-4; 6, 492c; 7, 492a; 8, P1; 9, PAK/3; 10, purified PAO pili; 11, purified PAK pili.

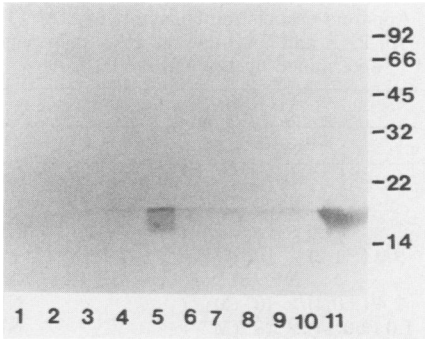

FIG. 4. Western immunoblot of whole-cell extracts of various strains of *P. aeruginosa* and purified pili probed with MAb PK41C (molecular mass markers [kilodaltons] are listed on the right). Lanes: 1, PAK/3; 2, PAO; 3, PAO 579; 4, HD1; 5, PAK; 6, 492c; 7, 492a; 8, P1; 9, K122-4; 10, purified PAO pili; 11, purified PAK pili.

blot was washed four times with TTBS, and 100 μl of a goat anti-mouse IgG (H+L)-alkaline phosphatase conjugate in TTBS was added to each well for 1 h. After washing the blot six times with TTBS, NBT/BCIP substrate was added and color development was stopped by aspiration and rinsing the membrane in distilled water.

RESULTS

Hybridoma production and screening. A library of 262 hybridoma clones that secreted antibody directed against PAK pili was produced. Some of these clones were subcloned and used to produce ascites tumors. Protein A affinity-purified MAbs were then screened against the pilin peptide fragments to determine presumptive specificities. Four of these antibodies, PK99H, PK34C, PK3B, and PK41C, were chosen for further study. These MAbs were immunoglobulin class G with κ light chains. PK99H, PK34C, and PK41C were of subclass 1, and PK3B was subclass 3.

Immunoblots of purified PAK and PAO pili revealed that PK99H (Fig. 2, lanes 11 and 10, respectively) and PK3B (Fig. 5, lanes 11 and 10, respectively) were specific for the PAK pilin subunit, while PK41C (Fig. 4, lanes 11 and 10, respectively) and PK34C (Fig. 3, lanes 11 and 10, respectively) reacted with both pilin types. This was confirmed by using whole-cell extracts from nine strains. PK41C (Fig. 4, lanes 1 through 9) and PK34C (Fig. 3, lanes 1 through 9) reacted with all strains examined, while PK99H (Fig. 2, lanes 1 through 9) and PK3B (Fig. 5, lanes 1 through 9) were PAK specific. Specificity was further examined by competitive ELISA. This methodology was found to be highly sensitive and allows for the calculation of the antibody affinity (15). Competitive ELISA confirmed that PK99H (molecular association constant [K_a] listed in Table 1) and PK3B (K_a for PAK pili = 1.0 [± 0.4] × 10^2 ml/g) were strain PAK specific. PK41C (K_a for PAK pili = 1.0 [± 0.2] × 10^4 ml/g and for PAO pili, 6.1 [± 1.1] ml/g) and PK34C (K_as listed in Table 1) reacted with both PAK and PAO pili, but with a lower affinity to PAO pili.

PK99H reacted with a high affinity to the cT-IV pilin fragment but did not react with cT-II or cT-III (Table 1). Since this MAb was specific for the C-terminal region, the ability of synthetic peptides corresponding to this region (Fig. 1) to react with PK99H was examined. This MAb reacted strongly with the synthetic peptides Ac17red, Ac17ox, and Ac11 of the C-terminal region but not with Ac9. This MAb had its highest affinity for the Ac17 peptide when the cysteine residues in the peptide were in the reduced state (Ac17red) (Table 1).

PK34C also reacted with a high affinity to the cT-IV fragment but did not react with cT-II or cT-III (Table 1). By

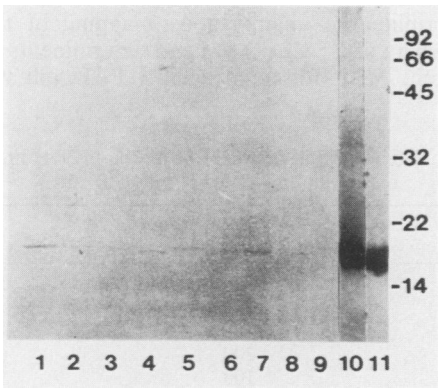

FIG. 3. Western immunoblot of whole-cell extracts of various strains of *P. aeruginosa* and purified pili probed with MAb PK34C (molecular mass markers [kilodaltons] are listed on the right). Lanes are as in the legend to Fig. 2.

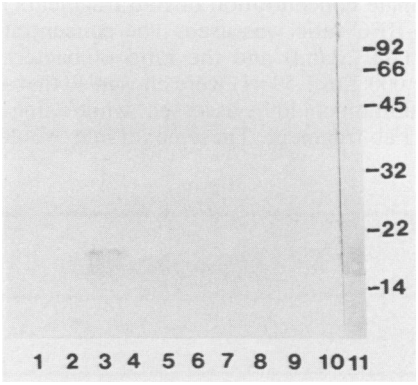

FIG. 5. Western immunoblot of whole-cell extracts of various strains of *P. aeruginosa* and purified pili probed with MAb PK3B (molecular mass markers [kilodaltons] are listed on the right). Lanes: 1, PAO; 2, PAO 579; 3, PAK; 4, PAK/3; 5, 492c; 6, 492a; 7, HD1; 8, P1; 9, K122-4; 10, purified PAO pili; 11, purified PAK pili.

TABLE 1. Apparent association constants of MAbs PK99H and PK34C for PAK and PAO pili and PAK pilin peptides, determined by competitive ELISA

Fragment	Association constant of MAb:	
	PK99H	PK34C
cT-I	ND[a]	ND
cT-II	NE[b]	NE
cT-III	NE	NE
cT-IV	9.9 (±1.2) × 10^{6c} (M^{-1})[d]	5.9 (±.7) × 10^7 (M^{-1})
Ac9	NE	7.5 (±1.7) × 10^5 (M^{-1})
Ac11	2.4 (±0.4) × 10^6 (M^{-1})	NE
Ac17re	1.0 (±0.14) × 10^8 (M^{-1})	NE
Ac17ox	3.4 (±2.2) × 10^7 (M^{-1})	7.6 (±2.5) × 10^5 (M^{-1})
PAK pili	3.0 (±1.0) × 10^2 (ml/g)[e]	3.8 (±1.7) × 10^2 (ml/g)
PAO pili	NE	5.6 (±2.3) (ml/g)

[a] Not done.
[b] No effect.
[c] Mean ± standard deviation.
[d] Molar association constants of the MAb were determined as described by Nieto et al. (15).
[e] Affinity constants for MAb binding to purified pili are expressed in milliliters per gram rather than as a molar affinity constant because of the polydispersed size distribution of purified pili.

using the synthetic peptides corresponding to the C-terminal region of the pilin subunit (Fig. 1), PK34C was found to react with synthetic peptides Ac9 and Ac17ox but with reduced affinities compared with cT-IV. Neither peptide Ac17red nor peptide Ac11 had any effect on this MAb (Table 1).

None of the pilin fragments competed with PK41C in a conventional competitive ELISA. However, this MAb reacted with the N-terminal peptide cT-I and synthetic peptide Ac12 when the fragments were coated on ELISA plates or in a dot blot (Fig. 6).

PK3B did not react with any of the pilin fragments or synthetic peptides with ELISA, competitive ELISA, or a dot blot methodology.

Effect of MAbs on adhesion. All antibodies were tested for their ability to inhibit PAK binding to BECs. Fab fragments of each antibody were used to avoid agglutination of the bacteria by the bivalent antibodies. PK99H and PK34C Fab fragments were found to inhibit PAK binding in a concentration-dependent manner (Fig. 7), while PK41C and PK3B had no effect.

In order to examine the effects of Fab fragments from PK99H and PK34C on the binding of different strains to BECs, a single concentration of Fab fragments and a fixed bacteria-to-BEC ratio was used. The concentration of Fab fragments (100 μg/ml) and the ratio of bacteria to BECs (between 1,000:1 to 1,500:1) were chosen so that a significant level of inhibition could be assessed, while using a minimum amount of Fab fragment. These conditions would not result

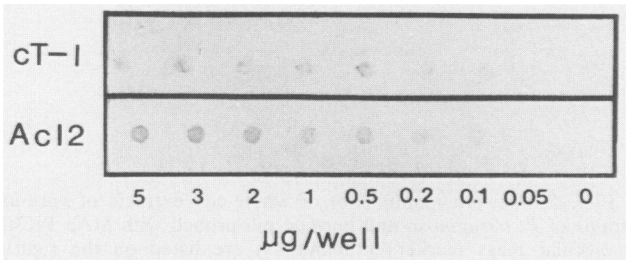

FIG. 6. Immunodot blot of pilin fragment cT-I and synthetic peptide Ac12 probed with MAb PK41C.

TABLE 2. Effect of Fab fragment of MAbs PK99H and PK34C on the binding of *P. aeruginosa* to BECs

Strain	Bacteria bound/BEC[a]		
	Control[b]	PK99H	PK34C
PAK	35.2 ± 1.4	25.6 (72.7) ± 1.0	23.8 (67.5) ± 1.7[c]
PAO	50.5 ± 2.0	55.5 (110) ± 11.1	45.9 (91) ± 0.7[c]
HD1	38.0 ± 3.6	31.0 (81.6) ± 3.5	31.3 (82.5) ± 1.1[c]
492c	30.9 ± 0.2	23.8 (77.1) ± 0.5[c]	26.2 (84.8) ± 1.4[c]
P1	36.3 ± 2.5	34.7 (95.6) ± 5.9	29.5 (81.3) ± 0.2[c]
K122-4	41.8 ± 1.5	38.3 (91.8) ± 2.8	28.0 (67.1) ± 0.4[c]
PAK/3	13.1 ± 1.4	12.0 (91.9) ± 0.7	12.2 (93.3) ± 0.6

[a] The concentration of PK99H and PK34C Fab used in the inhibition assays was 100 μg/ml and had a titer of 10^5 by ELISA, using PAK pili as the antigen (coated at 1 μg per well). Given is the mean ± the standard deviation. The percent of control is given in parentheses.
[b] Control value when 100 μg of Fab fragments per ml produced from normal mouse IgG was added. No difference was noted between these values and those from tubes to which no Fab fragments were added.
[c] The significant difference ($P < 0.05$) was determined by using the Student *t* test.

in maximal inhibition of binding. Fab fragments from PK99H had no effect on the binding of other bacterial strains tested, except strain 492c, while PK34C inhibited all pilus-producing strains examined, but to various extents (Table 2). Fab fragments produced from normal mouse polyclonal IgG had no effect on bacterial binding (the normal mouse IgG had no detectable specificity for PAK or PAO pili with ELISA).

Effect of MAbs on pilus binding. Fab fragments of PK99H and PK34C were found to inhibit the binding of PAK pili to BECs (Table 3). At a concentration of 200 μg/ml, pilus binding was virtually eliminated by either MAb. Fab fragments prepared from normal mouse IgG had no effect on binding.

DISCUSSION

One mechanism by which *P. aeruginosa* binds to an epithelial surface is mediated by pili (5, 19, 26), and pili have been shown to be a virulence factor in burn wound infections (23). Ramphal et al. (19) found that pili from heterologous strains could inhibit the binding of *P. aeruginosa* to epithelial cells, but polyvalent sera directed against heterologous pili could not. If pili are antigenically variable in the binding-domain region of the pilus, as suggested by Ramphal et al. (19), preventing pilus-mediated binding of multiple strains to an epithelial surface would be difficult.

To determine the unambiguous mapping of the pilus-binding domain of *P. aeruginosa* and determine its antigenic conservation, MAbs directed against PAK pili were pro-

TABLE 3. Effect of Fab fragment of MAbs PK99H and PK34C on the binding of PAK pili to BECs

Fab fragment	Concentration[a] (μg/ml)	% of control
PK99H	100	53.5 ± 3.3
PK99H	200	7.5 ± 0.6
PK34C	100	44.5 ± 0.1
PK34C	200	4.5 ± 0.7
IgG[b]	100	95.6 ± 0.9
IgG	200	94.9 ± 2.2

[a] Final concentration of Fab fragments. The final concentration of PAK pili used was 5 μg/ml.
[b] Fab fragments prepared from normal mouse IgG.

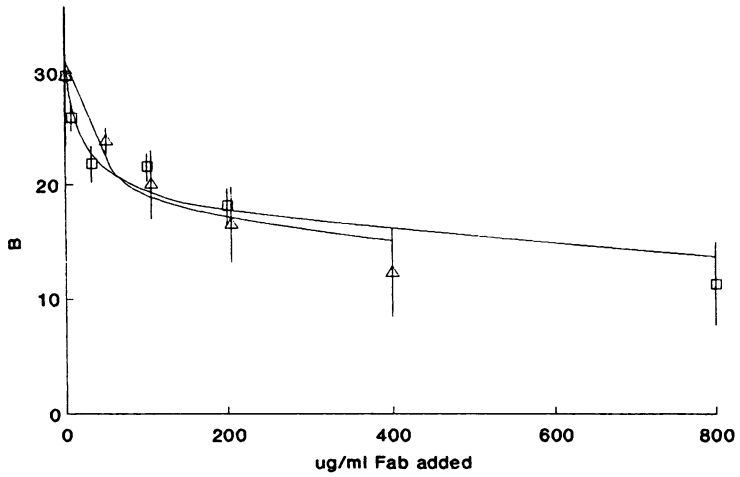

FIG. 7. Inhibition of PAK binding to BECs by Fab fragments of monoclonal antibodies PK99H (□) and PK34C (△) (ratio of bacteria to BECs was 1,101 to 1).

duced. Previously, the C-terminal region of pilin has been implicated as the region containing the binding domain for BECs (5, 16). This was done by using pilin fragments or synthetic peptides of the pilin and examining their effect on PAK binding. Small peptides, such as those used previously, do not maintain the exact three-dimensional structure that is found within the intact pilus. MAbs allow for the investigation of specific domains of the pilus.

Four MAbs to PAK pili were examined. PK99H was found to be specific for the C-terminal region of the pilin. This antibody reacted with synthetic peptides Ac11, Ac17red, and Ac17ox but not with Ac9, indicating that the antigenic epitope lay within the Ac17 region of the pilin and involved the Ac11 region. PK99H reacted with the highest affinity for Ac17red, demonstrating that the epitope likely does not rely on the three-dimensional shape produced by the disulfide bridge, but rather on the primary sequence. The lower affinity for Ac11 compared with Ac17 demonstrated that the epitope was not completely contained within this peptide.

Further information concerning the nature of the epitope was gained from the fact that PK99H did not react with the pilin produced by most of the strains examined. Examining the amino acid sequence of the Ac11 region in relation to that of other strains that have been sequenced reveals that the C-terminal amino acid residues of Ac11 are fairly conserved (17, 18, 21). This region would not be expected to be part of a strain-specific epitope. However, the region encompassing amino acid residues 130 to 138 (the N-terminal portion of Ac11 and the four amino acid residues immediately preceding Ac11) exhibits less conservation. This region fulfills all the criteria for the map location of the epitope recognized by PK99H on the basis of results presented here. Therefore, the epitope likely lies within this region.

PK99H reacted preferentially with the Ac17red peptide even though the mice from which the spleen cells were obtained for the fusion were immunized with intact pili. Two explanations would account for the production of such a clone. The first is that a small portion of the pilin monomers in the immunogen preparation possess cysteines in the reduced state, thus stimulating the production of a clone such as PK99H. The second explanation is that this clone was induced by pilin with oxidized cysteines, since PK99H recognizes the oxidized form.

PK34C also recognized the C-terminal region of the pilin. In addition to the cT-IV fragment, PK34C recognized Ac9 and Ac17ox. Part of Ac9 is located within the cT-IV fragment region. The epitope for PK34C would involve both a part of the Ac9 region and the Ac17 region. However, unlike PK99H, the disulfide bridge must be intact in order for PK34C to bind. The secondary-structure requirement for this epitope makes the prediction of the amino acid residues involved in antibody recognition more difficult, since the bending introduced by the disulfide bridge might bring more distantly located amino acid residues into a closer proximity. Ac9 and a number of residues in Ac17 (especially those in the Ac11 region) exhibit strain conservation (17, 18, 21). These residues are more likely to be involved in the epitope, since PK34C is cross-reactive.

PK41C was found to cross-react with the pilin from all strains examined and was N terminus specific. In this case the epitope was located at the C-terminal region of the cT-I pilin fragment, involving the amino acids between residues 22 through 33. This region is highly conserved between strains of *P. aeruginosa*, as well as other genera, including *Neisseria* and *Moraxella* (4, 16). Since this MAb reacts with the highly conserved N terminus of the pilin, it may be used as a probe to detect pilus production or for examination of pilus structure, assembly, and processing.

PK3B did not react with any of the fragments or synthetic peptides used. This indicated that the epitope recognized by this antibody is likely dependent on the three-dimensional structure of the pilin or the epitope is destroyed by the cleavage of pilin into smaller fragments.

Fab fragments of PK99H and PK34C inhibited binding of PAK to BECs (Fig. 7), while Fab fragments of PK41C, PK3B, or normal polyvalent mouse IgG had no effect on binding. Fab fragments of PK99H and PK34C did not affect the binding of PAK/3 (a nonpiliated mutant obtained from strain PAK).

PK99H was predominantly strain specific and may indicate that the pilus-binding domain is strain specific and immunologically poorly conserved. This MAb did inhibit the adherence of 492c to BECs. Strain 492c has a pilin that differs from PAK pilin, based on restriction polymorphisms of the pilin gene and whole-cell adherence kinetics, but the sequence of this pilin gene has not yet been determined (14, 16; unpublished data). However, since this MAb did not

react with strain 492c on immunoblots (Fig. 2), it likely has a lower affinity for 492c pilin than PAK pilin when denatured with sodium dodecyl sulfate. PK99H may have also inhibited the binding of HD1 to BECs to some extent; however, this inhibition was not statistically significant. PK34C inhibited the adhesion of all pilus-producing strains tested. Further, Fab fragments of PK99H and PK34C inhibited the binding of PAK pili to BECs. Binding of pili to BECs could virtually be eliminated under the conditions tested. The complete inhibition of binding was not obtained by using whole bacteria, even when relatively high concentrations of Fab fragments were used. This may have been due to proteolysis of the Fab fragments by the bacteria or poor epitope accessibility on the bacterium or both. Further, the inability to completely inhibit whole-cell binding, while completely inhibiting the binding of purified pili, raises the possibility that the bacterium can bind to BECs by a nonpilus adhesin.

Earlier studies in which pilin fragments were used indicated that the pilus-binding domain was affected by the state of oxidation of the intrachain disulfide bridge in the C-terminal region of the pilin (5). The oxidized disulfide bridge was more effective in affecting bacterial binding. PK99H apparently recognizes primary structure determinants on the pilin with reduced cysteine, increasing the affinity of the antibody. This MAb, therefore, likely does not recognize the three-dimensional structure of the binding domain but, rather, a region within or near the binding domain. However, PK34C binds more effectively to the oxidized form of the Ac17 peptide and thus may recognize the binding domain of the pilin. The cross-reactive nature of PK34C and the ability of this MAb to inhibit the binding of a number of strains of *P. aeruginosa* to BECs indicates considerable conservation of the pilin epithelial cell-binding domain between strains.

ACKNOWLEDGMENTS

This investigation was supported by the Canadian Cystic Fibrosis Foundation (R.T.I. and W.P.) and the Medical Research Council of Canada (W.P. and R.H.). P.D. was supported by a postgraduate fellowship from the Natural Sciences and Engineering Research Council, Canada.

LITERATURE CITED

1. **Adams, M. H.** 1959. Methods of study of bacterial viruses, p. 443–452. *In* M. H. Adams (ed.), Bacteriophages. Interscience Publishers, Inc., New York.
2. **Bradley, D. L.** 1980. A function of *Pseudomonas aeruginosa* PAO polar pili: twitching motility. Can. J. Microbiol. **26:** 146–154.
3. **Bradley, D. E., and T. L. Pitt.** 1974. Pilus-dependence of four *Pseudomonas aeruginosa* bacteriophages with non-contractile tails. J. Gen. Virol. **23:**1–15.
4. **Deal, C. D., J. A. Tainer, M. So, and E. D. Getzoff.** 1985. Identification of a common structural class for *Neisseria gonorrhoeae* and other bacterial pilins, p. 302–308. *In* G. K. Schoolnik (ed.), The pathogenic neisseriae. American Society for Microbiology, Washington, D. C.
5. **Doig, P., T. Todd, P. A. Sastry, K. K. Lee, R. S. Hodges, W. Paranchych, and R. T. Irvin.** 1988. Role of pili in the adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells. Infect. Immun. **56:**1641–1646.
6. **Hodges, R. S., A. K. Saund, P. C. S. Chong, S. A. St.-Pierre, and R. E. Reid.** 1981. Synthetic model for two-stranded o-helical coiled-coils. Design, synthesis, and characterization of an 86-residue analog of tropomyosin. J. Biol. Chem. **256:**1214–1224.
7. **Irvin, R. T., and H. Ceri.** 1985. Immunochemical examination of the *Pseudomonas aeruginosa* glycocalyx: a monoclonal antibody which recognizes L-guluronic acid residues of alginic acid. Can. J. Microbiol. **31:**268–275.
8. **Irvin, R. T., J. W. R. Govan, J. A. M. Fyfe, and J. W. Costerton.** 1981. Heterogeneity of antibiotic resistance in mucoid isolates of *Pseudomonas aeruginosa* obtained from cystic fibrosis patients: role of outer membrane proteins. Antimicrob. Agents Chemother. **19:**1056–1063.
9. **Kennett, R. H.** 1980. Fusion of cells suspended in polyethyleneglycol, p. 365–367. *In* R. H. Kennett, J. J. McKearn, and K. B. Bechtol (ed.), Monoclonal antibodies. Hybridomas: a new dimension in biological analyses. Plenum Publishing Corp., New York.
10. **Kennett, R. H.** 1980. Freezing of hybridoma cells, p. 375. *In* R. H. Kennett, J. J. McKearn, and K. B. Bechtol (ed.), Monoclonal antibodies. Hybridomas: a new dimension in biological analyses. Plenum Publishing Corp., New York.
11. **Kroprowski, H., W. Gerhard, and C. M. Groce.** 1977. Production of antibodies against influenza virus by somatic cell hybrids between mouse myeloma and primed spleen cells. Proc. Natl. Acad. Sci. USA **74:**2985–2988.
12. **Laemmli, U. K., and M. Favre.** 1973. Maturation of the head of bacteriophage T4. DNA packaging events. J. Mol. Biol. **80:** 575–599.
13. **Matsoukas, J. M., M. N. Scanlon, and G. J. Moore.** 1984. A cyclic angiotensin antagonist: [1,8-cysteine]angiotensin II. J. Med. Chem. **27:**404–406.
14. **McEachran, D. W., and R. T. Irvin.** 1985. Adhesion of *Pseudomonas aeruginosa* to human buccal epithelial cells: evidence for two classes of receptors. Can. J. Microbiol. **31:**563–569.
15. **Nieto, A., A. Gaya, M. Jansa, C. Moreno, and J. Vives.** 1984. Direct measurement of antibody affinity distribution by hapten-inhibition enzyme immunoassay. Mol. Immunol. **21:**537–543.
16. **Paranchych, W., P. A. Sastry, K. Vopel, B. A. Loh, and D. Speert.** 1986. Fimbriae (pili): molecular basis of *Pseudomonas aeruginosa* adherence. Clin. Invest. Med. **9:**113–118.
17. **Pasloske, B. L., B. B. Finlay, and W. Paranchych.** 1985. Cloning and sequencing of *Pseudomonas aeruginosa* PAK pilin gene. FEBS Lett. **183:**408–412.
18. **Pasloske, B. L., P. A. Sastry, B. B. Finlay, and W. Paranchych.** 1988. Two unusual pilin sequences from different isolates of *Pseudomonas aeruginosa*. J. Bacteriol. **170:**3738–3741.
19. **Ramphal, R., J. C. Sadoff, M. Pyle, and J. D. Silipigni.** 1984. Role of pili in the adherence of *Pseudomonas aeruginosa* to injured tracheal epithelium. Infect. Immun. **44:**38–40.
20. **Rivera, M., and M. B. Nicotra.** 1982. *Pseudomonas aeruginosa* mucoid strain. Its significance in adult chest diseases. Am. Rev. Respir. Dis. **126:**833–836.
21. **Sastry, P. A., B. B. Finlay, B. L. Pasloske, W. Paranchych, J. R. Pearlstone, and L. B. Smillie.** 1985. Comparative studies on the amino acid and nucleotide sequences of pilin derived from *Pseudomonas aeruginosa* PAK and PAO. J. Bacteriol. **164:** 571–577.
22. **Sastry, P. A., J. R. Pearlstone, L. B. Smillie, and W. Paranchych.** 1985. Studies on the primary structure and antigenic determinants of pilin isolated from *Pseudomonas aeruginosa* K. Can. J. Biochem. Cell Biol. **63:**284–291.
23. **Sato, H., K. Kinaga, and H. Saito.** 1988. Role of pili in the pathogenesis of *Pseudomonas aeruginosa* burn infection. Microbiol. Immunol. **32:**131–139.
24. **Towbin, H., P. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
25. **Watts, T. H., P. A. Sastry, R. S. Hodges, and W. Paranchych.** 1983. Mapping of antigenic determinants of *Pseudomonas aeruginosa* PAK polar pili. Infect. Immun. **42:**113–121.
26. **Woods, D. E., D. C. Straus, W. G. Johanson, Jr., V. K. Berry, and J. A. Bass.** 1980. Role of pili in the adherence of *Pseudomonas aeruginosa* to mammalian buccal epithelial cells. Infect. Immun. **29:**1146–1151.