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Evidence for Pili-Mediated Adherence of *Klebsiella pneumoniae* to Rat Bladder Epithelial Cells In Vitro

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The possible role of pili in the pathogenesis of urinary tract infections caused by *Klebsiella pneumoniae* was studied in an in vitro mixture of a phosphate-buffered saline suspension of rat bladder epithelial cells and phosphate-buffered saline-washed *K. pneumoniae*. Nonpiliated and piliated populations derived from a single *K. pneumoniae* strain were obtained by controlling the total time of growth in broth medium. The piliated phase demonstrated a significant increase in adherence when compared to the nonpiliated phase. Incubation of the bacteria and epithelial cell mixture at 4 and 37°C resulted in no differences in adherence; optimal adherence occurred at pH 5. Pretreatment of the bacteria with enzymes to destroy the pili resulted in a decrease in adherence, as did killing the bacteria by various means before adherence testing. Pretreatment of the epithelial cells with certain saccharides inhibited bacterial adherence. Finally, a 96% decrease in adherence was observed after coincubation of bacteria and epithelial cells with papain-treated antipili antibodies. Thus, it appears that pili on the surface of *K. pneumoniae* mediate attachment of the bacteria to rat bladder epithelial cells.

The adherence of bacteria to mammalian cell surfaces mediated by bacterial surface appendages called pili or fimbriae has long been recognized (2, 5-7). However, it is still a matter of some conjecture as to whether the pili-mediated attachment is correlated to the virulence of the organism. Silverblatt has demonstrated by electron microscopy that *Proteus* appears to attach to renal pelvis epithelium by pili (15). The attachment is followed by colonization of the tissue, eventually leading to intracellular penetration by the bacteria. Pili on the surface of gonococci have been shown to mediate initial attachment of the organisms to the urogenital tract (20), as well as to serve as an antiphagocytic mechanism (11). Piliated *Salmonella* have been demonstrated to be more virulent than nonpiliated strains in mice when the infection is initiated orally, but not when it is initiated by the intraperitoneal route (4).

Salit and Gotschlich have recently demonstrated that purified type I pili from *Escherichia coli* attach to monkey kidney cells in tissue culture (14) and hemagglutinate guinea pig erythrocytes (RBC) (13).

Gillies and Duguid have noted that piliation of *Shigella flexneri* strains occurred to a higher degree after serial passage of the organisms in broth culture medium and that piliation decreased, but did not totally disappear, after serial culture on solid agar medium (8). The study reported here has utilized this property, present also in many *Klebsiella* strains, to investigate the possibility of pili-mediated adherence of *Klebsiella pneumoniae* to rat bladder epithelial cells by the piliated and nonpiliated phases of a *K. pneumoniae* strain isolated from a human urinary tract infection.

MATERIALS AND METHODS

Bacteria. The *K. pneumoniae* strain was donated by the bacteriology laboratory in The John Sealy Hospital, Galveston, Tex. The strain was isolated from a urinary tract infection, and species identification was confirmed with the Minitek differentiation system (BBL Microbiology Systems [BBL], Cockeysville, Md.). The nonpiliated form of the organism was maintained on nutrient agar (BBL) and stored at 4°C. The piliated phase was produced by serial passage in broth medium over the course of 1 week. Piliation was confirmed by transmission electron microscopy (TEM) and by hemagglutination of guinea pig RBC. The piliated phase was maintained in Trypticase soy broth (BBL) with added dextrose (5 g/liter) and stored at −70°C. To prepare cultures for adherence testing, a loopful of the nonpiliated or piliated phase was inoculated into 10 ml of Trypticase soy broth with added dextrose and incubated at 37°C for 24 h. Before adherence testing, organisms were tested for hemagglutinating ability to assay the degree of piliation in the population.

Rats. Female Sprague-Dawley (ARS, Madison, Wis.) or male or female Sprague-Dawley (Timco, Houston, Tex.) rats were used to obtain bladder epi-

thelial cells. Rats were given water and standard rat chow ad libitum.

Adherence testing. Twenty-four-hour broth cultures of the piliated or nonpiliated phases of the *K. pneumoniae* isolate were pelleted three times by centrifugation in phosphate-buffered saline (PBS; 10 ml; 10 min, 260 × *g*) and resuspended in PBS (pH 7.2) to give approximately 10^9 bacteria per ml. A rat bladder was removed aseptically and cut longitudinally to expose the inner surface. The outer surface was spread evenly over a glass microscope slide, and the inner surface was scraped with the edge of a glass slide. The bladder cells were rinsed off the microscope slide with PBS, collected, and washed three times (10 ml of PBS, 10 min, 50 × *g*) before being resuspended in PBS (pH 7.2) to a concentration of 10^5 epithelial cells per ml as determined by quantitation in a Petroff-Hausser counting chamber. A mixture of 0.5 ml of the bacterial suspension and 0.5 ml of the epithelial cell suspension was incubated at 37°C for 1 h on a rotary shaker (150 rpm). After incubation, unattached bacteria were removed by a series of differential centrifugations (5 ml of PBS, 10 min, 50 × *g*). The final pellet, consisting of epithelial cells with attached bacteria, was resuspended in a drop of PBS, transferred by pipette, dropped (not spread) onto a glass microscope slide, air dried overnight, and stained with methylene blue. Adherent bacteria on 20 epithelial cells were counted, and the average number of bacteria per epithelial cell was determined. The counting process originated in the upper left corner of the slide and proceeded over, down one field, and back across in a grid pattern until 20 epithelial cells of approximately 3,500 μm^2, when viewed under ×1,000 magnification, were encountered. It was decided that 20 epithelial cells were representative of the population as a result of pilot studies in which quantitation of bacteria attached to as many as 80 epithelial cells showed no significant differences in the average number of adherent organisms per cell. These results are in agreement with studies by Svanborg Eden et al. on *E. coli* adherence to human uroepithelial cells (18). In experiments that required the sacrifice of more than one rat, epithelial cells from scraped bladders were pooled, washed three times in PBS, and adjusted to 10^5 cells per ml as described above before aliquots were taken for adherence studies. This ensured a uniform population of epithelial cells in each test suspension. Epithelial cells were utilized immediately after experimental preparation to ensure viability. Approximately 90% of the epithelial cells were viable, as shown by trypan blue exclusion tests, after the final centrifugation to remove unattached bacteria.

Electron microscopy. Negative stains of bacteria were performed by using the following technique. Bacteria were fixed for 2 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate at 37°C. One drop of the bacterial suspension was placed on a 0.25% Formvar-coated copper grid and allowed to stand for 5 min. The excess drop was removed with a pointed piece of filter paper, and a drop of distilled water was added to the grid for 30 s. This drop was also removed with filter paper, and a drop of freshly prepared 0.1% phosphotungstic acid was placed on the grid for exactly 30 s. Excess stain was removed with filter paper, and the grid was allowed to dry overnight before examination by TEM for the presence of pili. Pili diameters were determined by the use of a calibrated grid (no. 1002, Ernest F. Fullam, Inc., Schenectady, N.Y.), with measurements taken by micrometer directly from negatives of photomicrographs.

Hemagglutination tests. Heparinized guinea pig RBC were washed twice in 0.85% saline and resuspended to a concentration of 3% (vol/vol) in saline. One drop of the RBC suspension was added to 1 drop of a 24-h broth culture of *K. pneumoniae* (10^{10} to 10^{11} bacteria per ml) on a glass microscope slide or in depressions on a porcelain tile. The slide or tile was gently rocked by hand for 2 min, and if no hemagglutination was present at the end of that time, the slide or tile was transferred to a humidity chamber for an additional 30 min. If no hemagglutination appeared at the end of 30 min, the test was recorded as negative. Cultures demonstrating hemagglutination within 2 min were considered to be piliated; cultures with no hemagglutination at the end of 30 min were considered to be nonpiliated. No intermediate values were ever observed when cultures were assayed for hemagglutination before adherence testing. Treated bacteria were also tested for hemagglutinating ability before and after treatments. If hemagglutination was present after treatment, D-mannose (25 mg/ml in PBS) was added to the bacteria and guinea pig RBC suspension to determine if hemagglutination inhibition would occur.

Saccharide inhibition. D-Mannose, alpha-methyl-D-mannoside, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine (Sigma Chemical Co., St. Louis, Mo.), D-glucose, or maltose (BBL) was dissolved in PBS to a concentration of 25 μg/ml and used to pretreat scraped rat bladder epithelial cells for 20 min before the addition of bacteria for adherence testing.

Enzyme treatment of bacteria. PBS-washed bacteria were treated for 1 hour at 37°C with 2 mg of pepsin (Sigma Chemical Co.) per ml in PBS (pH 6.0) or 2 mg of trypsin (ICN Pharmaceuticals Inc., Cleveland, Ohio) per ml in PBS (pH 8.0). After treatment, the bacteria were washed three times in PBS to remove the enzyme before adherence testing.

Bacterial death. Bacteria were killed before adherence testing by the following procedures. Unwashed bacteria were autoclaved for 30 min at 121°C and 15-lb/in^2 pressure. PBS-washed bacteria were resuspended in 1% formaldehyde (Matheson, Coleman & Bell, Norwood, Ohio) and incubated with shaking for 48 h at 37°C. Unwashed bacteria were treated for 2.5 h in an ethylene oxide-filled chamber and removed without aeration. All treated cultures were washed in PBS as described above before adherence testing and also streaked on nutrient agar (BBL) plates to ensure sterility.

Antiserum. The piliated phase of the *K. pneumoniae* isolate was used to obtain antipili antibodies. The organisms were fixed overnight in 2.5% glutaraldehyde, washed twice in 0.85% saline, and finally resuspended to a concentration of 10^9 bacteria per ml. Antiserum to pili was obtained by the method of Gillies and Duguid (8). The course of immunization in adult rabbits consisted of six injections given at 3-day intervals. Two subcutaneous injections of 0.25 ml were followed

by two intravenous injections of 0.25 ml. The final two inoculations were 0.5 ml given intravenously. Boosters consisting of 0.5 ml were given intravenously at monthly intervals. Cardiac punctures were used to obtain blood from the rabbits. For 10 ml of antipili antiserum, a series of six absorptions with the nonpiliated phase of the organism was necessary. Bacteria from 75 nutrient agar (BBL) plates were harvested and washed in 0.85% saline. The bacteria were divided equally into six portions, two of which were boiled for 60 min at 100°C to disrupt the bacteria and expose thermostable interior antigens. The bacteria were pelleted by centrifugation (2,445 × *g*, 20 min), and the supernatant was discarded. The antiserum was added to the bacterial pellet and emulsified with a sterile glass stirring rod. The mixture was held at 37°C for 1 h and then centrifuged (2,445 × *g*, 20 min). The antiserum was transferred to the next pellet and the procedure was repeated. The final two pellets consisted of the disrupted bacteria. The resultant antipili antiserum was divided into 1-ml aliquots and stored at −70°C. The same procedures were used to obtain antiserum to the nonpiliated phase. This antiserum was absorbed with the piliated phase as described, except broth cultures were used to obtain piliated organisms for the absorption process.

Antiserum titers were determined before and after absorption by agglutination tests performed in microtiter plates (Cooke Engineering Co, Alexandria, Va.). Serial twofold dilutions of antiserum were carried out, after which 25 μl of a PBS-washed bacterial suspension (10^8 or 10^9 bacteria per ml) was added to each well. Plates were incubated for 1 h at 37°C and stored overnight at 4°C, and titers were determined the following morning. Titers are stated as reciprocals of the highest dilution resulting in positive agglutination. Serum protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). Absorption of antiserum to the piliated phase, with the nonpiliated phase, reduced antibody titers to the nonpiliated phase from 512 to 2. Antibody titer to the piliated phase remained at 1,024, the highest dilution tested, both before and after the absorption. Absorption also reduced the protein concentration of the antiserum to the piliated phase from 390 mg/ml before absorption to 97 mg/ml after absorption. Antiserum obtained to the nonpiliated phase contained a titer of 64 to the nonpiliated phase and 512 to the piliated phase. After absorption with the piliated phase, these titers were reduced to 4 for the nonpiliated phase and 2 for the piliated phase. Absorption reduced the protein concentration in the antiserum to the nonpiliated phase from 180 to 35 mg/ml after absorption. The results of these titer determinations indicate that the antiserum against the piliated phase, after absorption, contained antibodies directed mainly against pili and not against other bacterial surface antigens.

Aliquots of antiserum to pili were then treated with papain (Sigma Chemical Co.) to cleave the antibody molecules into Fc and Fab fragments before adherence testing. Aliquots of 1.0 ml were treated with 0.1 ml of papain (25 mg/ml) for 4 h at 37°C, after which 0.001 g of *p*-chloromercuribenzoic acid was added to each aliquot to stop the reaction (12). The antiserum was added to the bacteria and epithelial cell suspension and incubated as described above. To ensure that any decrease in adherence was due to Fab fragments and not to enzyme or inhibitor, controls consisting of papain and *p*-chloromercuribenzoic acid in saline and *p*-chloromercuribenzoic acid alone in saline were also incubated with suspensions of bacteria and epithelial cells.

RESULTS

Adherence of *K. pneumoniae* to bladder epithelial cells. In examining various clinical strains of *Klebsiella* isolated from human urinary tract infections, it was noted that a strain previously believed to be nonpiliated by a failure to hemagglutinate guinea pig RBC and by the lack of observable pili after TEM examination exhibited pellicle formation if left in broth culture for at least 1 week. The formation of a pellicle on the surface of broth medium is characteristic of piliated enteric bacteria (5). Consequently, a study was undertaken to determine the effects of daily passages in broth medium on the ability of the *K. pneumoniae* isolate to adhere to rat bladder epithelial cells in vitro. The results of this study are shown in Fig. 1. A daily increase in adherence was noted, whereas the control, a previously converted piliated phase of the strain, exhibited a constant level of adherence. Bacteria were examined daily by TEM to determine the degree of piliation. Although a few organisms on day 1 appeared to have thin-diameter pili present on the bacterial surface, the majority of organisms observed were nonpiliated (Fig. 2a and b). By day 3, the majority of organisms observed were either nonpiliated or piliated with thin pili. One organism on day 3 was observed to possess type I pili (7 nm). This organism is shown in Fig. 3a and b. Days 5 and 7 revealed a further increase in the number of organisms exhibiting type I pili, and by day 10 the majority of organisms examined were uniformly piliated and indistinguishable from the organism in Fig. 3. Thus, it appeared that the appearance of type I pili corresponded to the increase in bacterial adherence to bladder epithelial cells. It was also evident that extended passages in broth medium did not diminish the adherence capability of the previously converted piliated control and that the 24-h broth culture inoculated from agar medium contained a population of bacteria in a predominately nonpiliated phase.

The remaining experiments described below were performed with 24-h broth cultures of the *K. pneumoniae* isolate subcultured either from broth cultures to obtain the piliated phase or from solid agar to obtain the nonpiliated phase.

Adherence of piliated and nonpiliated

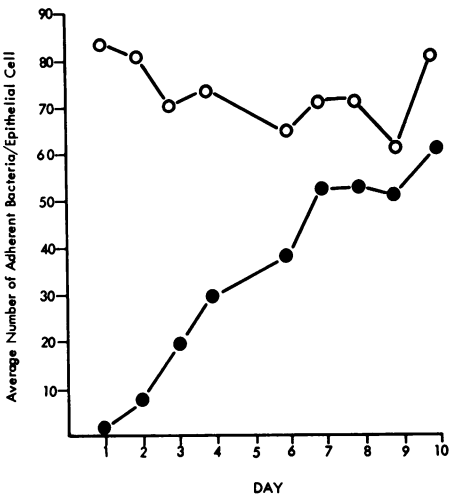

FIG. 1. *Effect of daily passage in broth medium on adherence of K. pneumoniae to bladder epithelial cells in vitro. PBS-washed K. pneumoniae (10^9 bacteria per ml) were mixed with PBS-washed, scraped rat bladder epithelial cells (10^5 cells per ml) and incubated for 1 h at 37°C with shaking. Unattached bacteria were removed by differential centrifugations. Epithelial cells with attached bacteria were fixed and stained, and the number of bacteria adhering to 20 epithelial cells was determined. Symbols: (○) On day 0, 1 ml of a broth culture of a previously converted piliated K. pneumoniae strain was inoculated into 10 ml of broth medium. (●) On day 0, nonpiliated-phase organisms from agar medium were inoculated into broth medium. On subsequent days, 1 ml of the bacterial suspension used for the adherence test was inoculated into 10 ml of broth medium for adherence testing the following day. On days 1 and 10, both the nonpiliated- and piliated-phase organisms gave identical biochemical reactions in speciation tests.*

***Klebsiella* to bladder epithelial cells.** The results of three studies on the adherence of the *Klebsiella* isolate to rat bladder epithelial cells are shown in Table 1. The piliated phase exhibited a significant increase in adherence when compared with the adherence observed with the nonpiliated phase ($0.005 < P < 0.01$). Figure 4a and b illustrates typical adherence patterns by the piliated and nonpiliated organisms.

Effect of temperature and pH on adherence to bladder epithelial cells. Studies to determine the effects of temperature and pH on adherence were then initiated. Comparable adherence with the piliated phase was noted at incubation temperatures of 4 and 37°C (Table 2). The nonpiliated phase exhibited little adherence at either temperature. The optimum pH for the adherence of piliated bacteria to the epithelial cells appeared to be pH 5.0, with decreasing amounts of adherence observed through a pH of 9.0. At a pH of 4.0, the bacteria agglutinated, resulting in a decrease in adherence. The upper range of pH tolerance for the epithelial cells appeared to be pH 9.0. Resuspension of the epithelial cells in a pH 10.0 solution resulted in total lysis of the bladder cells.

Effect of enzymatic alteration of pili on adherence to bladder epithelial cells. Trypsin and pepsin treatment of the piliated organisms for 1 h before incubation with the epithelial cells caused an 89% decrease in adherence for each treatment group (Table 3). Little adherence was again noted for the nonpiliated phase, and no appreciable differences in adherence were observed when treated and untreated nonpiliated organisms were compared with each other. Piliated phase organisms treated with pepsin or trypsin lost the ability to hemagglutinate guinea pig RBC immediately after enzyme treatment. However, hemagglutinating ability returned to each treatment group within 15 min of enzyme removal.

Effect of autoclaving and formaldehyde and ethylene oxide treatment on adherence to bladder epithelial cells. To determine if

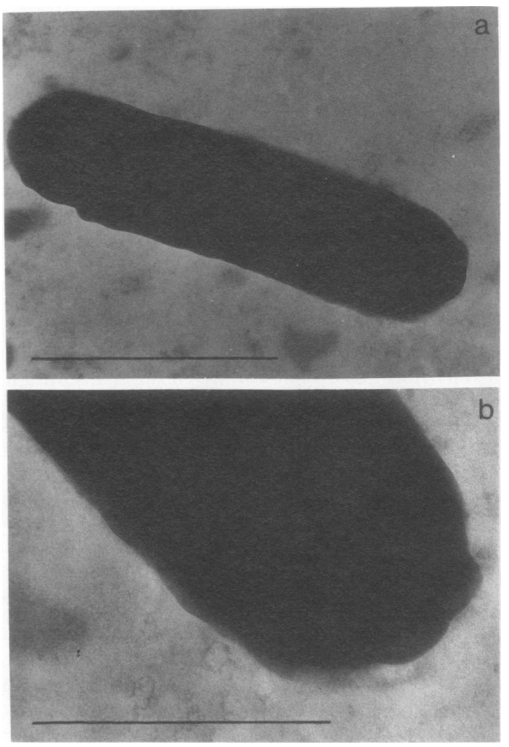

FIG. 2. *Negative stain of the nonpiliated phase of the K. pneumoniae isolate. (a) Bar, 1 μm; (b) bar, 0.5 μm.*

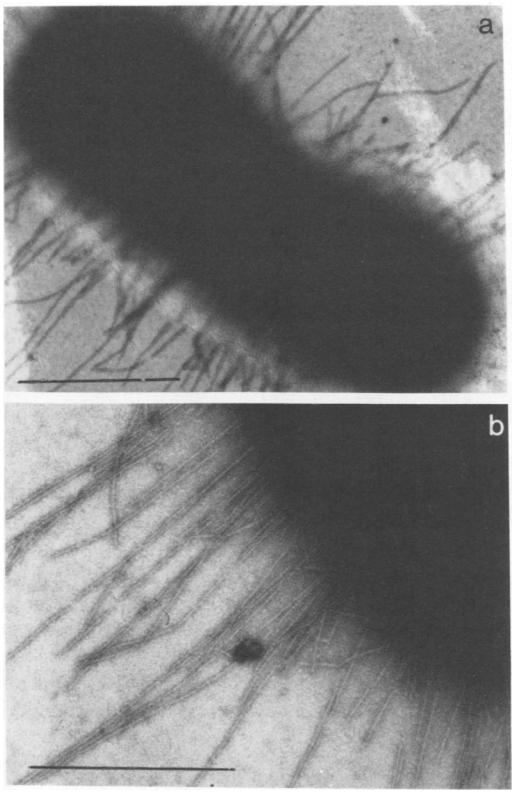

FIG. 3. *Negative stain of the piliated phase of the K. pneumoniae isolate. (a) Bar, 1 μm; (b) bar, 0.5 μm.*

TABLE 1. *Adherence of piliated and nonpiliated K. pneumoniae to bladder epithelial cells*[a]

Test no.	Phase of bacteria[b]	Bacteria/cell[c]
1	Piliated	147
	Nonpiliated	1
2	Piliated	81
	Nonpiliated	1
3	Piliated	97
	Nonpiliated	1
Avg	Piliated	108[d]
	Nonpiliated	1

[a] Adherence testing as described in the legend to Fig. 1.

[b] Piliation confirmed by hemagglutination tests with guinea pig RBC.

[c] Average number of adherent bacteria per epithelial cell.

[d] P value ($0.005 < P < 0.01$) determined by Student's t test.

bacterial adherence is an active process on the part of the organism, bacteria were killed by various means and examined for the ability to adhere to bladder epithelial cells. Autoclaved, piliated *Klebsiella* demonstrated a 99% decrease in adherence when compared with untreated control bacteria (Table 4). Formaldehyde-treated and ethylene oxide-treated bacteria also demonstrated a significant decrease in adherence, 93 and 76%, respectively. However, a few epithelial cells in the formaldehyde and ethylene oxide treatment tests exhibited bacterial adherence in control value ranges, indicating that the bacteria had not totally lost the ability to bind to the bladder cells. Autoclaved and formaldehyde-treated piliated organisms lost the ability to hemagglutinate guinea pig RBC, whereas ethylene oxide-treated bacteria retained hemagglutinating ability after treatment.

Effects of saccharides on adherence to bladder epithelial cells. Various investigators have demonstrated that bacterial adherence mediated by type I pili can be inhibited by various saccharides, specifically, D-mannose and alpha-methyl-D-mannoside (3, 10). A study was undertaken to determine if this inhibition could be observed in the *K. pneumoniae* and bladder cell system. D-Mannose and alpha-methyl-D-mannoside pretreatment of bladder epithelial cells resulted in 69 and 71% decreases in the adher-

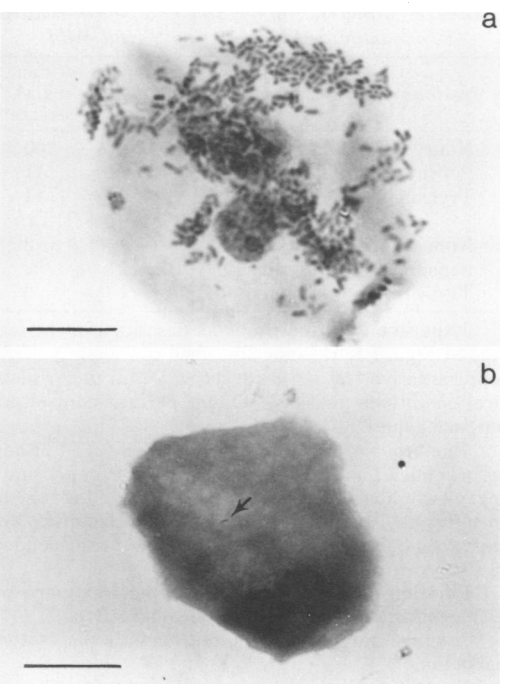

FIG. 4. *(a) Rat bladder epithelial cell with adherent bacteria after incubation with piliated-phase K. pneumoniae. Bar, 15 μm. (b) Rat bladder epithelial cell with one adherent bacterium (arrow) after incubation with nonpiliated-phase K. pneumoniae. Bar, 15 μm.*

TABLE 2. *Effects of temperature and pH on adherence of K. pneumoniae to bladder epithelial cells*[a]

Treatment	Phase of bacteria[b]	Bacteria/cell[c]
Temp (°C)[d]		
37	Piliated	35
4	Piliated	36
37	Nonpiliated	1
4	Nonpiliated	1
pH[e]		
5.0	Piliated	103
6.0	Piliated	72
7.0	Piliated	67
8.0	Piliated	55
9.0	Piliated	49

[a] Experimental procedures as described in the legend to Fig. 1.

[b] Piliation confirmed before adherence testing by hemagglutination tests with guinea pig RBC.

[c] Average number of adherent bacteria per epithelial cell.

[d] Temperature of incubation. Results are the average of two tests.

[e] pH of the final suspension medium for bacteria and epithelial cells. Results described are from a single test.

TABLE 3. *Adherence of trypsin- and pepsin-treated K. pneumoniae to bladder epithelial cells*[a]

Treatment[b]	Phase of bacteria[c]	Bacteria/cell[d]	% Control adherence[e]
None	Piliated	54	100
Pepsin	Piliated	6	11
Trypsin	Piliated	6	11
None	Nonpiliated	1	2
Pepsin	Nonpiliated	1	2
Trypsin	Nonpiliated	1	2

[a] Experimental procedures as described in the legend to Figure 1. Results described are from a single representative test. Other tests were run under identical conditions and gave similar percent control adherence values.

[b] Bacteria were washed twice in PBS (pH 7.2) and resuspended for 1 h with shaking at 37°C in pepsin (2 mg/ml in phosphate buffer, pH 6.0), trypsin (2 mg/ml in phosphate buffer, pH 8.0), or PBS. Bacteria were then washed to remove the enzymes and resuspended in PBS for use in adherence testing.

[c] Piliation confirmed before enzyme treatment by hemagglutination tests with guinea pig RBC.

[d] Average number of adherent bacteria per epithelial cell.

[e] Percent control adherence = (average number of adherent treated bacteria per cell/average number of adherent untreated bacteria per cell) × 100.

ence, respectively. Other saccharides tested did not result in any significant inhibition of adherence (Table 5).

Effects of *K. pneumoniae* antiserum on adherence to bladder epithelial cells. The results obtained thus far appeared to point to a pili-mediated attachment of *Klebsiella* to bladder epithelial cells. Antisera produced against both the piliated and nonpiliated phases of the isolate were then tested to determine the effect on bacterial adherence. When antiserum to pili was coincubated with bacteria and epithelial cells, a 97% decrease in adherence was noted. However, bacterial agglutination was present at the end of this incubation and may have been responsible for the observed decrease in adherence. When Fab fragments from antipili antiserum treated with papain were tested, a 96% decrease in adherence was noted. No bacterial agglutination was observed after the 1-h incubation. Antiserum to the nonpiliated phase, absorbed with the piliated phase, was unable to inhibit bacterial adherence (Table 6).

DISCUSSION

It is generally recognized that bacterial attachment to epithelial cell surfaces is often a prerequisite for the initiation of an infectious process. Many investigators have attempted to demonstrate that pili play an important role in bacterial

TABLE 4. *Adherence of killed K. pneumoniae to bladder epithelial cells*[a]

Test no.	Treatment	Phase of bacteria[b]	Bacteria/cell[c]	% Control adherence[d]
1	None	Piliated	131	100
	Autoclave[e]	Piliated	1	1
	None	Nonpiliated	1	1
	Autoclave	Nonpiliated	<1	<1
2	None	Piliated	58	100
	Formaldehyde[f]	Piliated	5	7
	None	Nonpiliated	2	4
	Formaldehyde	Nonpiliated	2	4
3	None	Piliated	41	100
	Ethylene oxide[g]	Piliated	10	24
	None	Nonpiliated	<1	<1
	Ethylene oxide	Nonpiliated	<1	<1

[a] Experimental procedures as described in the legend to Fig. 1. Treated cultures were streaked on solid agar to confirm sterility. Results described are the average of two tests.

[b] Piliation determined before treatment by hemagglutination tests with guinea pig RBC.

[c] Average number of adherent bacteria per epithelial cell.

[d] Percent control adherence = (average number of adherent treated bacteria per cell/average number of adherent untreated bacteria per cell) × 100.

[e] Bacteria were autoclaved for 30 min at 121°C and 15 lb/in^2.

PBS-washed bacteria were resuspended in 1% formaldehyde with shaking for 48 h at 37°C.

[g] Bacterial cultures were treated for 2.5 h in an ethylene oxide chamber and removed without aeration.

TABLE 5. *Saccharide inhibition of K. pneumoniae adherence to bladder epithelial cells*[a]

Test no.	Saccharide[b]	Bacteria/cell[c]	% Control adherence[d]
1	None	35	100
	D-Mannose	11	31
	Alpha-methyl-D-mannoside	10	29
	D-Glucose	45	129
2	None	77	100
	Maltose	71	92
	N-acetyl-D-glucosamine	88	114
	N-acetyl-D-galactosamine	88	114

[a] Experimental procedures as described in the legend to Fig. 1. Results described are from a single representative test. Other tests were run under identical conditions and gave similar percent control adherence values.

[b] PBS suspensions of bladder epithelial cells were pretreated for 20 min with 25 μg of the appropriate saccharide per ml before the addition of a PBS suspension of piliated *K. pneumoniae*.

[c] Average number of adherent bacteria per epithelial cell.

[d] Percent control adherence = (average number of adherent bacteria per treated cell/average number of adherent bacteria per untreated cell) × 100.

adherence and colonization of urinary tract epithelium leading to urinary tract infection; however, the results of these studies have been inconclusive (15–17). In this study, we have provided strong evidence for pili-mediated attachment of the *K. pneumoniae* isolate to rat bladder epithelial cells. In addition, we have described an in vitro adherence system characterized by the ease in which epithelial cells are obtained and by the high viability of bladder cells after incubation with bacteria. This system provides an easy and reliable method for the study of bacterial adherence to bladder epithelial cells.

In an initial study with this system, piliated and nonpiliated bacteria were resuspended in filter-sterilized urine to more closely approximate the environmental conditions under which bacteria encounter epithelial cells in the bladder and to determine the validity of the PBS suspension medium used throughout the study. No differences in adherence were noted between piliated *K. pneumoniae* suspended in PBS and those suspended in urine. The nonpiliated phase was not adherent in either suspension medium.

Cultural conditions play an important role in determining adherence capabilities of bacteria (3, 18). Growth in broth medium will influence pili production, as we have demonstrated, and may have accounted for some variability in numbers of adherent bacteria noted between different experiments thoughout the study.

A certain amount of variability was also noted in the receptiveness of epithelial cells to bacterial adherence between rats and even between epithelial cells from the same bladder. In adherence studies with piliated-phase organisms, a small percentage of the epithelial cell population possessed only a few adherent organisms as compared with the majority of the cells, which exhibited higher levels of adherence. This variability has also been noted in similar test systems (18; A. E. Avots-Avotins, R. C. Fader, and C. P. Davis, unpublished data). Although at times this variability lowered the average number of adherent bacteria per cell, it did not alter the overall significance of the study. In tests requiring the sacrifice of more than one rat to obtain bladder cells, the cells were pooled before ali-

TABLE 6. *Effects of K. pneumoniae antiserum on adherence to bladder epithelial cells*[a]

Test no.	Antiserum	Bacterial agglutination[b]	Bacteria/cell[c]	% Control adherence[d]
1	Control	−	62	100
	Antipili antibody[e]	+	2	3
2	Control	−	85	100
	Nonpiliated antibody[f]	−	101	119
3	Control	−	46	100
	Antipili antibody (Fab)[g]	−	2	4

[a] Experimental procedures as described in the legend to Fig. 1. In tests 1 and 2, 0.5 ml of antiserum was coincubated with bacteria and epithelial cells. Controls were 0.5 ml of saline added to bacteria and epithelial cells. In test 3, 0.25 ml of antiserum treated with papain was mixed with 0.5 ml of saline and added to the bacteria and epithelial cells. Controls consisted of 0.75 ml of saline added to the bacteria and epithelial cells.

[b] +, Bacterial agglutination present after 1 h of incubation; − = no bacterial agglutination present.

[c] Average number of adherent bacteria per epithelial cell.

[d] Percent control adherence = (average number of adherent bacteria per cell with antiserum/average number of adherent bacteria per cell in control) × 100.

[e] Antiserum to piliated-phase *K. pneumoniae* absorbed with nonpiliated phase. Results described are the average of two tests.

[f] Antiserum to nonpiliated phase absorbed with piliated phase. Results described are from a single test.

[g] Antipili antiserum treated with papain for 4 h at 37°C, after which *p*-chloromercuribenzoic acid was added to halt the reaction. Results described are the average of two tests.

quots were divided for adherence testing to negate any differences in receptiveness between epithelial cell populations. Controls were also performed for each experiment to ensure that differences in adherence levels observed were a result of the treatments and not due to variations in epithelial cell receptiveness.

We have demonstrated that a significant increase ($P < 0.01$) in bacterial adherence occurred with the piliated phase of the urinary tract infection isolate when compared with that observed with the nonpiliated phase. The nonpiliated phase did not at any time exhibit more than 10 adherent organisms on any one given epithelial cell. These few adherent bacteria may represent organisms that produced pili early in broth cultures or organisms that had not completely lost their pili after serial passage on agar medium.

The capsule present on the *K. pneumoniae* isolate can be discounted as playing a significant role in adherence due to the fact that both the piliated and nonpiliated phases possessed the capsule under all growth conditions utilized in this study. Capsules present on certain *E. coli* strains do not appear to play a role in bacterial adherence to human uroepithelial cells (19). However, it has been suggested that by covering pili, capsules may play an indirect role in adherence by determining pili availability (19).

Our results obtained from temperature and pH studies are in close agreement with results reported by Salit and Gotschlich in their study of purified *E. coli* type I pili adherence to monkey kidney cells (14). These workers found no differences in pili binding at incubation temperatures of 4 and 37°C and reported optimal binding at pH 4 to 5. We have demonstrated similar results with piliated *K. pneumoniae*. The bacterial clumping noted in our study at pH 4 is probably the result of pili aggregation on the surface of adjacent bacteria since it has been reported that pili aggregate at pH 3.92 (1).

Depiliation of gonococci by enzymatic treatment with trypsin or by mechanical shearing eliminated the ability of the organisms to adhere to human epithelial cells and to hemagglutinate rabbit RBC (11). The depiliated gonococci recovered hemagglutinating ability within 15 min after enzyme removal if resuspended in growth medium. However, if antibiotics that inhibit protein synthesis were added to the growth medium, hemagglutinating activity was not recovered. It has also been reported that *E. coli* regenerated pili to 90% of control lengths within 30 min of mechanical depiliation if resuspended in growth medium; however, no experiments were performed to determine if the restored pili were functionally active (9). In our studies, treatment of the *K. pneumoniae* isolate with trypsin or pepsin reduced, but did not eliminate, attachment to bladder epithelial cells. In addition, hemagglutination of guinea pig RBC by the organism was not noted immediately after trypsin or pepsin treatment, although weak hemagglutinating activity was noted within 15 min after removal of the enzymes. In light of these observations, the low adherence levels noted in our study after enzyme treatment could be attributed to organisms that successfully regenerated pili within the 60-min incubation period with the epithelial cells.

Heat-killed or chemically killed *K. pneumoniae* either completely or partially lost the ability to adhere to bladder epithelial cells, respectively. When organisms were autoclaved, no evidence of pili could be observed either by hemagglutination of guinea pig RBC or by TEM. Treatment with formaldehyde produced low levels of adherence, although no pili were observed by TEM and these organisms were also unable to hemagglutinate guinea pig RBC. Ethylene oxide-treated bacteria exhibited higher levels of adherence and were able to weakly hemagglutinate guinea pig RBC. Pilus-like structures were observed by TEM and probably represent pili altered by ethylene oxide treatment. These results indicate that chemically treated bacteria are able to adhere to epithelial cell surfaces, although in reduced numbers, when the treatment leaves pili on the bacterial surface relatively intact, but destruction of pili by heat denaturation eliminates adherence capabilities. In general, these results are in agreement with studies by Svanborg Eden et al. on the attachment of *E. coli* to human uroepithelial cells (18, 19).

The inhibition of type I pili-mediated adherence by mannose and mannose analogs has been noted for a number of years (3, 10). D-Mannose and alpha-methyl-D-mannoside inhibited the adherence of *K. pneumoniae* to bladder epithelial cells, whereas none of the other saccharides tested caused any significant decrease in adherence. D-Mannose also inhibited the hemagglutination of guinea pig RBC by the *K. pneumoniae* isolate. These results provide further evidence that pili mediate the attachment of *K. pneumoniae* to bladder epithelial cells.

Our studies have demonstrated that antiserum produced against pili inhibited attachment of the piliated-phase *K. pneumoniae* to bladder epithelial cells. Salit and Gotschlich have reported that antiserum produced against purified *E. coli* type I pili blocked attachment of these pili to monkey kidney cells (14). In addition,

Punsalang and Sawyer reported that adherence of piliated gonococci to human epithelial cells and rabbit RBC was inhibited by antiserum produced against purified gonococcal pili (11). It is clear from these studies that the attachment of different genera of bacteria to mammalian cells is blocked by antibodies to pili. Our results are the first report that the adherence of *K. pneumoniae* to mammalian cells can be blocked by antiserum produced against *Klebsiella* pili.

It appears that the adherence to rat bladder epithelial cells by the *K. pneumoniae* strain utilized in this study is mediated by pili. However, this statement is not to be construed as meaning that pili are the sole mechanism of bacterial attachment. For example, in vitro or in vivo, it may be possible that in the shift from nonpiliated to piliated phases, additional alterations of the bacterial surface may generate other adhesive factors in addition to the production of pili. These factors may also play a role in attachment, but in light of the absence of any evidence pointing to other factors, we conclude that pili can mediate attachment of *K. pneumoniae* to bladder epithelial cells in the in vitro system used in this study.

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