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Adherence of *Escherichia coli* to Human Urinary Tract Epithelial Cells

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The adherence of *Escherichia coli* to human uroepithelial cells obtained from midstream urine specimens of healthy women was studied. Bacteria labeled with [^{3}H]uridine were used, and unattached organisms were separated from the epithelial cells by vacuum filtration with 5-μm-pore-size Nucleopore membrane filters. These techniques allowed adherence to be measured in large numbers of epithelial cells and overcame the problem of distinguishing experimental bacteria from the indigenous organisms present on uroepithelial cells. Adherence was not appreciably affected by temperature. Adherence was maximal at pH 4 to 5 and at bacterial-to-epithelial-cell ratios of 5,000 or more. The latter observation suggested that there are a limited number of receptors on the epithelial cell surface, an idea which was supported by competition experiments. Adherence occurred within 1 min and then decreased gradually or quickly, depending on the type of bacterial growth medium, to a stationary level of adherence, approximately 50% of that observed initially. The ability of epithelial cells from a single individual to bind *E. coli* varied in a cyclical and repetitive pattern. Adherence tended to be higher during the early phase of the menstrual cycle and diminished shortly after the time of expected ovulation; adherence frequently correlated with the value obtained on the same day of the menstrual cycle during the preceding months. Adherence was markedly enhanced by bacterial incubation in broth for 72 h and inhibited by α-D-mannose. These results suggest that adherence is a complex phenomenon perhaps mediated in part by bacterial pili and mannose residues on uroepithelial cells.

Adherence of bacteria to epithelial cells has recently been shown to play an important role in the colonization of mucous membranes (6, 18, 20). The role of bacterial adherence in the pathogenesis of urinary tract infections is not clear, but colonization of the urogenital epithelium of susceptible individuals by specific strains has been associated with successful microbial invasion of the urinary tract. Most recurrent urinary infections are caused by reintroduction of bacteria, usually *Escherichia coli,* from the fecal reservoir (21). It has been well established in longitudinal studies that colonization of the vaginal mucosa with bacteria from the gastrointestinal tract precedes bacteriuria (1, 24). Moreover, cultures taken at frequent intervals between episodes of bacteriuria showed both a higher incidence and greater density of vaginal carriage of urinary pathogens in patients when compared to similar cultures from women (19, 22) or girls (1) who never had a urinary infection.

Studies on the adherence of *E. coli* to human urogenital cells have supported the concept that bacterial-epithelial cell specificity may be involved in the adherence process. Svanborg Edén et al. (27) demonstrated that *E. coli* isolated from urine of patients with acute pyelonephritis adhered more avidly to uroepithelial cells from normal women than did strains isolated from patients with asymptomatic bacteriuria. Fowler and Stamey (4, 5), however, showed no correlation between the adhesion of a bacterial strain to vaginal epithelial cells from normal women and its clinical pathogenicity. Other studies suggest that uroepithelial cells vary in their ability to accept bacteria. *E. coli* adhered more readily to vaginal cells of women (4) and girls (8) with recurrent urinary infections than to similar cells from controls with no history of bacteriuria.

Sugar residues on the surface of epithelial cells may serve as receptors for the binding of some *E. coli* strains (14). D-Mannose has been shown to inhibit the adherence of intact bacteria to epithelial cells (15). The possibility that the mannose-binding ligand on *E. coli* is associated with pili is supported by the observation that the binding of purified pili to monkey cells is specifically inhibited by D-mannose (17). In ad-

dition, other studies have shown that the fraction of organisms which adhered to epithelial cells and exhibited a high degree of mannose-binding activity were heavily piliated. The nonadherent fraction of organisms, on the other hand, lacked detectable mannose-binding activity and were nonpiliated (13). The presence of pili on *E. coli* isolated from the urine of patients with urinary infection has recently been correlated with the ability of the bacteria to bind to human uroepithelial cells, but adherence was not inhibited by D-mannose (26).

In most of the studies described above, adherence was assayed by counting by light microscopy the number of bacteria binding to 40 to 50 epithelial cells. This report describes a sensitive and reproducible technique which assessed adherence of radioisotopically labeled *E. coli* to large numbers of epithelial cells from voided urine of healthy individuals. The use of labeled bacteria eliminates the problem of distinguishing the experimental bacteria from the indigenous organisms on the epithelial cells. The results of this study suggest that in vitro adherence of *E. coli* to uroepithelial cells is influenced not only by the conditions of incubation but also by the surface components of both cell types.

MATERIALS AND METHODS

Bacteria. *E. coli* serotypes O1, and O18 were isolated from urine. Strains 1-9, 4454, and 4476 were obtained from Thomas Stamey, Stanford University, Stanford, Calif. All bacteria were maintained on nutrient agar slants (Difco). Bacteria were transferred to agar plates before use in adherence experiments.

Growth and labeling of bacteria. Bacteria were prepared for isotopic labeling by growing the cells overnight at 37°C in yeast nitrogen base (YNB; Difco) buffered to pH 7 with phosphate buffer (Na_2HPO_4, 8 mM; KH_2PO_4, 2 mM). The bacteria were washed and inoculated to an optical density of 0.03 at 540 nm as determined by a spectrophotometer (Coleman model 44) into YNB supplemented with 20 μCi of [^{3}H]uridine per ml (New England Nuclear Corp.). Log-phase bacteria (optical density = 0.15) were harvested by centrifugation, washed once in phosphate-buffered saline (NaCl, 137 mM; KH_2PO_4, 2 mM; Na_2HPO_4, 8 mM; pH 7.2), and suspended to a concentration of 10^8 colony-forming units per ml in minimal essential medium containing Earle's salts (MEM; International Scientific Industries) at pH 5. Bacteria were incubated in MEM at 37°C for 30 min before use in the assay. The ratio of colony-forming units to disintegrations per minute was determined by conventional plate count techniques and by collecting an aliquot of bacteria on a 0.4-μm-pore-size polycarbonate membrane filter (Nuclepore). To stimulate pili production, *E. coli* were grown in brain heart infusion broth (Difco) for 72 h before transfer to YNB.

Epithelial cells. Uroepithelial cells were obtained from freshly voided midstream urine specimens provided by premenopausal women who had no history of urinary or vaginal infections and were not taking contraceptive or antimicrobial agents.

The epithelial cells were harvested by centrifugation, washed once in phosphate-buffered saline, and suspended in MEM at pH 5 to a concentration of 10^5 cells per ml using a hemocytometer.

Bacterial adherence. Aliquots of 0.2 ml of bacteria and 0.2 ml of uroepithelial cells were combined and incubated at 37°C for 30 min. Control tubes containing bacteria plus MEM or epithelial cells plus MEM were also prepared. After incubation, 0.2 ml of each suspension was filtered under vacuum through a 5-μm-pore-size polycarbonate membrane filter (Nuclepore). The filters were washed with 15 ml of phosphate-buffered saline and placed in scintillation vials containing 10 ml of 3a70B scintillation fluid (Research Products International). The vials were counted in a Packard model 3385 Tri-Carb liquid scintillation spectrophotometer.

Adherence was calculated by dividing the number of bacteria adhering by the number of epithelial cells counted in the assay. The number of epithelial cells was determined by hemocytometer counts of the suspension of MEM plus epithelial cells. The number of adhering bacteria was calculated by subtracting the number of bacteria attaching non-specifically to the filter in the absence of epithelial cells from the number of bacteria present on the filter in the assay. The final value is expressed as the number of bacteria adhering per epithelial cell.

Mannose treatment. α-D-Mannose was added to *E. coli* suspensions in MEM to achieve a final concentration of 2.5% (wt/vol) when combined with epithelial cells. Mannose-treated bacterial suspensions were compared with nontreated controls to assess the role of mannose as an inhibitor of bacterial adherence.

RESULTS

Bacterial labeling and adherence procedures. *E. coli* were labeled by growing the cells in YNB containing 20 μCi of [^{3}H]uridine per ml. This procedure labeled 1:30 to 1:60 colony-forming units assuming random incorporation of the label. More complex media provided maximum rates of bacterial growth but resulted in a 25 to 50% decrease in labeling efficiency. Excess label was sufficiently removed by a single wash; multiple washes decreased adherence by 10 to 25%. Approximately 90% of the uroepithelial cells obtained from fresh midstream urine specimens were squamous (from the bladder trigone, urethra, or vaginal vestibule), and the remainder were transitional (from the renal pelvis, ureters, or bladder). The cells maintained their viability and integrity as determined by trypan blue exclusion and light microscopy for up to 5 h in MEM.

Initial attempts to separate labeled bacteria from epithelial cells utilizing microporous membranes (Millipore Corp.) proved ineffective because of entrapment of unattached bacteria.

Control experiments revealed, however, that 99% of the unattached bacteria passed through 5-μm-pore-size Nuclepore polycarbonate filters, whereas all of the epithelial cells were retained. The possibility that significant numbers of unattached bacteria in the suspensions might be trapped on the epithelial cells retained by the filter was also examined. Epithelial cells were collected on the filters, and then labeled *E. coli* were filtered under vacuum through the epithelial cells. The number of bacteria trapped by the epithelial cells was negligible. The possibility that epithelial cells might incorporate uridine initially present in the supernatant or lost from bacteria during the incubation with the epithelial cells was excluded by experiments in which isotopically labeled bacteria were incubated in MEM for 60 min; the filtrate (0.4-μm-pore-size filter) was then incubated with epithelial cells. These experiments demonstrated that epithelial cells did not incorporate radioactive label that may have been present in the supernatant. Rather, the increase in radioactive counts observed after incubation was due to bacteria adhering to the epithelial cells.

Optimal conditions for bacterial adherence. Initial experiments were designed to determine the optimal conditions for adherence of *E. coli* to uroepithelial cells. To determine the effect of temperature, bacteria and epithelial cells were incubated at 0, 10, 23, 37, or 41°C for 30 min. No significant difference in adherence was noted. To assess the effect of pH, bacteria were suspended in MEM adjusted to a pH range of 2 to 10 (Fig. 1). Maximum adherence occurred at pH 4; a significant decrease in adherence was noted only at pH 1.

The effect of incubation time on adherence was determined for *E. coli* grown in enteric minimal medium (NH_4Cl, 93.4 mM; NH_4NO_3, 12 mM; Na_2SO_4, 14 mM; K_2HPO_4, 17 mM; KH_2PO_4, 7 mM; $MgSO_4 \cdot 7H_2O$, 0.4 mM; glucose, 27 mM) or YNB (Fig. 2). Adherence occurred within 1 min in both instances. A gradual decline in adherence over 30 min to a stationary level occurred for bacteria grown in enteric minimal medium. A more precipitous drop in adherence was detected when bacteria were grown in YNB. The stationary level of adherence was approximately 50% of that observed initially.

The relationship between bacterial concentration and adherence was examined by adding increasing concentrations of *E. coli* to a constant number of uroepithelial cells (Fig. 3). With increasing concentrations of bacteria, the number of bacteria adhering per uroepithelial cell increased, but, at high concentrations, adherence tended to stabilize. These experiments suggested

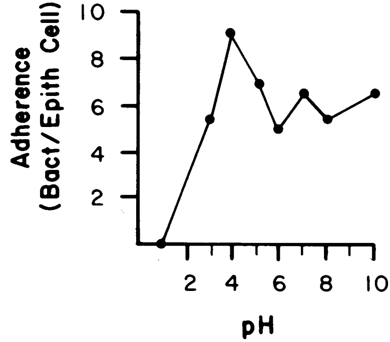

FIG. 1. *Effect of pH of incubation on the adherence of E. coli (O1) to uroepithelial cells. Incubation was at 37°C.*

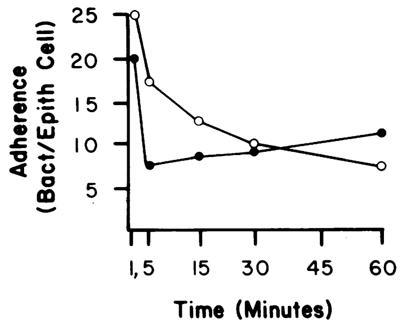

FIG. 2. *Effect of incubation time on the adherence of E. coli (O1) grown in YNB (●) and enteric minimal medium (○) to uroepithelial cells. Incubation was at 37°C.*

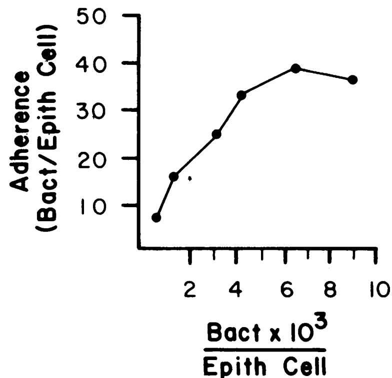

FIG. 3. *Effect of varying concentrations of E. coli (O1) on their adherence to 10^5 uroepithelial cells per ml. Incubation was at 37°C.*

that adherence was a specific interaction between bacteria and a limited number of receptor sites on the epithelial cells; at high concentrations the epithelial cells were saturated. This concept was further supported by competition experiments. When epithelial cells were prein-

cubated with high concentrations of homologous unlabeled *E. coli*, the subsequent adherence of labeled bacteria was reduced by 90%.

Variability of assay technique. The reproducibility of the technique was assessed by determining the adherence of eight separate samples prepared from the same pools of bacteria and uroepithelial cells. The coefficient of variation was 22%. Subsequent results were reported as the mean of duplicate assays.

Influence of variation in epithelial cells on adherence. The possibility that uroepithelial cells differ in their ability to bind *E. coli* was investigated by experiments in which cells collected at frequent intervals from two healthy women were incubated with two *E. coli* isolates (O1, O18) (Fig. 4). Although there was no appreciable difference in the mean adherence either between strains or between individuals, a striking day-to-day variation in the receptivity of the epithelial cells was noted. To further define the nature of this variability, adherence was correlated with the days of the women's menstrual cycles (Fig. 5). Adherence ranged from 1 to 17 bacteria per cell and appeared to be both cyclical and repetitive. Higher values were noted in the early phase of the cycle and diminished shortly after the time of expected ovulation (day 14). The number of bacteria per epithelial cell frequently correlated with the value obtained on the same day of the menstrual cycle 1 or 2 months previously.

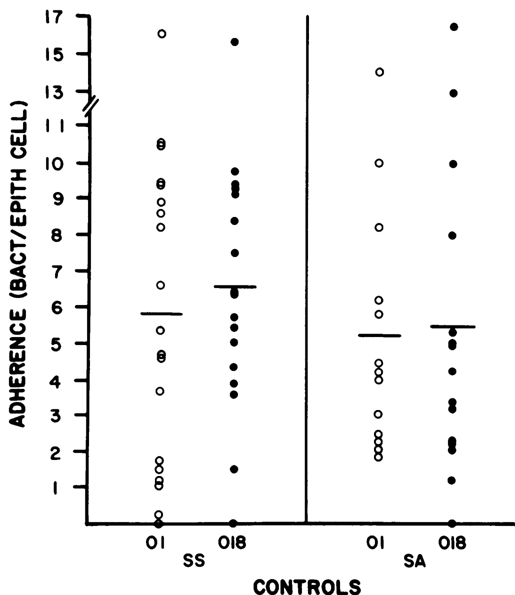

FIG. 4. *Adherence of E. coli (O1, O18) to uroepithelial cells from two women with no history of urinary infection. Each point represents a separate observation.*

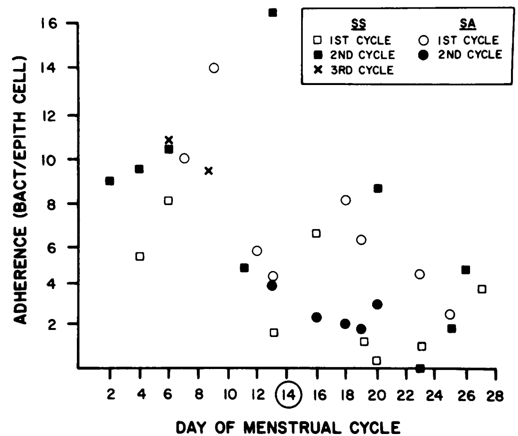

FIG. 5. *Relationship between the adherence of E. coli (O1) and the day of the menstrual cycle on which uroepithelial cells were obtained from two women with no history of urinary infections. Adherence was measured on the same day that the cells were collected.*

Adherence of different *E. coli* strains to pooled uroepithelial cells. A comparison of the adherence capabilities of different bacterial isolates can only be meaningful if the fluctuation in the receptivity of uroepithelial cells is taken into consideration. Since it was not practical to test a large number of bacteria simultaneously, we utilized a reference strain to compare the adherence capabilities of different bacteria. In each experiment we determined the adherence of the reference strain as well as test strains to pooled uroepithelial cells. A ratio was formed comparing the mean adherence of each test strain to the simultaneously determined mean adherence of the reference strain. The adherence of nine strains to pooled uroepithelial cells and the adherence relative to that simultaneously determined for the reference strain is presented in Table 1. It is apparent that comparisons of the adhesive properties of bacteria, even on the basis of multiple observations, may be misleading. The bacteria which demonstrated the highest mean adherence actually closely approximated the adhesive characteristics of the reference strain. Other isolates with low mean adherence were considerably more adherent relative to the reference strain and probably would have registered higher mean adherence if tested at a time of increased epithelial cell receptivity.

Effect of *E. coli* (O1) growth conditions on adherence. Piliation has traditionally been increased by prolonged growth in broth (3). Bacteria grown for 72 h in brain heart infusion broth

demonstrated markedly increased adherence to uroepithelial cells from some individuals when compared to the adherence of the same strain grown for 18 h on MacConkey agar (Table 2). Adherence was increased even further if the organisms incubated in brain heart infusion broth were grown in YNB to stationary rather than log phase. Strikingly less adherence was detectable, however, for the same 72-h-broth-grown organisms when epithelial cells from different individuals were utilized.

Mannose inhibition. The concept that sugar residues on the surface of epithelial cells serve as receptors for the binding of *E. coli* was investigated by adding α-D-mannose (2.5%) to suspensions of epithelial cells and several strains of *E. coli* (Table 3). Bacteria were grown in agar and in broth to stimulate pilus formation. Adherence was inhibited by 76 to 100%, even when large numbers of bacteria were attached to the epithelial cells.

DISCUSSION

This study describes a reproducible and sensitive technique for assaying adherence of *E. coli* to human uroepithelial cells. In addition to permitting rapid assessment of adherence in large numbers of cells, the use of radioisotopically labeled bacteria eliminates the problems associated with identifying or removing indigenous bacteria that frequently colonize epithelial cells. Other investigators, who relied on visual examination by light microscopy to determine the number of bacteria adhering to uroepithelial cells, have reported considerably higher values (20 to 130 bacteria per cell) (4, 25) than were usually found in our experiments (10 to 20 bacteria per cell). Failure to differentiate experimental bacteria from those already present on epithelial cells or modification of the cell surface through vigorous washes required to remove indigenous bacteria prior to incubation could account in part for this discrepancy. The use of repeated centrifugation rather than filtration under vacuum may also artificially enhance adherence by packing bacteria onto the cells (7).

The impact of physical characteristics on adherence was variable. The fact that adherence was not affected by the temperature of incubation suggests that enzymatic processes or the fluidity of the epithelial cell membranes are not important. The possibility, however, that the rate of adherence is affected by temperature has yet to be investigated. The finding that adherence was maximal at pH 4 is consistent with the observations of Mårdh and Weström (12), who noted that adherence of piliated strains of *Neisseria gonorrhoeae* to vaginal epithelial cells was significantly greater at pH 4.5 than at 5.5 or 7.2. Pili have their lowest net charge at pH 4.5 and have already been demonstrated to attach to mammalian cells more avidly at this pH range (17). Although colonization of the vaginal vestibule with *E. coli* occurs at a pH of 4 to 4.4, vaginal carriage is more often associated with an introital pH of more than 4.4 (23).

Experiments to determine the effect of incu-

TABLE 1. *Mean adherence to pooled uroepithelial cells for nine E. coli strains*

E. coli strain	Bacteria/cell[a]	(Test bacteria/cell)/(reference bacteria/cell)[b]
1	23.3 ± 51	1.09
2	22.9 ± 46	1.48
3	16.3 ± 52	0.85
4[c]	14.8 ± 28	1.00
5	6.6 ± 1.6	0.66
6	6.4 ± 3.9	0.76
7	4.4 ± 4.2	5.90
8	3.4 ± 3.4	0.25
9	2.8 ± 2.5	3.80

[a] Adherence (mean ± standard deviation) based on different cell pools.
[b] Mean adherence of the test strain relative to the simultaneously determined mean adherence of the reference strain based on common cell pools.
[c] Reference strain.

TABLE 2. *Effect of E. coli (O1) growth conditions on adherence to uroepithelial cells*[a]

Individual	A	B	
	6-h YNB bacteria/cell	6-h YNB bacteria/cell	18-h YNB bacteria/cell
SA	2	69	138
SS	2	41	126
AA	0	1	4
CA	—	5	8
MO	—	1	2

[a] A, 18-h MacConkey agar prior to YNB; B, 72-h brain heart infusion broth prior to YNB.

TABLE 3. *Inhibition of E. coli adherence to uroepithelial cells by D-mannose (2.5%)*

Strain	Growth conditions	Bacteria/cell[a]	Mannose inhibition[a] (%)
4454	6-h YNB	10.5 ± 5.7[b]	94 ± 8.4
4476	6-h YNB	8.8 ± 9.9[b]	100 ± 0
AC	6-h YNB	7.29 ± 10.6[b]	95 ± 7.9
AC	18-h YNB	3.16 ± 0.02[b]	76 ± 12.0
AC	6-h YNB	42[c]	100
AC	18-h YNB	96.2[c]	100

[a] Mean ± standard deviation.
[b] 18-h MacConkey agar prior to YNB.
[c] 72-h brain heart infusion broth prior to YNB.

bation time on adherence provided several unexpected results. Maximum adherence occurred much more rapidly (within 1 min) than previously described (60 to 90 min) (17, 25). Furthermore, rather than increase, adherence decreased gradually (bacteria grown in enteric minimal medium) or quickly (bacteria grown in YNB) to a stationary level of adherence approximately 50% of that observed initially. These data suggest that adherence may be a dynamic phenomenon which can be influenced in part by some bacterial characteristic(s) acquired during growth in different media. A similar phenomenon has been noted in adherence of chicken embryonic neural cells and liver cells (16). In these systems, loose aggregates were formed immediately and then dissociated over 30 min until a non-dissociable aggregate was formed. It is possible that two different attachment mechanisms are reflected in these results—one may provide early temporary binding whereas the second may be a more stable form of attachment.

A striking degree of variation has been observed in the adherence of *E. coli* to epithelial cells. Regardless of the adherence characteristics of an *E. coli* strain, we and others (25) have repeatedly observed that some cells from a single individual were covered with bacteria whereas other cells from the same sample were free of bacteria. Furthermore, the same *E. coli* strain which adhered avidly to uroepithelial cells from some individuals (>100 bacteria per cell) barely adhered to cells from other women (<10 bacteria per cell) (Table 2). Variation in the receptivity of epithelial cells was also noted when we determined adherence of the same *E. coli* strains to uroepithelial cells obtained on different days from several women who had never had a urinary infection (Fig. 4). When adherence was correlated with the day of the menstrual cycle, a repetitive, cyclical pattern became apparent (Fig. 5). Adherence from cycle to cycle appears to be maximal during the estrogen-dependent phase and diminishes after ovulation. The impact of hormones on bacterial colonization of rat vaginal epithelium has been studied by Larsen et al. (9, 10). Colonization varied cyclically and peaked during the proestrus and estrus phases. Additional studies demonstrated that a peak in estrogen secretion was reflected by maximum colonization (11). Taken together, these observations may suggest that receptor sites on uroepithelial cells are more available during certain stages of cell development or hormonal influence.

Our studies also indicate a wide variation in the mean adherence of different *E. coli* strains (Table 1). Because of the striking difference in the ability of epithelial cells to bind bacteria, we routinely compared adherence of the experimental strain to that of a reference strain. Some strains, which consistently adhered more or less avidly than the reference strain, also exhibited an expected level of mean adherence. For other strains, however, the observed adherence appeared to be more of a reflection of the epithelial cell receptivity than the adhesive ability of the organism. Previous studies have shown increased adherence of *E. coli* strains associated with renal infection when compared to isolates from the bladder and rectum (27). Others, however, have shown no statistical difference between adherence values for anal and vesical isolates (5). The wide day-to-day variation we have noted in the ability of a single strain to adhere to epithelial cells may explain some of this controversy.

The ability of bacteria to adhere to uroepithelium is probably influenced in part by the surface characteristics of the epithelial cell. Uroepithelial cells appear to have a limited number of receptors. As the number of bacteria per epithelial cell in the incubation mixture increased, a corresponding rise in adherence was initially noted. At ratios of 5,000 or more bacteria per epithelial cell, however, the system appeared to be saturated. Further support for the idea of limited receptor sites on uroepithelial cells is provided by competition experiments. Adherence of isotopically labeled bacteria to uroepithelial cells that had previously been incubated with homologous *E. coli* was greatly reduced. Recent reports indicate that some bacteria may bind to carbohydrate molecules on epithelial cell surfaces; if specific carbohydrates are added to the incubation mixture, adherence is inhibited (13, 15, 17). Our studies have shown that adherence of *E. coli* to uroepithelial cells can be completely inhibited by α-D-mannose, suggesting that a mannose-containing carbohydrate on the host epithelial cell is involved in adherence. This is in accordance with previously reported results on mannose-sensitive adherence of *E. coli* to kidney cell monolayers (17) and buccal mucosal cells (13, 15). Contrary results have been reported by Svanborg Edén and Hansson (26), who demonstrated mannose-sensitive *E. coli* hemagglutination but mannose-insensitive adherence to uroepithelial cells.

Our studies also suggest that pili may be associated with the ability of *E. coli* to adhere to uroepithelial cells. Surface structures such as pili can be washed off the bacterial surface (2); we noted that adherence was reduced by 10 to 25% when bacteria were subjected to several washings prior to incubation. Conversely, bacteria grown in broth for 72 h to stimulate pilus

formation adhered much more avidly than organisms grown on agar, a medium not conducive to piliation. Although the identity of the mannose-sensitive binding substance on *E. coli* is not fully defined, Salit and Gotschlich (17) showed that binding of purified *E. coli* pili to kidney cell monolayers could be specifically inhibited by D-mannose. Ofek and Beachey (13) subsequently demonstrated that the ability of *E. coli* to adhere to human buccal epithelial cells is directly related to its piliation and ability to bind mannose residues. Svanborg Edén and Hansson (26) noted a relationship between the presence of pili on *E. coli* and its ability to adhere to human uroepithelial cells, but they could not demonstrate inhibition of adherence by D-mannose.

We have shown that adherence of *E. coli* to uroepithelial cells is a very rapid and dynamic phenomenon that appears to be subject to a variety of determinants. Adherence can be easily altered by varying the conditions in which bacteria are prepared or incubated with epithelial cells. Naturally occurring changes in the surface characteristics of epithelial cells and bacteria also seem to play an important role in their ability to bind to one another. It would appear that any assessment of the role of bacterial adherence in the pathogenesis of urinary infections should take these variables into consideration.

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