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Mannose-Sensitive and Mannose-Resistant Adherence to Human Uroepithelial Cells and Urinary Virulence of *Escherichia coli*

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The adherence to human uroepithelial cells of 23 *Escherichia coli* strains belonging to three groups with different levels of virulence was investigated, and the mechanism of adherence was studied. It was found that strains belonging to the most virulent group adhered better to human uroepithelial cells than did avirulent strains. Adherence of less virulent but supposedly nephropathogenic strains was more variable. These results suggest that adherence is an important virulence factor, especially in the group of strains with the highest but a more general virulence. Piliated strains adhered better than did nonpiliated strains. We found strong evidence for the existence of at least two different mechanisms of adherence: (i) mannose-sensitive adherence by piliated strains, very likely mediated by type I pili because this mannose-sensitive adherence was associated with mannose-sensitive hemagglutination of guinea pig erythrocytes by broth cultures of the strains; (ii) mannose-resistant adherence by piliated strains, very likely mediated by non-type I pili because this mannose-resistant adherence was invariably associated with mannose-resistant hemagglutination of human group A erythrocytes by the strains, whether grown in broth or on plates. Additionally, one strain without pili and without hemagglutinating activity adhered well. Thus in most cases adherence seemed to be mediated by bacterial pili, although different types might be involved.

Studies have shown that adherence to mammalian cell membranes is important in the initiation of several bacterial infections. The ability of *Escherichia coli* to adhere to uroepithelial cells may be a decisive virulence factor. Strains from urinary tract infections have been shown to possess fimbriae more often than strains from the feces of healthy persons (12, 26). Svanborg-Edén et al. have shown that *E. coli* strains isolated from the urine of patients with acute pyelonephritis had a high capacity to adhere to human uroepithelial cells, strains isolated from patients with asymptomatic bacteriuria and from feces of healthy persons had a low capacity, and strains isolated from patients with acute cystitis occupied an intermediate position (28, 29, 31). They suggested that adherence is mediated by type I pili. The adherence was not inhibited by D-mannose (30), yet type I pili cause hemagglutination (HA) of guinea pig erythrocytes, which is sensitive to D-mannose (mannose-sensitive HA, MSHA; 2, 5, 6, 23). In contrast, Ofek et al. found that D-mannose inhibited the adherence of *E. coli* to human epithelial cells, and that there was strong evidence for a positive link between adherence, presence of pili, and mannose-binding activity by *E. coli* (21, 22). Salit and Gotschlich demonstrated mannose-sensitive binding of type I pili to Vero cell monolayers (24), and Schaeffer and co-workers described mannose-sensitive adherence of *E. coli* to human uroepithelial cells (25). Other studies demonstrated that type I pili were also responsible for the mannose-sensitive adhesion of *E. coli* to bovine epithelial cells (13), and methyl α-D-mannopyranoside prevented colonization of the urinary tract of mice with *E. coli* by blocking the bacterial adherence (1).

In a previous study we found, by means of a mouse model, differences in virulence between different *E. coli* strains (32). By following the kinetics of the viable count in the mouse kidney and other organs after intravenous injection, together with the results of measurements of 50% lethal dose values and killing rates, we found it possible to divide *E. coli* strains into three main virulence groups: group I strains were avirulent; group II strains were specifically virulent for the mouse kidney; group III strains were the most virulent, with a more general virulence for mice.

We also found that *E. coli* strains isolated

from patients with acute pyelonephritis were more often virulent in our mouse model than cystitis strains, suggesting a relationship between our mouse model and human infection (J. F. van den Bosch, P. L. Oe, P. Postma, J. de Graaff, and D. M. MacLaren, manuscript in preparation).

In the present study we have investigated adherence to uroepithelial cells as a possible virulence factor, the mannose sensitivity of this adherence, and the role of bacterial pili.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains used in this study were the same as in our previous study and belonged to one or another of the three main virulence groups as described (32).

Adherence to human uroepithelial cells. Throughout all experiments, human uroepithelial cells were collected from the freshly voided urine of one healthy woman without a history of urinary tract infection. To collect these cells, 30 ml of urine was filtered slowly under low vacuum on a 12-μm Sartorius Membranfilter (diameter, 5 cm; Göttingen, West Germany) until a small volume was left. This residue was washed three times with phosphate-buffered saline (PBS, pH 7.0). After the third washing, the residue was filtered down completely, allowing the epithelial cells to settle on the filter at random. The epithelial cells were transferred to a glass cover slip (9 by 32 mm) by pressing the cover slip on the filter. The cover slip with epithelial cells was placed in a Leighton tube and dried for 15 min. The adherence assays were performed in these tubes. Bacteria were grown overnight in nutrient broth (Oxoid, London, England) with gentle agitation at 37°C. Cells were spun down and suspended carefully in PBS to a concentration of 10^8/ml.

For adherence assays, 5 ml of the bacterial suspension was added to a Leighton tube containing a cover slip with attached epithelial cells, and incubated for 1 h with gentle shaking at 37°C. The cover slip was removed from the Leighton tube and washed twice with PBS to remove bacteria that had not adhered. The epithelial cells were fixed for 15 min in methanol, washed twice again with PBS, and stained for 20 min with 30% filtered Giemsa stain (Giemsas-Lösung; Merck, Darmstadt, West Germany). After two washings with distilled water, the cover slip was dried in the air and mounted upside down on a glass slide.

To determine the inhibition of adherence by mannose, the assay was carried out in the presence of 1% D-mannose, together with an assay on the same strain without mannose to serve as a control. In each assay a control experiment was included without added bacteria, to determine the possible presence of extraneous bacteria on the epithelial cells. Each strain was tested at least twice, on different days. Adherence was examined with a light microscope at ×400 magnification and expressed as the percentage of epithelial cells with more than 25 adherent bacteria out of a total of 50 cells counted. The counted cells were of about equal size and were regarded as randomly chosen.

HA activity. Guinea pig blood (Albino random bred; National Institute of Public Health, Bilthoven, The Netherlands) and human group A blood were collected 1:1 in Alsever solution (2.05% glucose, 0.8% sodium citrate, 0.42% NaCl, 0.055% citric acid; pH 6.1); blood cells were washed three times with PBS and suspended to a final concentration of 0.5%. Bacteria were grown overnight either in nutrient broth with gentle agitation or on nutrient agar plates at 37°C. Cells grown in broth, as well as cells grown on agar plates, were suspended in PBS to a concentration of 10^9 bacteria per ml. Serial dilutions of the bacterial suspensions in 100 μl of PBS were made in a Multiwell Disposo-tray with a well capacity of 1 ml (Libro Division, Flow Laboratories, Inc., Hamden, Conn.). To each well, 100 μl of the 0.5% erythrocyte suspension was added. For testing mannose sensitivity, 100 μl of 0.5% erythrocyte suspension containing 1% D-mannose was added to 100 μl of undiluted bacterial suspension (10^9/ml). The agglutination was read after incubation for 30 min with gentle agitation at 37°C, followed by storage overnight at 4°C.

Electron microscopy. Bacteria were grown overnight in nutrient broth with gentle agitation at 37°C, spun down, and carefully resuspended in the same volume of PBS containing 0.25% Formalin. After 1 h of fixation, a drop of the bacterial suspension was placed on a Formvar-coated copper grid and allowed to stand for 3 min; excess suspension was drained off, and the adherent bacteria were negatively stained with 1% phosphotungstic acid (pH 7.2) for 10 s. Grids were examined in an electron microscope at 60 kV (Philips EM 301). The percentage of piliated bacterial cells out of 100 cells examined was determined for each strain.

RESULTS

Adherence to human uroepithelial cells and virulence of strains. The results of the adherence tests are given in Table 1. When the epithelial cell population and the bacterial suspension were unchanged in each test, the reproducibility of the test could be fully derived from the binomial distribution. The possibile influence of time and of extraneous bacteria on the test results was found to be small in comparison with the expected variation from the binomial distribution. Further statistical analysis of the counted numbers was performed on duplicate experiments.

Figure 1 shows the adherence of strains arranged according to the virulence group to which they belong (32). By statistical analysis with the Kruskal-Wallis test at a significance level α = 5%, we rejected the hypothesis that the mean level of adherence for group I, II, and III strains is equal. According to the method of Dunn for multiple comparisons, a significant difference in adhesive capacity was found only between group I and group III strains (14). Thus strains belonging to the most virulent group, III, adhered better to human uroepithelial cells than did avirulent group I strains, which suggests that

TABLE 1. *E. coli strains used: summarized results of various tests*

E. coli strain	Virulence group[a]	Serotype	Adherence[b]	Inhibition by mannose of adherence[c]	Piliation[d]	Hemagglutination pattern[e]					
						Broth culture				Plate culture	
						Gp	Gp+M	Hu	Hu+M	Hu	Hu+M
AD101	I	O1:K51:H7	18		0	–	–	–	–	–	–
AD102	I	O117:K–:H–	8		30	–	–	–	–	–	–
AD103	I	Rough:K–:H27	15		23	–	–	–	–	–	–
AD104	I	O75:K95:H5	34		98	8	–	32	–	–	–
AD105	I	O75:K100:H5	37		0	–	–	–	–	–	–
AD106	I	O75:K100:H–	16		0	–	–	–	–	–	–
AD107	I	Rough:K+:H–	42		99	16	–	–	–	–	–
AD108	I	O75:K100:H5	71	38	0	–	–	–	–	–	–
AD109	II	O75:K–:H5	23		76	8	–	–	–	–	–
AD110	II	O6:K2:H1	90	10	97	16	–	32	32	16	16
AD111	II	O6:K2:H1	64	0	98	–	–	64	32	32	16
AD112	II	O6:K2:H1	49		99	32	–	16	–	–	–
AD113	II	O4:K3:H?	13		93	–	–	–	–	–	–
AD114	II	O75:K95:H?	40		100	16	–	–	–	–	–
AD115	II	O6:K2:H1	59	14	100	–	–	64	32	64	32
AD116	III	O33:K+:H8	44	91	98	16	–	–	–	–	–
AD117	III	O6:K23:H1	82	93	94	32	–	64	–	–	–
AD118	III	O6:K23:H1	81	55	100	32	–	8	–	–	–
AD119	III	O18ac:K+:H–	73	14	95	–	–	32	32	16	32
AD120	III	O6:K23:H?	84	73	96	32	–	64	–	–	–
AD121	III	O6:K23:H1	89	75	96	32	–	–	–	–	–
AD122	III	O6:K+:H–	85	40	99	16	–	–	–	–	–
AD123	III	O18ac:K–:H–	63	17	71	–	–	32	32	32	32

[a] Virulence group as determined in hematogenous pyelonephritis in mice (32).
[b] Adherence to human uroepithelial cells; mean percentage of epithelial cells with more than 25 adherent bacteria.
[c] Mean percentage inhibition of adherence in the presence of 1% D-mannose. A control experiment without mannose was used as the 100% reference point.
[d] Percentage of piliated bacteria.
[e] Hemagglutination activity, given as reciprocals of titers, of guinea pig (Gp) and human group A (Hu) erythrocytes, together with controls in the presence of D-mannose (+M). Minus (–) means no agglutination.

adherence is an important virulence factor in group III strains. The adhesive capacity of the nephropathogenic group II strains was more variable.

Adherence inhibition by mannose. It has often been suggested that the adherence of *E. coli* is mediated by type I pili and can be inhibited by D-mannose, although contradictory results appear in other studies. Therefore we decided to study the inhibitory activity of 1% D-mannose on the adherence of strains with high adhesive capacity. The results of these inhibition experiments are shown in Table 1 and Fig. 2. For each strain separately, statistical analysis was performed on one pair of experiments. We tested the hypothesis of no inhibition by the one-sided 2×2 test of Fisher at a significance level $\alpha = 5\%$. It was shown that the adherence of strains AD 108, 116, 117, 118, 120, 121, and 122 was inhibited by mannose ($P < 0.05$). No inhibition of adherence in the presence of mannose was shown for the strains AD 110, 111, 115, 119, and 123 ($P > 0.05$).

From Fig. 2 we can see that adherence of the only group I strain tested (AD 108) and of two group III strains (AD 122, 118) was only moderately inhibited, whereas adherence of the other group III strains (AD 120, 121, 116, 117) was almost completely inhibited by mannose. The results suggest that different mechanisms of adherence to uroepithelial cells may be involved.

Electron microscopy. To find out the possible role of bacterial pili in adherence and in the suggested different mechanisms of adherence, we first determined the piliation of strains by electron microscopy. The results are shown in Table 1. Only four strains (AD 101, 105, 106, and 108), confined to the avirulent group I, had no pili at all. Of two other group I strains, the majority of cells were not piliated, and the piliated cells possessed only a few pili per cell (AD 102 and 103). If we regard these two strains as nonpiliated, statistical analysis of the hypothesis of no difference in adherence between piliated and nonpiliated strains, by the one-sided two-sample test of Wilcoxon (14) at a significance level $\alpha = 5\%$, showed that in general, piliated strains adhered better to uroepithelial cells than did nonpiliated strains ($P = 0.006$). The most

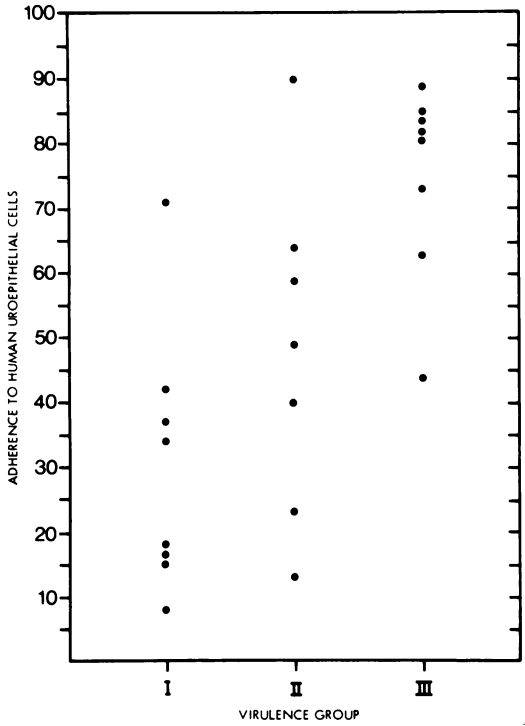

FIG. 1. *Adherence to human uroepithelial cells of E. coli strains with different levels of virulence. Group I strains are avirulent, group II strains are proposed to be nephropathogenic, and group III strains are the most virulent (32). Adherence is given as the percentage of epithelial cells with more than 25 adherent bacteria.*

obvious exceptions were strain AD 108, with no pili but with a considerable adhesive ability which was moderately inhibited by mannose, and strain AD 113, which was heavily piliated but did not show adherence.

HA patterns of strains. To investigate possible differences in pilus types, we measured the hemagglutinating activities of the strains with guinea pig and human group A erythrocytes, and the mannose sensitivity of these agglutinations (Table 1). HA of guinea pig erythrocytes was observed only with bacteria grown in broth; only very weak or no HA was observed with bacteria grown on agar plates. The HA of guinea pig erythrocytes by bacteria grown in broth was always mannose sensitive, indicating the presence of type I pili (5, 6, 23). Indeed, all strains with MSHA were piliated, as shown by electron microscopy. However, five heavily piliated strains (AD 111, 113, 115, 119, and 123) did not agglutinate guinea pig erythrocytes, which indicates that these strains have pili different from type I pili.

Figure 3 shows the adherence of strains in relation to MSHA activity for guinea pig erythrocytes and to the presence of pili. Strains showing MSHA tended to adhere better when their MSHA activity was higher, which indicates a relation between adherence and the degree of piliation with type I pili. Nonpiliated strains without any MSHA activity showed little adherence, with one exception (AD 108). The other strains, which adhered well but showed no MSHA (AD 111, 115, 119, and 123), were piliated with pili other than type I pili. Only one piliated strain without MSHA activity showed no adherence (AD 113). Thus adherence seems to be mediated by bacterial pili, mainly of type I, but other types of pili may be involved.

When the HA of human group A erythrocytes is considered (Table 1), the four strains AD 111, 115, 119, and 123 stand out because of the following properties: they showed mannose-resistant HA (MRHA) of human group A erythrocytes, whether grown in broth or on solid medium; they showed, however, no MSHA of guinea pig erythrocytes, but adhered well and were piliated. Moreover, these were the same strains that showed mannose-resistant adherence (Fig. 2). The other mannose-resistant adhering strain (AD 110) showed both MRHA of human group A erythrocytes and MSHA of guinea pig erythrocytes. Some strains that showed MSHA of guinea pig erythrocytes also showed MSHA of human group A erythrocytes when cells were grown in broth (Table 1).

DISCUSSION

In the present study we have shown that *E. coli* strains belonging to the most virulent group (group III) (32) adhered better to human uroepithelial cells than did avirulent group I strains. The adherence of the nephropathogenic group II strains was more variable (Fig. 1). This suggests that, especially for group III strains, adherence is an important virulence factor. Although it is difficult to compare mouse virulence with human virulence, we have shown that strains isolated from the urine of patients with acute pyelonephritis belonged mainly to the virulent groups, in contrast to strains isolated from patients with cystitis (van den Bosch et al., manuscript in preparation), which indicates that the mouse model may be used to discriminate between strains from human infections. Furthermore, Aronson et al. (1) suggested a relationship between adherence to human epithelial cells and colonization of the urinary tract of mice, both of which were mannose sensitive. We must, however, be careful, because several investigators have shown species-specific adherence (18) as well as individual susceptibility of women (11, 17, 27). It should be emphasized that

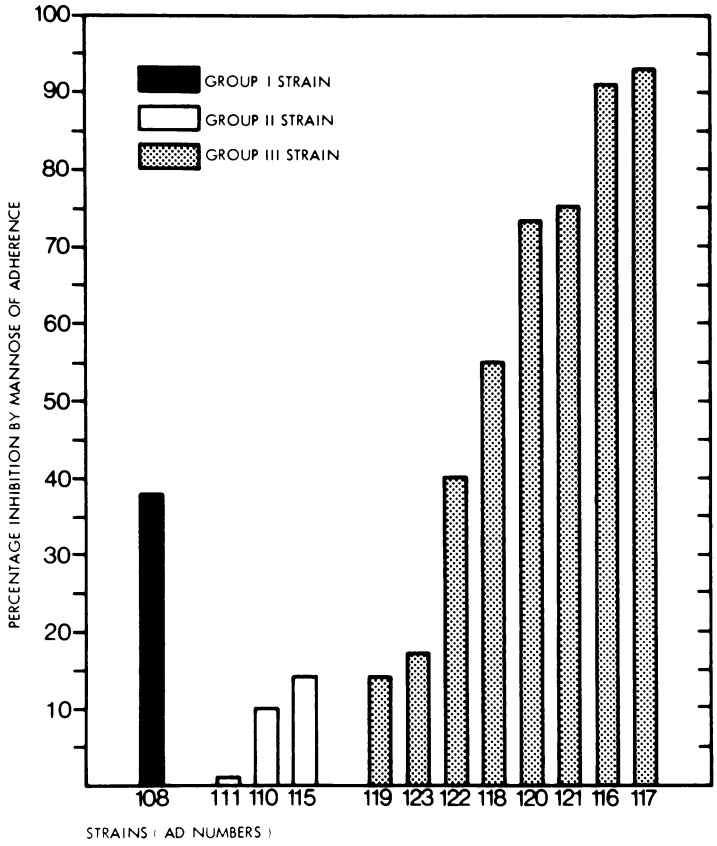

FIG. 2. *Percentage inhibition of adherence to human uroepithelial cells of well-adhering E. coli strains in the presence of 1% D-mannose. A control experiment without mannose was used as the 100% reference point.*

in the present study we used uroepithelial cells from one healthy woman, and that the results might have been different had cells from an infection-prone woman been used (11, 17).

An additional problem is that we measured the capacity of the strains to adhere to human uroepithelial cells, whereas virulence was determined in a mouse model where bacteria were injected intravenously. Therefore, a direct relationship between adherence and mouse virulence seems inherently improbable. Nevertheless, we found such a relationship between mouse virulence and adherence to human uroepithelial cells. We can conclude that adhesive capacity is associated with some other factor, which determines virulence after intravenous injection. Indeed, Minshew et al. (19) have recorded a prima facie improbable association between HA of human erythrocytes by extraintestinal *E. coli* and virulence for chicken embryos. This brings to mind the well-known association between adhesive capacity and the enterotoxigenicity of enteropathogenic *E. coli* strains.

However, the possibility is not excluded that group III strains are extremely virulent because they adhere to other cells throughout the mouse body, a concept that is supported by the finding that group III strains gave high viable counts in kidney as well as in blood, spleen, and liver after intravenous injection, and thus differed from group II strains (32). By the same line of reasoning, group II strains, which we consider nephropathogenic in view of their high counts in the kidney after 8 h, should adhere specifically to uroepithelial cells. However, there is no further evidence for this hypothesis, because, first, several group II strains did not adhere well to uroepithelial cells, and second, the mechanism of adherence found in group II strains was similar to that of some group III strains. It is likely, therefore, that other virulence factors are involved as well, e.g., hemolysis, amount of K-antigen, and susceptibility to phagocytosis.

The most striking result of the present study is the strong evidence we found for the existence of different mechanisms of adherence. The first mechanism found is the mannose-sensitive adherence, very likely mediated by type I pili be-

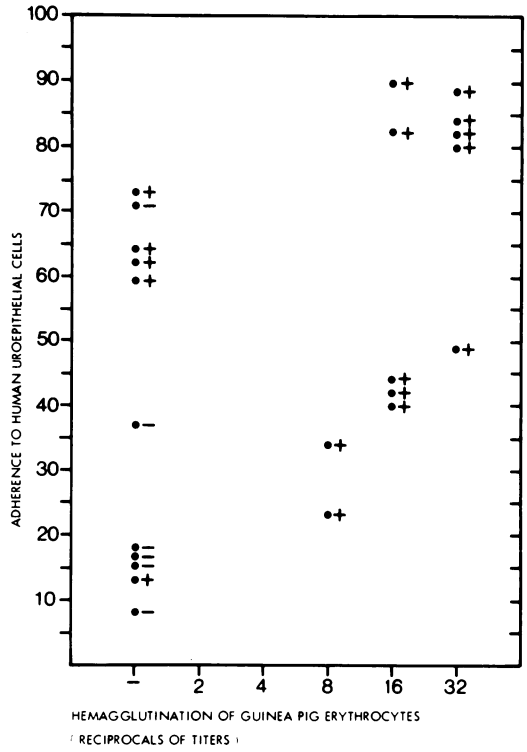

FIG. 3. *Adherence to human uroepithelial cells of E. coli strains, in relation to their HA activity with guinea pig erythrocytes and to their piliation. Adherence is given as the percentage of epithelial cells with more than 25 adherent bacteria. Piliation of strains as judged by electron microscopy is designated as + (piliated) or – (nonpiliated).*

cause this adherence was associated with piliation and MSHA of guinea pig erythrocytes by broth cultures of the strains. This mannose-sensitive adherence, mediated by type I pili, has been found in several earlier studies (1, 13, 21–25). Some strains, when grown in broth, showed, besides MSHA of guinea pig erythrocytes, MSHA of human group A erythrocytes, possibly also caused by common pili (10). Furthermore, we found some evidence that strains with higher guinea pig MSHA capacity adhered better to human uroepithelial cells (Fig. 3), which suggests a relationship between the degree of piliation and adherence capacity.

The second mechanism of adherence found is the mannose-resistant adherence, which was very likely mediated by other than type I pili for the following reasons: (i) this mannose-resistant adherence was invariably associated with piliation and with MRHA of human group A erythrocytes; (ii) this MRHA was always found with both broth and plate cultures; (iii) with one exception, mannose-resistant adhering strains did not show HA of guinea pig erythrocytes. If these pili indeed are responsible for the mannose-resistant adherence found, they resemble the specific virulence-associated bacterial pili K88, K99, CFA/I, and CFA/II, all responsible for the MRHA of erythrocytes from different species, especially by bacteria grown on solid media (3, 7, 8, 10, 15). The non-type I pili found resembled most closely the CFA/I, both exhibiting MRHA of human group A erythrocytes. Although we have not as yet tested possible antigenic similarity, we do not think that these non-type I pili are similar to the CFA/I, because it has recently been shown that some 50% of *E. coli* strains isolated from extraintestinal infections can also cause MRHA of human erythrocytes (4, 20). Carvioto et al. (4) have not found any similarity between these extraintestinal isolates showing MRHA of human erythrocytes and the CFA/I. Furthermore, Minshew et al. (19) could not relate MRHA of human erythrocytes by extraintestinal isolates to a specific plasmid, whereas CFA/I was shown to be plasmid controlled (9).

Källenius and Möllby (16) recently showed an association between MRHA of human erythrocytes and mannose-resistant adherence to periurethral cells, but they only tested one strain of serotype O75:K nontypable:H–. Our strains showing MRHA of human group A erythrocytes, together with mannose-resistant adherence to human uroepithelial cells, belonged to serotypes O6:K2:H1 (three strains), O18ac:K+:H–, and O18ac:K–:H– (Table 1). Strain AD 110 caused MSHA of guinea pig erythrocytes when grown in broth, as well as MRHA of human erythrocytes when grown either in broth or on plates. It is possible that this strain possesses type I pili as well as another pilus type. We are currently investigating this possibility. We have not yet succeeded in showing by electron microscopy any structural difference between type I pili (see Fig. 4) and non-type I pili (see Fig. 5).

Besides the two mechanisms of adherence described above, others may exist that are not necessarily dependent on pili, since one avirulent strain (AD 108) without pili and without HA activity showed a high degree of adherence which was moderately inhibited by mannose. Another strain (AD 113), nephropathogenic, which was heavily piliated without any HA activity or adherence to uroepithelial cells, may possess another type of pilus which played no part in our adherence experiments.

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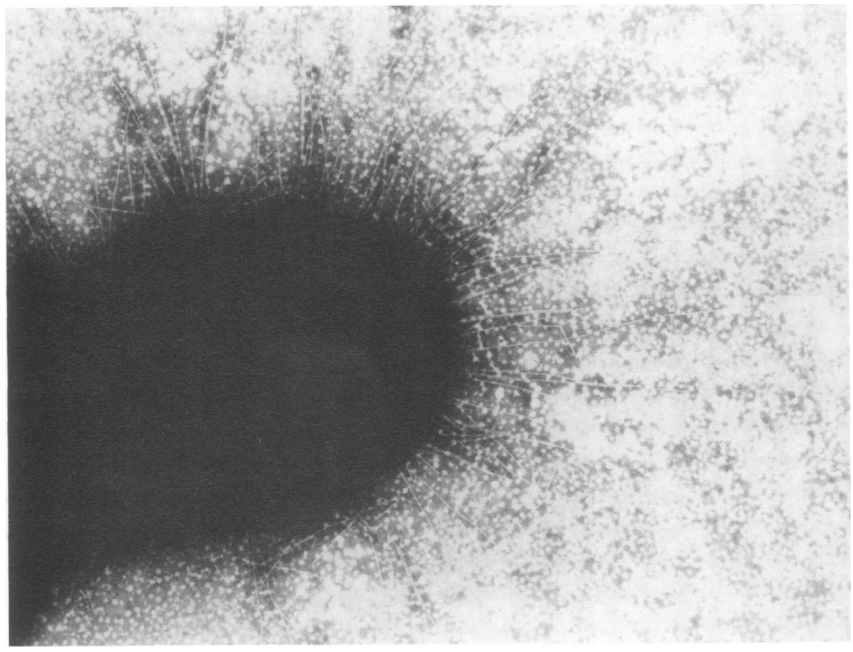

FIG. 4. *Electron micrograph of E. coli strain AD 122, showing type I pili. Negatively stained with phosphotungstic acid, ×41,000.*

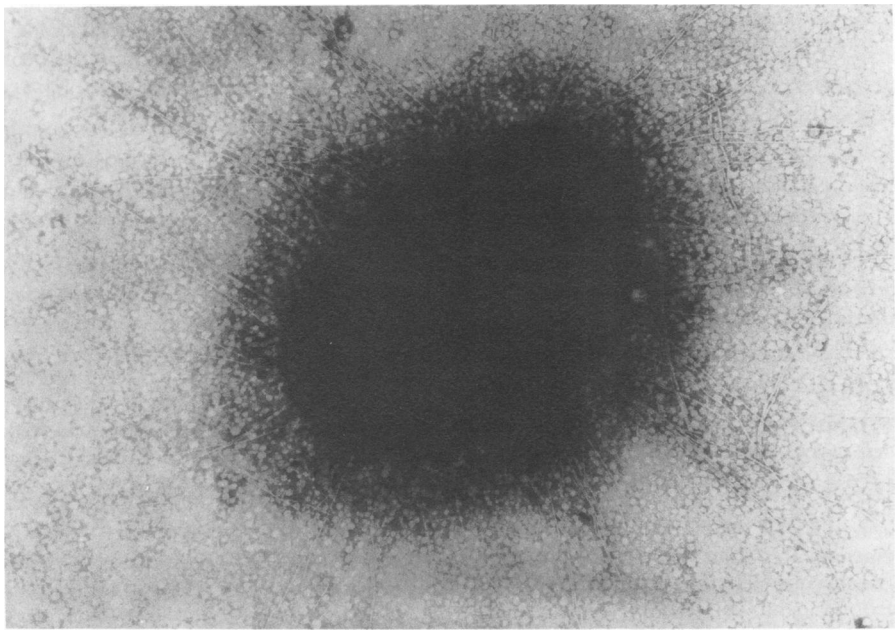

FIG. 5. *Electron micrograph of E. coli strain AD 111, showing non-type I pili (see text). Negatively stained with phosphotungstic acid, ×41,000.*

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