Effect of Pili on Susceptibility of *Escherichia coli* to Phagocytosis

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The degree of piliation of 20 clinical isolates of *Escherichia coli* was correlated with their susceptibility to phagocytosis by human polymorphonuclear leukocytes. Piliation was quantitated by negative staining, and phagocytosis was quantitated by a monolayer technique. Ingestion was confirmed by electron microscopy. In the absence of source of opsonins, there was a positive correlation between the degree of piliation and susceptibility to phagocytosis ($y = 0.83x + 19.58$; correlation coefficient = 0.65; $P < 0.01$). Heavily piliated strains were no longer phagocytized after their pili were removed by ultraviolet irradiation. Phagocytosis was reduced 75% in the presence of 0.1 M D-mannose, an agent which competitively inhibits binding of pili to cell surfaces. L-Mannose, D-glucose, and D-galactose were much less inhibitory. The viability of piliated organisms was reduced by 1 log after 1 h of incubation with polymorphonuclear leukocytes. Addition of 10% fresh human serum increased both the rate and completeness of killing. These observations suggest that polymorphonuclear leukocytes may interact with the pili of *E. coli* to promote phagocytosis. This phenomenon may have clinical relevance in situations where normal opsonic activity is poor, such as the renal medulla.

Polymorphonuclear leukocytes (PMNs) are believed to recognize bacteria prone to phagocytosis by the presence of opsonins on the surface of the microorganisms. Interaction of these opsonins, usually specific antibodies or complement, with receptors on the leukocyte membrane initiates the events which result in ingestion and killing of the pathogen (26, 27). It has long been known that certain particles can be phagocytized in the absence of opsonins (7, 14). These include senescent erythrocytes (2, 19), latex spheres (6), paraffin oil droplets (28, 29), and nonpathogenic bacteria (33). Although the mechanism for recognition of these particles is not well understood, speculation has centered on the role of hydrophobic or electrostatic interactions (33, 35). We have reported previously that the presence of pili enhances the susceptibility of *Proteus mirabilis* to phagocytosis in the absence of opsonins (25). In this study we present evidence that there are receptors on the membranes of PMNs for the pili of *Escherichia coli* and that binding of the pili to the leukocyte membrane appears to induce ingestion and killing of the bacteria.

MATERIALS AND METHODS

Bacteria. All strains were clinical isolates obtained from blood and urine. Stocks of each strain were stored in out-dated human blood at −78°C. Before use, each strain was cultivated in Trypticase soy broth for 48 h and passed serially three times, which favored development of maximal piliation. For some experiments, organisms were depiliated by exposure to supralethal doses of ultraviolet irradiation. Two Sylvania G15T8 germicidal lamps (principal wave length, 254 nm) were used as the ultraviolet source. Bacterial suspensions were placed in petri dishes on ice 3 cm below the lamp and irradiated for 40 min.

Evaluation of the extent of piliation. All inocula were examined by negative staining with 0.5% uranyl acetate before use. At least 100 bacteria of each strain were evaluated. The degree of piliation was expressed as the percentage of bacteria with 50 or more pili. To assess the reproducibility of this method, triplicate samples of 20 strains were coded and examined by a microscopist who was unaware of the sequence. Overall, there was less than 15% variation among the triplicate samples.

Assessment of phagocytosis. The degree of bacterial cell association, was quantitated by the monolayer method of Ofek et al. (15). Human PMNs were obtained from heparinized blood by sedimentation with dextran. The cells were washed in Hanks balanced salt solution (HBSS) and allowed to adhere to glass cover slips placed in 60-mm tissue culture petri dishes. The cells formed a layer of approximately 200 cells per mm^2. After the unattached leukocytes were washed off, approximately 10^8 organisms, suspended in 1 ml of HBSS, were placed on each cover slip, and the monolayers were incubated at 37°C for 30 min on

a platform shaker. When the effect of potential inhibitors was evaluated, HBSS without glucose was used. After incubation, the monolayers were rinsed twice with HBSS to remove bacteria not associated with leukocytes, stained with Giemsa stain, and examined by light microscopy. At least 200 PMNs on each cover slip were evaluated, and the percentage of cells which had two or more associated bacteria was determined. Assays were done in triplicate. Percent inhibition was calculated by the following formula: inhibition = 1 − (phagocytosis in the presence of inhibitor/phagocytosis in the absence of inhibitor) × 100.

Electron microscopy. Selected monolayers were fixed in 2.5% glutaraldehyde for 1 h at 0°C, washed overnight in 0.01 M sodium cacodylate, and postfixed in 1% osmium tetroxide for 1 h at 0°C. They were dehydrated in a graded series of ethanol and proplylene oxide and embedded in Epoxy resin. Removal of the monolayer from the cover slip was facilitated by immersion in liquid nitrogen. Pieces of the monolayers, 1 to 2 μm square, were mounted on aluminum chucks, and ultrathin sections were prepared. Sections were stained in uranyl acetate and lead citrate and photographed with a Phillips 201 electronmicroscope.

Bactericidal assay. The ability of PMNs to kill piliated bacteria was assessed by the Hirsch-Strauss modification of the Maaløe bactericidal assay (12). Bacteria were suspended in HBSS and incubated with PMNs for 1 h. The ratio of bacteria to cells was 2:1. At intervals, a sample of the incubation mixture was removed, mixed with distilled water to lyse the PMNs, and, after appropriate dilution, suspended in melted agar for enumeration of colony-forming units.

RESULTS

The relationship between the presence of pili and the degree of leukocyte association of two representative strains is shown in Table 1. Strain 3781 was heavily piliated with 70% of the bacteria having 50 or more pili per cell. A total of 73% of the leukocytes incubated with this strain had two or more associated bacteria. On the other hand, there was no significant leukocyte association with the nonpiliated strain 3915. When strain 3781 was depiliated by ultraviolet light, leukocyte association was abolished. Addition of 10% normal human serum enhanced the leukocyte association of both nonpiliated strain 3915 and depiliated strain 3781. These results suggest that pili promote the ability of *E. coli* to become associated with human PMNs but that the difference between piliated and nonpiliated organisms is not seen in the presence of serum.

Examination by electron microscopy of monolayers that had been incubated for 1 h with heavily piliated *E. coli* revealed that most leukocytes had one or more bacteria lying within membrane-bound vacuoles (Fig. 1). The few bacteria that were adherent to the leukocyte surface appeared to be in the process of being ingested

TABLE 1. *Effect of serum opsonins on degree of leukocyte association of piliated and depiliated E. coli*

Strain	Organisms depiliated[a]	Normal serum present	% Of organisms piliated[b]	% Of leukocyte association[c]
3781	No	No	70	73
	Yes	No	0	1
	No	Yes	70	86
	Yes	Yes	0	60
3915	No	No	0	1
	Yes	No	0	0
	No	Yes	0	100

[a] Bacteria were depiliated by ultraviolet irradiation for 40 min.

[b] Percentage of bacteria with 50 or more pili.

[c] Percentage of PMNs in monolayers with two or more associated bacteria after 30 min of incubation.

(Fig. 2). This observation suggests that bacteria which were associated with the monolayer cells by light microscopy were indeed engulfed.

Figure 3 shows the correlation between the degree of piliation and susceptibility to phagocytosis of 20 different clinical isolates of *E. coli*. None of the five isolates which completely lacked pili was ingested. In contrast, all of the heavily piliated strains were susceptible to phagocytosis. Overall, there was a positive correlation between piliation and phagocytosis ($y = 0.83x + 19.58$; correlation coefficient = 0.65; $P < 0.01$).

It is known that binding of piliated *E. coli* to the surface of several types of cells is inhibited in the presence of carbohydrates that contain a D-mannopyranoside linkage (5, 16, 17, 20). As Fig. 4 shows, phagocytosis of the heavily piliated strain 3781 was markedly suppressed by the presence of as little as 0.01 M D-mannose. Alphamethyl mannoside had a similar effect (data not shown), but D-galactose and D-xylose did not inhibit phagocytosis even when they were present in concentrations 10-fold higher than that of mannose. Phagocytosis was only partially inhibited by L-mannose. These observations are compatible with the concept that phagocytosis of piliated *E. coli* is induced by the interaction of the pili with stereo-specific, mannose-containing binding sites on the leukocyte membrane.

Concanavalin A, like pili, also binds to mannose-containing receptor sites (21) and induces membrane perturbations, such as endocytosis (9, 18) and capping (32). As Fig. 5 shows, the addition of as little as 5×10^{-4} mM concanavalin A (50 μg/ml) profoundly inhibited ingestion of heavily piliated *E. coli*. It is not likely that this effect resulted from nonspecific suppression of phagocytosis, as concanavalin A did not inhibit
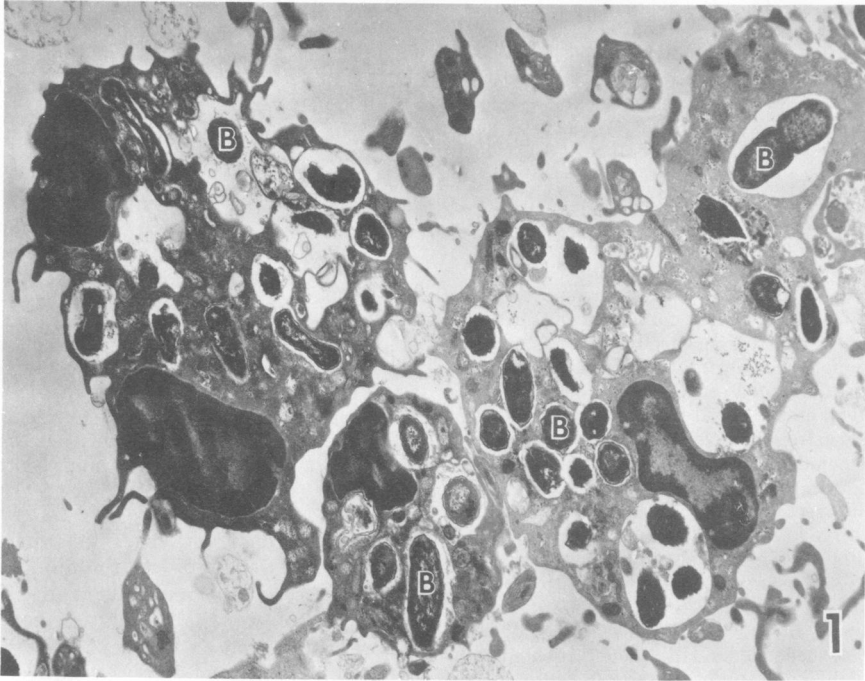
FIG. 1. *Electron micrograph of PMNs from a monolayer that was incubated with a heavily piliated strain of E. coli. Bacteria (B) lie within intracellular vacuoles. No surface adherence is seen.* × 13,300.

opsonin-mediated phagocytosis (Fig. 5). This observation suggests that pili and concanavalin A may share common binding sites.

To determine whether pili-dependent ingestion results in bacterial killing, heavily piliated bacteria were incubated for 1 h with leukocytes in the presence or absence of serum, and the number of surviving colony-forming units was determined. As Fig. 6 shows, *E. coli* incubated without leukocytes slightly increased in number, whereas bacteria that were incubated with leukocytes alone decreased by about 1 log (87%). In the presence of both leukocytes and 10% normal human serum, killing was even more prompt, and titers fell by 2 logs (98%) in 1 h. This experiment suggests that pili-dependent ingestion does result in significant bacterial killing but that the bactericidal activity of PMNs is more efficient when ingestion is mediated by conventional opsonins.

DISCUSSION

The mechanism whereby leukocytes identify a particle prone to phagocytosis in the absence of a source of opsonins is not completely understood. Van Oss and colleagues have proposed that leukocytes may recognize and phagocytize particles which are more hydrophobic than themselves by processes which involve changes in free energy of the interface between phagocyte and bacterium (33). The monomeric pilin molecule is known to have prominent hydrophobic regions (3), and it is possible that pili facilitate phagocytosis by increasing the net hydrophobicity of the bacterial surface. Pili might also promote engulfment by enabling the leukocyte to form more intimate contact with the bacteria. The surfaces of both bacteria and leukocytes are negatively charged, and the resultant electrostatic repulsion might theoretically be expected to prevent adequate contact for engulfment. However, cells capable of forming protrusions of sufficiently narrow radius of curvature, such as pili, are able to penetrate this electrical bilayer (35). In this regard, a similar explanation has been proposed to account for the absorption of certain viruses onto plasma membranes (8) and for the enhanced stickiness of platelets after exposure to adenosine diphosphate, an agent which causes platelets to assume a spiculated shape (34, 36).

The fact that pili-mediated phagocytosis can be inhibited by D-mannose suggests that PMNs recognize piliated *E. coli* by a more selective mechanism. Mannose is known to inhibit binding of piliated *E. coli* to the surface of other cell types (5, 16, 17, 20), and Bar-Shavit and colleagues have recently shown that in the absence of serum opsonins, mannose blocks the ability of

FIG. 2. *Electron micrograph of another PMN from the same monolayer as shown in Fig. 1. The cell appears to be engulfing a heavily piliated bacteria. Arrows point to pili. ×42,000.*
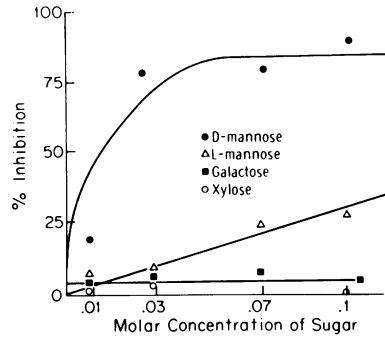

FIG. 4. *Comparison of the inhibitory effects of four sugars on the phagocytosis of a heavily piliated strain of E. coli. Percent inhibition was calculated as described in the text.*
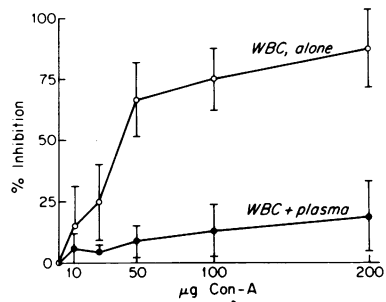

FIG. 5. *Effects of increasing concentration of con-canavalin A (Con-A) on the phagocytosis of a heavily piliated strain of E. coli. The experiment was done in the presence and absence of 10% fresh plasma. WBC, Leukocytes.*
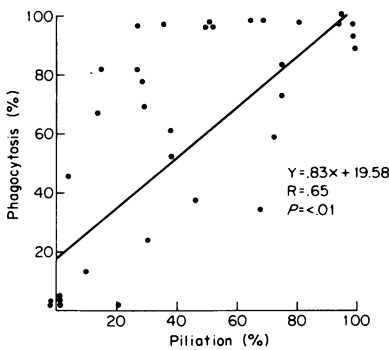

FIG. 3. *Correlation between the degree of piliation and susceptibility to phagocytosis of 20 strains of E. coli. Piliation was quantitated by negative staining and represents the percentage of bacilli with 50 or more pili. Susceptibility to phagocytosis was determined by incubating bacteria with a leukocyte monolayer and represents the percentage of PMNs with two or more associated bacteria. R, Correlation coefficient.*
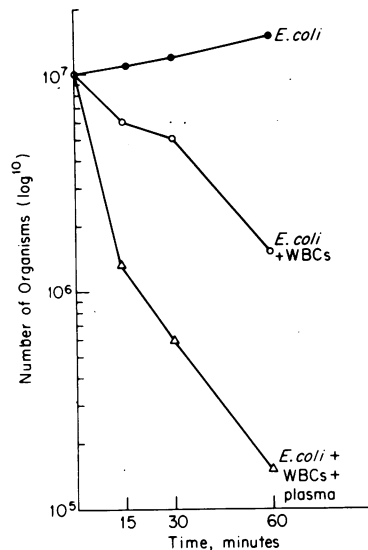

FIG. 6. *Killing of heavily piliated E. coli by PMNs in an in vitro system: a comparison of the bactericidal activity in the presence and absence of 10% fresh plasma. WBC, Leukocytes.*

PMNs and macrophages to ingest *E. coli* (1). Presumably, the sites responsible for pilus recognition and binding are mannose-containing carbohydrates on the leukocyte surface (16). We do not mean to imply that the mannose-containing binding sites are specific phagocytic receptors for pili. Ingestion may well be more a consequence of the manner in which piliated bacteria interact with the phagocyte surface than the particular receptor to which they bind. Many cells, including PMNs, are believed to respond to the binding of diverse ligands by undergoing stereotypic membrane perturbations (37). One such response is endocytosis of the ligand-receptor complex (9, 18). An important requisite for a ligand to initiate these conformational changes is its ability to cross-link adjacent binding sites, i.e. serve as a multivalent ligand (11, 31). We suggest that heavily piliated bacteria, because they establish many points of attachment with the leukocyte surface, may also function as multivalent ligands and thus stimulate their own endocytosis. The circumferential distribution of pili over the *E. coli* surface may also facilitate ingestion. Griffin and Silverstein have shown recently that for ingestion to occur enveloping phagocytic membranes must interact with the entire surface of a particle in a stepwise manner (10). Contact with only a portion of the surface of a particle results in binding but not ingestion.

Like the type I pili of *E. coli*, type IV pili of *P. mirabilis* also enhance susceptibility to phagocytosis (25). Both pilus types have a helical configuration, are 7 nm in diameter, and mediate attachment to cell surfaces (3, 22, 23, 25). It is likely that they bind to different sites however, as adherence of piliated type IV *P. mirabilis* is resistant to mannose (22).

Not all bacterial pili enhance phagocytosis. For instance, *Proteus* type III pili, which are thinner than type IV pili (23), appear to neither mediate attachment to epithelial cells nor promote phagocytosis (23, 25). Moreover, gonococcal pili have been reported to confer resistance to phagocytosis (15). Although subsequent studies have suggested that surface factors other than pili may be more important in influencing the outcome of the interaction of gonococci with leukocytes (30), Jones and co-workers have recently presented evidence indicating that gonococcal pili are the major antigens against which opsonic antibody is directed (13).

Although we found a direct correlation between degree of piliation and susceptibility to phagocytosis, individual strains often deviated from their expected values. In all, only 40% of the variation in phagocytosis could be accounted for by the difference in piliation (correlation coefficient $[r] = 0.65$; $r^2 = 0.39$). Although an inherent lack of precision in the method used to quantitate phagocytosis may have accounted for some of the lack of correlation, it is also possible that the pili of individual strains may vary with respect to their avidity for the leukocyte surface. Information currently available does not permit us to distinguish between these possibilities.

Although both piliated and nonpiliated bacteria were readily ingested in the presence of fresh serum, the ability of leukocytes to recognize and ingest piliated *E. coli* may be important for host defense in body sites where normal opsonic activity is impaired. For example, the extreme hypertonicity of the renal medulla is known to be inimical to the activity of both antibody and complement, as well as to normal leukocyte function (4). In this regard we have reported previously that heavily piliated bacteria are cleared more rapidly from the rat renal medulla than are lightly piliated organisms (24).

In conclusion, although there is increasing recognition that in some infectious processes pili may enhance bacterial virulence by facilitating adherence to and colonization of mucosal surfaces, the experiments reported here suggest that once the bacteria penetrate the parenchyma, pili may, in fact, impair virulence by facilitating phagocytosis. It thus seems warranted to consider both potential outcomes when ascribing a role for pili in the pathogenesis of an infectious process.

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