Complement-independent Adherence of *Escherichia coli* to Complement Receptors In Vitro

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ABSTRACT We studied adherence to human cells by a strain of *Escherichia coli*. Adherence to erythrocytes was assessed directly by phase-contrast microscopy and indirectly by hemagglutination; adherence to peripheral blood leukocytes, using radiolabeled bacteria and subsequent determination of leukocyte-associated radioactivity; and adherence to renal glomeruli, by microscopy of fluoresceinated bacteria and of Gram-stained nonfluoresceinated bacteria.

In serum-free systems, *E. coli* of this strain adhered to human erythrocytes, which have surface receptors for the third component of complement (C3), but not to erythrocytes from species lacking this receptor. 1 mM trypan blue, a reagent that inhibits complement receptor function, inhibited adherence to human erythrocytes, as well as adherence to leukocytes and glomeruli. Preincubation of erythrocytes and leukocytes with complement-coated zymosan particles partially blocked subsequent bacterial adherence. Incubation of human erythrocytes with aging human serum, with trypsin-cleaved C3, or with C3 cleaved by the classical pathway convertase (EAC142)—all of which treatments deposited C3 on the erythrocyte surface, presumably at C3 receptors—inhibited subsequent *E. coli* adherence. Finally, incubation of *E. coli* with rabbit antiserum to human C3 blocked adherence to erythrocytes.

Bacterial hemagglutination and erythrocyte adherence were not inhibited by mannose in concentrations up to 2.5%. And this strain of *E. coli* did not adhere to or agglutinate guinea pig erythrocytes, the usual test particle used for demonstration of common pili. Finally, electron microscopy of adherent bacteria showed only rare surface pili. In contrast, adherence to and agglutination of guinea pig erythrocytes by a

stock piliated *E. coli* was inhibited by mannose but not by trypan blue.

We conclude that organisms of this strain of *E. coli* adhere to human erythrocytes, leukocytes, and glomeruli at complement receptors. Complement is not required for this interaction. Adherence apparently involves a C3-like structure on the bacterial surface, but bacterial surface pili play no role. The physiological or pathological role of this adherence is not apparent, but study of this phenomenon may elucidate functions of complement receptors on various cells.

INTRODUCTION

Serum-mediated adherence of bacteria to mammalian phagocytic cells (opsonization) is a critical part of normal host defenses. Serum factors required for opsonization include IgG and complement. Such factors function as ligands to bind bacteria to specific cellular receptors for the Fc fragment of IgG and for the third component of complement (C3).¹ Some bacteria may also adhere to mammalian cells, including cells other than phagocytes, by serum-independent mechanisms. In some circumstances, this nonopsonic adherence enhances bacterial virulence; alternatively, host defenses may actually depend upon this adherence. Several general concepts apply to nonopsonic adherence: (a) attachment is tissue specific for each bacterium; (b) attachment is mediated by pili (fimbriae) or other bacterial surface components; and (c) attachment involves specific ligand-receptor interactions.

While studying opsonization of various bacteria, we observed that organisms of a strain of *Escherichia coli* adhered to various human cells in the absence of opsonins but in a pattern characteristic of opsonic adherence. This paper presents our investigations of

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¹ Abbreviations used in this paper: BHI, brain-heart infusion; BHS, Hanks' balanced salt solution; C3, third component of complement; M-H, Mueller-Hinton; TS, trypticase soy.

that organism. The results indicate that nonopsonic adherence of the strain of *E. coli* investigated involves opsonic receptors for C3.

METHODS

Bacteria. Laboratory strains of various bacteria were lyophilized or stored at -70°C . After reconstitution, cultures were maintained on trypticase soy (TS) agar containing 5% sheep blood, on Mueller-Hinton (M-H) agar, or in brain-heart infusion (BHI) broth. For individual experiments, organisms were grown 18–24 h on TS agar, M-H agar, or blood agar, or in TS, M-H, or BHI broth; scraped from the agar or centrifuged from the broth; and washed once in Hanks' balanced salt solution (BHS) without phenol red. Organisms were suspended to $\sim 1 \times 10^9$ organisms/ml as determined by absorption at 541 nm (absorbance = 1.5) and confirmed by colony counts.

Fluorescent bacteria were prepared according to the method of Gelfand et al. (1). Radiolabeled bacteria were prepared by suspending a loopful of bacteria grown on M-H agar in 0.5 ml BHS containing 50 μCi of ^{14}C -labeled amino acids (New England Nuclear, Boston, Mass.) and mixing vigorously. The suspension was spread evenly over the surface of M-H agar and incubated 18 h at 37°C in 5% CO_2 . Bacteria were harvested from the plate, washed twice in BHS by centrifugation at 1,500 g, and finally resuspended in BHS to a concentration of 1×10^9 bacteria/ml and $78,980 \pm 4,300$ cpm/ml (Beckman liquid scintillation spectrometer, Beckman Instruments Inc., Fullerton, Calif.).

A serum-resistant strain of *E. coli*, designated *E. coli*¹, isolated originally on blood culture, was used for studies of adherence to human cells. In initial studies, organisms grown on TS agar were adherent (see below for methods of study of adherence). Subsequently, the bacteria lost that ability; even frozen bacteria from the original adherent sample were no longer adherent when grown on TS agar. Multiple new lots of TS agar were tested, but none supported adherence. *E. coli*¹ grown on M-H agar remained adherent. Adherence studies with erythrocytes reported in this paper were performed originally with organisms grown on TS agar, then confirmed in later experiments using bacteria grown on M-H agar. Studies with polymorphonuclear leukocytes were all performed using M-H agar-grown organisms.

A different strain of *E. coli*, designated *E. coli*³³⁹, was not adherent when grown on any agar or broth tested. This strain was used in some experiments as a control. A stock laboratory strain of commonly pilated *E. coli*, *E. coli* 07K1 (ATCC 23503), was also used as a control and was maintained in BHI broth.

Mammalian cells. Human erythrocytes were obtained by venipuncture or finger stick; rabbit, rat, and guinea pig cells, by cardiac puncture. Sheep erythrocytes were purchased from Cordis Laboratories, Inc., Miami, Fla. All cells were washed twice in BHS and suspended to a concentration of 1×10^8 cells/ml. Human peripheral blood leukocytes were obtained by sedimentation in 3% dextran (J. T. Baker Chemical Co., Phillipsburg, N. J.). The resultant supernatant fluid was centrifuged at 200 g, the erythrocytes were lysed in 0.87% ammonium chloride, and the leukocyte-containing pellet was washed twice in BHS and suspended to a concentration of 5×10^6 cells/ml. The cell pellet obtained after lysis of erythrocytes contained no visible hemoglobin; contamination of the leukocytes with erythrocytes and platelets was <1% (phase-contrast microscopy); and >75% of the leukocytes were polymorphonuclear leukocytes.

Renal glomeruli. Human renal cortical tissue was snap-

frozen in isopentane previously cooled to -70°C . Tissue sections were prepared as described by Gelfand et al. (2). Fluoresceinated and unfluoresceinated bacteria were incubated with renal glomeruli.

Trypan blue. Trypan blue was prepared in 10-mM stock solutions and used as previously described (3).

Mannose. D-Mannose (Sigma Chemical Co., St. Louis, Mo.) was dissolved in BHS at a 10% stock concentration. Final reaction mixtures contained mannose at 0.5, 1, 2, or 2.5% concentrations (4).

Complement-coated zymosan. 1 mg of zymosan, prepared as previously described (5), was resuspended in 1 ml of normal human serum or 1 ml of serum that had been heated at 56°C for 30 min. Both tubes were incubated for 15 min at 37°C , washed twice in BHS, and resuspended in BHS to final concentrations of 0.2 or 1.0 mg zymosan/ml.

C3b. C3 was cleaved in three different ways to produce the fragment C3b, which could react with C3b receptors. First, 0.2 ml normal human serum was incubated at 37°C for 2–4 h, during which time spontaneous decay of C3 to C3b is known to occur (6). During this decay, autologous human erythrocytes (1×10^9 /ml, 0.2 ml) were suspended in the serum; after the incubation, the erythrocytes were washed twice in BHS and resuspended to a concentration of 1×10^9 /ml. Second, 0.2 ml partially purified human C3 (Cordis Laboratories, Inc.) was incubated with 25 μg trypsin (Worthington Biochemical Corp., Freehold, N. J.) at 37°C for 1 min. The reaction was stopped by addition of 50 μg soybean trypsin inhibitor (Sigma Chemical Co.) (7). Third, sensitized sheep erythrocytes coated with the components C1, C4, and C2 ($\text{E}^{\text{sh}} \text{A}^{\text{sg}} \text{C142}$) were prepared according to the method of Rapp and Borsos (8) and brought to a final concentration of 1×10^9 /ml, total volume 10 ml. The cells were sedimented, the supernatant fluid discarded, and the packed cells resuspended in 0.2 ml purified human C3. The mixture was incubated for 30 min at 37°C , then centrifuged, and the supernatant fluid containing C3b was saved. As control, C3 was incubated in a similar fashion with $\text{E}^{\text{sh}} \text{A}^{\text{sg}} \text{C14}$ cells (9).

0.2 ml C3, cleaved by trypsin or $\text{E}^{\text{sh}} \text{A}^{\text{sg}} \text{C142}$, was added to 2 ml human erythrocytes. After incubation at 37°C for 30 min, the erythrocytes were washed twice in BHS and resuspended to 1×10^8 cells/ml. Presence of surface adherent C3 (C3b) was confirmed by fluorescence microscopy, using fluorescent goat anti-human C3 (N. L. Cappell Laboratories Inc., Cochranville, Pa.), and by agglutination with rabbit anti-human C3 (Behring Diagnostics, Inc., Woodbury, N. Y.).

Treatment of *E. coli* with antiserum to C3. In some experiments, bacteria were incubated with antisera to human C3/C3c or heavy chain-specific IgG (Behring Diagnostics). The antiserum to C3 had an antibody titer of 1.30 mg/ml; antiserum to IgG, 1.80 mg/ml. In these experiments, 0.2 ml *E. coli*¹ was mixed with 0.2 ml of undiluted antiserum for 30 min at 0°C , then washed and resuspended in BHS. Human erythrocytes were then added and adherence studied as outlined below. The specificity of each antiserum was verified by immunoelectrophoresis.

Adherence. Adherence of bacteria to human erythrocytes was studied as follows. 0.2 ml erythrocytes was combined with 0.2 ml bacteria (1×10^9 or 1×10^6 /ml) in a 12×75 -mm polypropylene tube and rotated (10 rpm) for 30 min at 37°C . 10 μl , on a glass slide, was examined under phase-contrast at $\times 1,000$ magnification. Slides were coded and the percentage of 100 erythrocytes with three or more adherent bacteria was counted. When inhibitors were used, erythrocytes were first incubated briefly at 37°C with 0.2 ml of inhibitor (1 mM trypan blue, 0.5% mannose, or 0.02 mg zymosan) before addition of bacteria.

To study adherence of bacteria to leukocytes, we first

attempted to use techniques similar to those used with erythrocytes. Bacteria and leukocytes were incubated, washed, placed on glass slides, and stained with methylene blue, Wright's stain, or Gram's stain. We could not accurately or reliably quantify adherence because of nearby but non-adherent bacteria, and because we could not always distinguish leukocyte granules from bacteria. These problems were encountered also with polymorphonuclear leukocyte monolayers on coverslips. Phase-contrast microscopy was unsuccessful for similar reasons, and because neutrophils adhered to glass and became polarized. We also attempted to study adherence using classic bacterial culturing techniques. Bacteria were incubated with leukocytes at 4° or 37°C, after which the mixtures were centrifuged at low speed (150–200 g) to sediment leukocytes but not bacteria. The leukocytes were washed, and aliquots (diluted in distilled water to lyse the leukocytes) were cultured quantitatively. Presumably because the degree of adherence was low (see below), because some free bacteria sedimented even at low speeds, or because quantitative cultures are not sensitive enough, no adherence above "background" could be demonstrated.

Therefore, we used ¹⁴C-labeled bacteria to demonstrate adherence to leukocytes. In this procedure, 0.4 ml of radiolabeled bacteria was incubated with 0.5 ml leukocytes on a rotator (10–12 rpm) at 37°C. Tubes were removed from the incubator at either 1 or 30 min, and either of two methods was used to separate adherent from nonadherent bacteria. First, contents of the tube were passed through 3- μ m polycarbonate filters, 13 mm in diameter (Nuclepore Corp., Pleasanton, Calif.). The filters were then washed with 20 ml 0.1 M phosphate-buffered saline, pH 7.4, using 90–120 mm Hg suction. The filters were removed, added to 2 ml Hyamine hydroxide (New England Nuclear), and digested overnight. Scintillation fluid (8 ml; Liquefluor and toluene, New England Nuclear) was added and cell-associated radioactivity was determined in a Beckman liquid scintillation spectrometer. The second method to separate adherent bacteria utilized differential centrifugation. Tubes were centrifuged at 150 g for 3 min, after which the pellets were washed twice in BHS and resuspended in Hyamine hydroxide for scintillation spectrometry. For inhibition of adherence, 0.1 ml trypan blue (1 mM) or 0.1 ml mannose (1%) was added to the suspension of leukocytes and radiolabeled bacteria. When complement-coated zymosan was used as an inhibitor, 0.5 ml leukocytes was incubated for 30 min at 4°C with 0.25 mg zymosan, then washed in BHS and resuspended to 0.5 ml. Radiolabeled bacteria were then added for the 37°C incubation. All inhibition experiments were then processed as above.

We also evaluated adherence of fluoresceinated bacteria to leukocytes. Equal volumes of fluoresceinated *E. coli* and human leukocytes were rotated at 37°C for 30 min; a drop of the mixture was then removed and examined with a fluorescent microscope. Alternatively, a monolayer of adherent leukocytes (>95% polymorphonuclear leukocytes) was overlaid with a drop of bacterial suspension, incubated in a moist chamber at 37°C for 30 min, rinsed with BHS, and examined with a fluorescent microscope. Trypan blue was added to some tubes (final concentration 1 mM) or was dropped onto the leukocyte monolayer before addition of fluoresceinated *E. coli*.

Adherence of bacteria to renal glomeruli was studied as described by Gelfand et al. (2). Tissue sections were overlaid with 20 μ l of unfluoresceinated or fluoresceinated *E. coli*¹. After 15 min incubation at room temperature, sections were washed thoroughly in 0.1 M phosphate-buffered saline, pH 7.2. The sections were then mounted with Evanol (DuPont Co., Wilmington, Del.) and examined with a fluorescence microscope or stained with Gram's stain and examined

under a light microscope. Before incubation with bacteria, some sections were first overlaid with 20 μ l trypan blue (10 mM) for 1 min, washed briefly in phosphate-buffered saline, then overlaid with *E. coli*, in which case the bacterial suspension contained 1 mM trypan blue (10).

Hemagglutination. Hemagglutination was assessed by mixing 0.05 ml erythrocytes and 0.05 ml bacteria on a glass slide with shallow wells. The slide was gently rotated for 5–10 min at room temperature and observed for macroscopic hemagglutination.

Electron microscopy. To identify pili, adherent *E. coli* grown on M-H agar were negatively stained by two techniques. In one method, organisms scraped from agar plates were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. One drop of the bacterial suspension was placed on a Formvar-coated grid (Ernest F. Fullam, Inc., Schenectady, N. Y.) and allowed to stand for 1–10 min. Then the drop was blotted with a pointed piece of filter paper and a drop of 0.1% aqueous phosphotungstic acid was placed on the grid for exactly 30 s. Excess stain was removed and the grid was allowed to dry overnight before examination under a transmission electron microscope. The second method used unfixed bacterial cells, scraped from agar and suspended in sterile water. A drop of each bacterial suspension was placed on a sheet of dental wax and a Formvar-coated grid floated on the drop's surface for 30 min. The grid was rinsed twice by flotation on distilled water drops, then floated on a drop of 1% aqueous uranyl acetate for 30 min and again rinsed twice by flotation on sterile distilled water. After being allowed to dry overnight, the grid was examined with a transmission electron microscope. Controls used for comparison were *E. coli*¹ grown on TS agar (nonadherent) and the known piliated *E. coli* 07K1 grown in BHI broth.

Statistical analysis. Statistical analyses were performed using Student's *t* test.

RESULTS

Adherence of *E. coli*¹ to human erythrocytes. Although there was some variation from experiment to experiment, results were consistent: 30–90% of erythrocytes had three or more adherent bacteria. Under phase-contrast microscopy, erythrocytes and bacteria were in constant motion so that the observer could readily distinguish adherent from simply adjacent bacteria. When the concentration of bacteria was decreased 10-fold, to 1 $\times$ 10⁸/ml, adherence was markedly diminished to 10% or less of cells. The adherent bacteria could also agglutinate human erythrocytes, but reproducible hemagglutination generally required higher concentrations of bacteria (~10¹⁰ bacteria/ml). Erythrocytes of all blood groups behaved similarly. Organisms of our strain (*E. coli*¹) were never adherent when grown in broth.

*E. coli*³³⁹, which did not adhere nonopsonically to human erythrocytes, was made adherent by preincubation for 15 min at 37°C with normal human serum (Fig. 1). Adherence of *E. coli*¹ to human erythrocytes in the absence of any serum factors was quantitatively comparable to the opsonic adherence by *E. coli*³³⁹ (Fig. 1). Neither strain of *E. coli* adhered to sheep erythrocytes. *E. coli*¹ also did not adhere to or ag-

FIGURE 1 Adherence of *E. coli*¹ to human erythrocytes (E^{hu}) and sheep erythrocytes (E^{sh}). Organisms (1×10^9 /ml) were incubated with an equivalent volume of erythrocytes (1×10^9 /ml) for 30 min at 37°C, then examined by phase-contrast microscopy. Results are expressed as the percentage of erythrocytes with three or more adherent bacteria. For comparison, an otherwise nonadherent *E. coli*³³⁹ was incubated with serum to activate complement, then washed and used in identical adherence assays.

glutinate guinea pig ($1 \pm 0.3\%$ adherence, mean $\pm$ SEM), rat ($4 \pm 0.8\%$), or rabbit ($4 \pm 1.4\%$) erythrocytes.

When human erythrocytes were preincubated with 1 mM trypan blue, which blocks the complement receptor (3, 10), adherence of *E. coli*¹ was reduced from 43 ± 6 to $6 \pm 2\%$ (Fig. 2). More specifically, preincubation of the erythrocytes with complement-coated zymosan also reduced subsequent adherence of *E. coli*¹, in this case to $12 \pm 2\%$ (Fig. 2). Inhibition pro-

FIGURE 2 Adherence of *E. coli*¹ to human erythrocytes (E^{hu}); inhibition by trypan blue (TB) and complement-coated zymosan (ZC3). Erythrocytes (0.2 ml, 1×10^9 /ml) were incubated with 1 mM trypan blue, 0.02 mg ZC3, or 0.02 mg ZCΔ (zymosan preincubated in heat-inactivated human serum) for 30 min at 37°C. Then 0.2 ml bacteria (1×10^9 /ml) were added and incubation continued an additional 30 min. Results are expressed as the percentage of erythrocytes with three or more adherent bacteria as determined by phase-contrast microscopy. * $P < 0.02$.

duced by trypan blue and by complement-coated zymosan was significant ($P < 0.02$). Zymosan previously incubated in heated serum did not inhibit adherence ($P > 0.05$).

The effect of occupying C3 receptors on human erythrocytes with C3b is illustrated in Fig. 3. In these experiments, control adherence was $56 \pm 6\%$. Treatment of erythrocytes with normal human serum or trypsin-cleaved C3 reduced adherence to 23 ± 5 and $23 \pm 3\%$, respectively. These results are highly significant ($P < 0.001$). Similarly, C3 cleaved by EAC142 (but not EAC14) produced significant inhibition of adherence as well ($P < 0.025$).

We reasoned that, if adherence involved C3b receptors on erythrocytes, then C3b-like structures must be present on the bacterial surface. This was not demonstrable with fluorescent antisera to C3; however, anti-serum to C3 inhibited adherence. Preincubation with anti-C3c reduced adherence from 29 ± 6 to $11 \pm 5\%$ ($P < 0.05$). Preincubation with rabbit anti-IgG (γ -chain specific) did not inhibit adherence ($23 \pm 8\%$, $P > 0.3$); this control excluded nonspecific inhibition by the rabbit immunoglobulins.

Comparison with piliated E. coli, effect of mannose. The piliated *E. coli* 07K1 strongly agglutinated guinea pig erythrocytes; human erythrocytes were weakly but definitely agglutinated. (Agglutinability was com-

FIGURE 3 Adherence of *E. coli*¹ to human erythrocytes (E^{hu}); inhibition by normal human serum (NHS) and by trypsin-cleaved C3 (C3b). Erythrocytes (0.2 ml, 1×10^9 /ml) were incubated with NHS at 37°C for 30 min, then washed and resuspended in BHS (E^{hu}/NHS). Other erythrocytes were incubated with trypsin-cleaved C3 (0.2 ml C3/2.0 ml erythrocytes) for 30 min at 37°C, washed, and resuspended in BHS at the same concentration of 1×10^9 /ml (E^{hu}/C3b). For comparison, untreated erythrocytes were washed and suspended in BHS at 1×10^9 /ml (E^{hu}/BHS). 0.2 ml bacteria were then incubated with 0.2 ml erythrocytes at 37°C for 30 min and adherence determined. Results are expressed as the percentage of erythrocytes with three or more adherent bacteria. ** $P < 0.01$.

pletely destroyed by one washing in BHS; therefore, all further studies with *E. coli* 07K1 were done using undiluted organisms in broth.) Agglutination of both kinds of erythrocytes was completely inhibited by 1% mannose. This pattern of hemagglutination and its inhibition by mannose is characteristic of bacteria bearing common pili (11–13).

Data in Table I demonstrate that adherence characteristics of *E. coli* 07K1 were identical to agglutination characteristics in that adherence to human and guinea pig erythrocytes was inhibited by 1% mannose. Furthermore, trypan blue had no effect on adherence of *E. coli* 07K1. (Hemagglutination also was unaffected by trypan blue.) In marked contrast, *E. coli*¹ did not adhere to guinea pig erythrocytes, and adherence to human erythrocytes was inhibited by trypan blue but not by mannose, even in concentrations of 2.5%.

Adherence to renal glomeruli. Human renal glomeruli overlaid with fluoresceinated or nonfluoresceinated *E. coli*¹ had >100 bacteria/glomerulus. Preincubation of the tissue sections with trypan blue reduced that number to <10 bacteria/glomerulus. Studies with other complement receptor inhibitors were not performed.

Adherence of *E. coli*¹ to leukocytes. Using the ¹⁴C-labeled bacteria method, we determined that a small fraction of the bacteria adhered to leukocytes, but results were highly reproducible. Table II summarizes adherence of *E. coli*¹ as determined by the filtration method. Approximately 7% of labeled bacteria became cell-associated after 30 min incubation. Trypan blue inhibited adherence to ~5% ($P < 0.025$); mannose did not inhibit adherence ($P > 0.05$). The centrifugation

TABLE II
Adherence of [¹⁴C]*E. coli*¹ to Peripheral Blood Leukocytes: Inhibition by Trypan Blue

Inhibitor	Incubation time	Bacteria adherent†	P*	
			vs. no inhibitor	vs. trypan blue
	min	%		
None	1	3.6±0.5§	ND	ND
	30	6.7±0.6	ND	<0.025
Trypan blue (1 mM)	1	3.0±0.5	ND	ND
	30	4.9±0.5	<0.025	ND
Mannose (0.5%)	1	5.5±0.4	ND	ND
	30	8.6±1.2	>0.05	<0.02

* Student's paired *t* test.

† Determined by filtration method.

§ Mean±SEM.

^{||} ND, not done.

method yielded higher percentages of bacteria associated but essentially the same results: trypan blue inhibited adherence ($P < 0.05$), mannose did not ($P > 0.1$). The results of studies of inhibition of adherence by complement-coated zymosan particles are summarized in Table III. After 1 min incubation of ¹⁴C-labeled *E. coli*¹ and human leukocytes, 9.5% of bacteria were adherent; the value increased to 12.7% after 30 min. Where leukocytes had first been incubated with zymosan alone, values were identical. When complement-coated zymosan was used in the preincubation, subsequent adherence values were slightly diminished: 7.6% at 1 min., 10.2% at 30 min. The 1-min

TABLE I
Adherence Characteristics of *E. coli*¹ and the Piliated *E. coli* 07K1: Effects of Trypan Blue and Mannose on Adherence to Human and Guinea Pig Erythrocytes

Erythrocytes	Inhibitor	Adherence	
		<i>E. coli</i> ¹	<i>E. coli</i> 07K1
		%	
Human	None*	50±3.3‡	19±2.4
	Trypan blue 1 mM	2±0.8	21±5.1
	Mannose 1%	56±4.6	1±0.6
	Mannose 2%	57±5.4	ND§
	Mannose 2.5%	59±3.1	ND
Guinea pig	None	2±0.5	96±2.2
	Trypan blue 1 mM	ND	95±3.5
	Mannose 1%	ND	1±0

* Equivalent volume BHS.

‡ Mean±SEM.

§ ND, not done.

TABLE III
Adherence of [¹⁴C]*E. coli*¹ to Peripheral Blood Leukocytes: Inhibition by Complement-coated Zymosan

Inhibitor	Incubation time	Bacteria adherent†	P*	
			vs. no inhibitor	vs. Z-C3b
	min	%		
None	1	9.5±1.7§	ND	<0.025
	30	12.7±1.7	ND	>0.1
Zymosan	1	9.2±1.0	>0.5	<0.05
	30	12.1±0.8	>0.5	<0.001
Zymosan-C3b¶	1	7.6±1.2	<0.025	ND
	30	10.2±0.9	>0.1	ND

* Student's paired *t* test.

† Determined by centrifugation method.

§ Mean±SEM.

^{||} ND, not done.

¶ Zymosan preincubated with serum, then washed.

value was significantly different from both control 1-min values; the 30-min value was not significantly different from adherence at 30 min with no inhibitor, but was different from adherence at 30 min in the presence of zymosan alone.

Fluoresceinated *E. coli*¹ grown on TS agar did not adhere to leukocytes in suspension or monolayer. The same strain grown on M-H agar adhered well ($45 \pm 7\%$ adherence). The leukocytes to which the bacteria adhered in suspension appeared to be primarily neutrophils; on monolayers, fluoresceinated M-H agar-grown *E. coli*¹ clearly were adherent to polymorphonuclear leukocytes. Incorporation of trypan blue in the mixture reduced adherence to $11 \pm 5\%$ ($P < 0.01$).

Electron microscopy. Adherent and nonadherent *E. coli*¹ had very similar morphology in both negatively stained (phosphotungstic acid and uranyl acetate) preparations. Phosphotungstic acid-stained bacteria had no detectable pili, whether the organisms had been grown on M-H agar or TS agar. Almost all organisms grown on M-H agar and stained with uranyl acetate had no identifiable pili (Fig. 4a). A few organisms grown on M-H agar had small numbers of pili demonstrable with uranyl acetate (Fig. 4b); of 200 cells counted, only 5 were piliated (2.5%) and these 5 cells had only a few pili each (range, 1 to 20). Organisms grown on TS agar had no observable pili with uranyl acetate staining (Fig. 4c). In contrast, almost every *E. coli* 07K1 cell examined (>200) possessed numerous pili (range, 5 to >50/cell) (Fig. 4d).

DISCUSSION

Evidence that *E. coli*¹ adheres specifically to C3 receptors may be summarized as follows: (a) *E. coli*¹ adhered to tissues having C3 receptors (human erythrocytes, human peripheral blood leukocytes, human glomeruli), but not to tissues lacking that receptor (sheep, guinea pig, rat, and rabbit erythrocytes); (b) adherence to human erythrocytes, leukocytes, and glomeruli was abolished by 1 mM trypan blue, a reagent that inhibits complement receptor function (3, 10); (c) adherence to human erythrocytes and leukocytes was inhibited by particles (in this case, zymosan) that were coated with C3; (d) preincubation of receptor-bearing human erythrocytes with C3b blocked subsequent attachment of *E. coli*¹; (e) preincubation of *E. coli*¹ with antiserum to C3 blocked subsequent adherence to human erythrocytes.

Only trypan blue completely abolished adherence. More specific agents (e.g., C3b, antiserum to C3) never produced complete inhibition. Thus, the possibility exists that *E. coli*¹ adherence involved two or more receptors, only one of which was the complement receptor. Whatever the explanation, the observation

FIGURE 4 Transmission electron microscopy of negatively stained bacteria. (a) *E. coli*¹ grown on M-H agar (adherent) showed no pili on the majority (>95%) of 200 cells examined. (b) A rare *E. coli*¹ grown on M-H agar was sparsely piliated (arrow). (c) *E. coli*¹ grown on TS agar (nonadherent) lacked pili. (d) *E. coli* 07K1 (ATCC 23503) grown in BHI had numerous pili on almost every organism. Uranyl acetate staining. Bar, 0.5 μ m.

remains that *E. coli*¹ adherence involves a ligand resembling C3b and a receptor extremely close to or identical to the complement receptor.

There is precedent for this finding. Jondal et al. (14) noted an association between Epstein-Barr virus receptors and complement receptors on human lymphoid cells. They concluded that the two receptors were not identical but were closely related sterically, perhaps on the same molecule. Other complement receptors (e.g., those on human neutrophils) could not serve as virus receptors; the specificity for lymphoid complement receptors represented, they suggested, a viral adaptation "particularly favorable for viral replication." The observation by Mayer et al. (15) may also relate to our studies: *Brucella melitensis* adhered to all B lymphocytes; other bacteria adhered only to sub-

sets of B or T cells. However, patterns were not correlated with complement receptors on the lymphocytes.

The adherence of gram-negative bacteria to epithelial cells generally involves bacterial surface structures called pili or fimbriae. The pili of Enterobacteriaceae (to which family *E. coli* belongs) can be divided into at least four types: common pili, K88, K99, and colonization factor antigen. Most bacteria can, under appropriate in vitro cultural conditions, express the chromosomal common pilus. Its presence can readily be detected by the ability of piliated organisms to agglutinate erythrocytes, especially guinea pig erythrocytes, and adhere to epithelial cells; these abilities are inhibited by 0.5% mannose (11–13). Other pili are plasmid mediated. K88 and K99 pili mediate adherence to tissues from animals other than man (16, 17). Colonization factor antigen mediates both hemagglutination of human erythrocytes and epithelial cell adherence and is mannose resistant (18). Adherence of our *E. coli*¹ appeared not to involve any of these surface structures since (a) the *E. coli*¹ adherence factor survived washings that destroyed common pili activity; (b) *E. coli*¹ neither agglutinated nor adhered to guinea pig erythrocytes; (c) mannose, even in concentrations up to 2.5% did not inhibit adherence; and (d) only a rare adherent bacterium had pili demonstrable by electron microscopy. Furthermore, adherence of the heavily piliated *E. coli* 07K1 was not inhibited by trypan blue. Whatever its nature, the adherence factor of *E. coli*¹ had specific cultural requirements, as evidenced by its expression only when organisms were grown on certain solid media.

Bacterial hemagglutination or adherence to erythrocytes has no established pathological or physiological importance. However, Minshew et al. (19) have demonstrated an intriguing correlation among the properties of hemolysin production, human erythrocyte agglutination, and virulence for chick embryos in *E. coli* isolated from extraintestinal infections in humans. Adherence to epithelial cells is a critical requirement for virulence of a number of bacteria, including Enterobacteriaceae (20–22), gonococci (23, 24), and streptococci (25). Alternatively, other investigators have suggested that bacterial adherence may in some circumstances subserve host defenses by the elimination of attached bacteria on sloughing epithelial cells (26, 27).

Nelson, in his original articles regarding immune adherence (28, 29), suggested that this phenomenon was an important factor in bloodstream clearance of bacteria by phagocytes. He postulated and presented evidence that bacteria, once coated with antibody and complement, adhere to erythrocytes and thereby become easier for phagocytes to ingest. Viruses might also be so cleared, he suggested, not only by the antibody-complement mechanism but also by their innate adherence to erythrocytes. It is conceivable that some

bacteria may also adhere nonopsonically to erythrocytes or other cells and be more rapidly cleared.

Cells or tissues known to have C3 receptors include primate erythrocytes, neutrophils, macrophages, B lymphocytes, human renal glomeruli, and rabbit platelets (30, 31). The neutrophil and macrophage receptors are critically important in phagocytosis of microorganisms (32–34). The functions of receptors on other cells are less clearly defined, though they are thought to affect cellular cooperation and activation during the immune response (30) and renal localization of immune complexes (2).

Speculation is hazardous regarding adherence of uncertain importance and receptors of incompletely defined function. On the one hand, bacterial adherence involving complement receptors may represent bacterial adaptation for enhanced virulence. For example, Waisbren (35) has suggested that bacterial adherence to various host cells might be important in initiation or perpetuation of gram-negative shock. On the other hand, such adherence might represent a host defense mechanism, perhaps of low efficiency, perhaps primitive. It is even conceivable that this kind of adherence is a vestigial phenomenon that provides clues to earlier functions of complement receptors, especially on non-phagocytic cells.

We conclude that at least one strain of *E. coli* can adhere to complement receptors without mediation of complement proteins. This phenomenon involves bacterial surface structures which are antigenically related to C3. The physiological or pathological importance of this finding is speculative at this time. Evaluation of other *E. coli* and Enterobacteriaceae from a variety of sources may answer some of the questions raised by this study.

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