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Mannose Binding and Epithelial Cell Adherence of *Escherichia coli*

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The mannose-binding activity of several isolates of *Escherichia coli* was monitored by aggregometry with mannan-containing yeast cells. The velocity of yeast cell aggregation was found to correlate with the ability of the organisms to adhere to human epithelial cells. Mannose or its derivatives specifically inhibited or reversed epithelial cell adherence and yeast cell aggregation. Most of the adherent bacteria could be displaced within 30 min from the epithelial cells with methyl α-D-mannopyranoside, but not with other sugars tested. Cultures of *E. coli* were fractionated into nonadherent and adherent populations by adsorption with epithelial cells followed by elution of the adherent bacteria with methyl α-D-mannopyranoside. When the methyl α-D-mannopyranoside-displaced organisms were washed free of the sugar, they exhibited a high degree of mannose-binding activity and were heavily piliated. In contrast, the nonadherent fraction of organisms lacked detectable mannose-binding activity and were devoid of pili. Our results suggest that the binding activity of a mannose-specific lectin on the surface of *E. coli* can be quantitated directly on intact organisms, and the observed variations in the amount of mannose-binding activity among human isolates accounts for the variation in adherence of the organisms to mannose residues on epithelial cells.

It has become apparent that adherence of bacteria to host tissue plays a central role in the ability of bacterial pathogens to colonize mucosal (10) and endothelial (11) surfaces. Recent studies (17) suggest that sugar residues on the surfaces of epithelial cells serve as receptors for the binding of certain human and domestic animal pathogens. A mannose-specific ligand on the surface of *Escherichia coli* binds the organisms to mannose residues on human epithelial cells (18). L-Fucose may act similarly as a receptor for the attachment of *Vibrio cholerae* (12). The mannose-specific ligand of *E. coli* may be associated with pili, since it has been shown that the binding of purified pili to monkey kidney cells is specifically inhibited by D-mannose (19). D-Mannose also was shown to inhibit the adherence of intact organisms to epithelial cells (4, 18). It is conceivable that one might be able to determine the relative ability of a particular isolate of *E. coli* to adhere to epithelial cells by monitoring its mannose-binding activity.

In the present investigation, we studied the interaction of intact *E. coli* with mannose residues on yeast cells by aggregometry. The rate of reaction with the mannose residues on yeast cells was found to correlate with the ability of *E. coli* isolated from human specimens to adhere to epithelial cells. The specificity of the interaction was proven by showing that the mannose-binding activity was markedly increased in those bacteria which were displaced from the surface of human epithelial cells by addition of an excess of methyl α-D-mannopyranoside (αMM) but not of other sugars. Examination of the displaced organisms by electron microscopy demonstrated that adsorption to epithelial cells was selective for fully piliated organisms.

MATERIALS AND METHODS

Microorganisms. *Candida albicans* organisms were maintained on slants of Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) and periodically subcultured. To obtain a standard suspension, the organisms were subcultured in nutrient broth (Difco) for 24 h at 37°C with constant shaking.

Ten human isolates of *E. coli* were kept on deep agar slants. They were periodically checked for purity and identity by standard methods. Antibiotic susceptibility patterns of the isolates indicated that they represented different strains of *E. coli.* Before testing, the bacteria were transferred to nutrient broth and incubated for 48 h at 37°C. The organisms were harvested by centrifugation, washed three times in 0.02 M phosphate-0.15 M NaCl, pH 7.2 (PBS) and resuspended in the same buffer. The concentration of bacteria in the suspensions was determined by direct

counts in a Petroff-Hausser chamber (C. A. Hausser and Son, Philadelphia, Pa.).

Yeast cell aggregation tests. Candida yeast cells harvested from 2 liters of broth were washed twice with PBS and suspended in 100 ml of PBS containing 0.1% glutaraldehyde. The mixture was stirred for 1 h at room temperature, sedimented, washed again with PBS, suspended in 100 ml of PBS containing 0.1 M glycine, and stirred for 30 min at 23°C. The cells were then harvested, washed twice, and resuspended in PBS to a concentration of 2×10^7 yeast cells per ml, using a hemacytometer for counting. After adding sodium azide (0.02%) as preservative, the yeast cell suspension was stored at 4°C and used for up to 2 months. Aggregation of yeast cells was monitored in a Payton aggregometer, model 300A, according to a turbidimetric method (2). Aliquots (0.5 ml) of the standard yeast cell suspension were pipetted into glass cuvettes (8 by 45 mm) and stirred at a constant speed of 800 rpm at room temperature. The aggregometer was adjusted to read 30% transmission for the yeast cell suspension and 90% for PBS. After establishing a stable base line, test organisms, 1×10^7 to 20×10^7 bacteria, in 10-μl volumes, were added. As aggregation proceeded, the change in percentage of transmission was continuously recorded as a function of time with a Goertz (Gelman Instrument Co., Ann Arbor, Mich.) recorder. The rate of yeast cell aggregation was measured as a function of the tangent of the steepest slope in the curve produced by increasing light transmittance according to the method of Baumgartner and Born (2). The results are expressed as the natural logarithm of the aggregation velocity in millimeters of needle deflection per minute.

Adherence tests. The adherence tests were performed by methods described previously (16). Briefly, 0.5 ml of a suspension of epithelial cells (2×10^5 cells per ml in PBS) from the buccal mucosa of one of us (I.O.) was mixed with 0.5 ml of washed *E. coli* adjusted to 2×10^8 bacteria per ml. The mixture was rotated end-over-end for 30 min at room temperature. The epithelial cells were separated from the nonadherent bacteria by repeated differential centrifugation, resuspended in 0.05 ml of PBS, and smeared on a glass microscope slide. After drying, the preparation was stained with gentian violet and examined with a bright-field microscope. Adherence was recorded as the number of bacteria per epithelial cell of 30 counted cells.

In some experiments, *E. coli* were fractionated into adherent and nonadherent fractions by the adsorption of larger numbers of organisms (5×10^9) to 10^6 epithelial cells in a total volume of 4 ml. After incubation for 1 h, the mixture was centrifuged at $100 \times g$, and the nonadherent fraction of bacteria was collected from the supernatant. The epithelial cell pellet, resuspended and washed repeatedly by differential centrifugation, was finally suspended in 2 ml of 0.02 M αMM and incubated for 1 h at 25°C. The epithelial cells were sedimented at $100 \times g$, and the αMM-displaced (adherent) organisms were collected from the supernatant. Control experiments were performed by substituting αMM with PBS alone or a variety of other sugars as indicated. Timed experiments of the displacement of *E. coli* from epithelial cells by αMM were performed by incubating epithelial cells with adherent bacteria (see above) at various times in the presence of 0.02 M αMM. The bacteria retained on the epithelial cells were enumerated after differential centrifugation of each of the timed samples.

Reagents. All sugars used in this study were products of Pfanstiehl. Other materials were from commercial sources and of the highest purity available.

Preparation of rabbit antisera. One of the strains of *E. coli* (M10) was harvested from a 48-h culture in nutrient broth, washed three times, and suspended in PBS. Rabbits were injected intravenously with 0.5 ml of the *E. coli* suspension (2×10^9 bacteria per ml) three times weekly for 2 consecutive weeks. Immune sera were collected at 4 weeks. In some experiments the antisera were adsorbed by suspending 10^{10} nonadherent bacteria in 0.2 ml of serum diluted 1:100 and incubating the suspension at 37°C for 30 min. After centrifugation at $15{,}000 \times g$ for 20 min, the absorbed serum was removed from the cell pellet.

Enumeration of pili by electron microscopy. Bacterial suspensions were negatively stained with phosphotungstic acid and examined by electron microscopy by methods described previously (15). Pili were enumerated by the method of Novotny et al. (14). A cell was scored as piliated when it possessed at least 50 or more type 1 pili of any length attached to the cell surface. The limitations of this technique have been discussed, and we followed the precautions suggested by Novotny et al. (14) to improve the accuracy of the results.

Immunofluorescence tests. Immunofluorescence tests were performed by incubating 10^7 bacteria in 0.1 ml of the absorbed antiserum (see above) for 30 min at 23°C. The bacteria were then washed twice with PBS and suspended in 0.1 ml of a 1:20 dilution of goat anti-rabbit immunoglobulin G (Kalstead) and further incubated for 15 min at 23°C. The organisms were then washed thrice in PBS, smeared on a glass slide, mounted in a drop of Elvanol at pH 6.65, and examined with a fluorescence microscope.

RESULTS

Previous studies have shown that adherence of intact *E. coli* to epithelial cells of the oral cavity (18) and of the intestine (4) is specifically inhibited by D-mannose and its derivatives. The adherence of each of several human isolates of *E. coli* was similarly inhibited by αMM but not by methyl α-D-glucopyranoside (Table 1), indicating that the ability to bind to mannose residues on epithelial cells is a characteristic shared by certain human strains of *E. coli*.

Aggregation of mannan-containing yeast cells induced by *E. coli*. To assess the mannose-binding activity of the intact organisms, the interaction of the *E. coli* strains with mannan-containing yeast cells was studied by aggregometry. In this way the rate of *E. coli*-yeast cell interaction could be monitored (Fig. 1). αMM (or D-mannose, not shown in Fig. 1), but

not methyl α-D-glucopyranoside, either inhibited the reaction or reversed it (Fig. 1). Similarly to methyl α-D-glucopyranoside, the following sugars tested at the same molarity were without effect on aggregation: methyl α-D-galactoside, D-glucose, D-glucosamine, *N*-acetyl-D-glucosamine, rhamnose, maltose, galactose, 2-deoxy-D-glucose, L-fucose, L-arabinose, methyl β-L-arabinoside, D-arabinose, L-xylose, D-xylose, and *N*-acetylneuraminic acid. Yeast cells pretreated with sodium metaperiodate, a reagent which cleaves vicinal hydroxyl groups of sugars, no longer agglutinated with *E. coli*. The treatment with sodium metaperiodate did not cause any loss in number or integrity (as judged by phase-contrast microscopy) of the yeast cells.

The rate of the *E. coli*-yeast cell interaction was dependent on the dose of *E. coli* added to the system (Fig. 2). To see the relationship between yeast cell aggregation and mannose binding more clearly, the rates of aggregation induced by the various human isolates were plotted against the concentrations of αMM needed to achieve 50% inhibition of aggregation (Fig. 3). It is clearly shown that the concentration of αMM needed to achieve 50% inhibition was directly proportional to the rate of aggregation induced by the strain tested. These results indicate that yeast cell aggregation is mediated by the interaction of a ligand(s) on the surface of *E. coli* with mannose residues on the yeast cells.

TABLE 1. *Inhibition of adherence to epithelial cells of various strains of E. coli by αMM*[a]

E. coli strain	No. of adherent bacteria/epithelial cell in presence of:		
	PBS	αMM	αMG
M1	79 ± 19	4 ± 2	86 ± 10
M2	51 ± 7	7 ± 4	56 ± 8
M3	79 ± 7	4 ± 2	75 ± 10
M4	23 ± 6	5 ± 3	23 ± 6
M5	30 ± 3	4 ± 1	28 ± 3
M6	41 ± 5	3 ± 1	44 ± 6
M7	56 ± 7	5 ± 2	60 ± 8
M8	76 ± 7	6 ± 4	70 ± 10
M9	89 ± 10	4 ± 1	91 ± 11
M10	25 ± 3	3 ± 1	26 ± 5
M11	77 ± 11	7 ± 3	77 ± 8
M12	70 ± 12	6 ± 3	68 ± 7

[a] Adherence tests were performed as described in the text. PBS (0.1 ml) without or with a 0.02 M final concentration of αMM or methyl α-D-glucopyranoside (αMG) was added to each test tube before adding 0.4 ml of bacteria and 0.4 ml of epithelial cell suspensions. Nonadherent bacteria were separated from epithelial cells by four differential centrifugations (100 × *g* for 5 min), using either PBS alone or PBS containing 0.02 M concentrations of the respective sugars. Results given are means ± standard error.

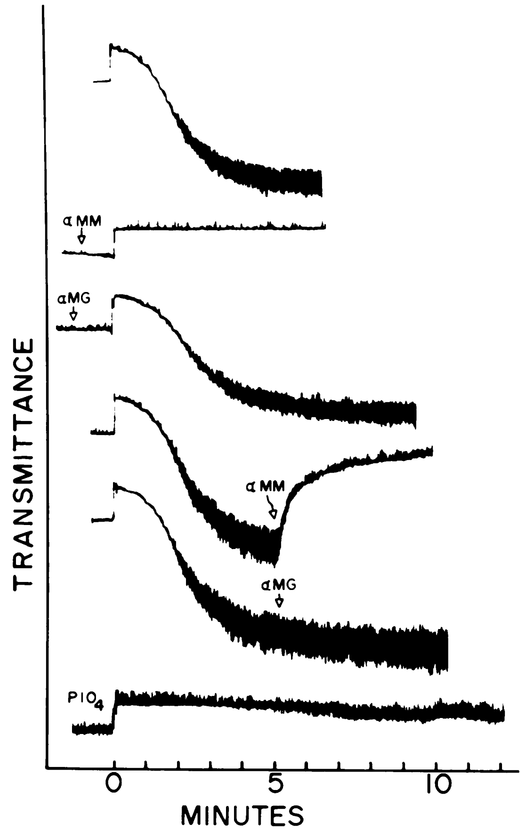

FIG. 1. *Aggregation of mannan-containing yeast cells by E. coli (top curve) was completely inhibited by preincubation of the yeast cells with αMM (second curve), but not with methyl α-D-glucopyranoside (αMG) (third curve). When added 5 min after the addition of E. coli to the yeast cells, αMM (fourth curve), but not αMG (fifth curve), reversed aggregation. Yeast cells treated with sodium metaperiodate (PIO_4) lost their ability to aggregate with E. coli (bottom curve).*

Relation between yeast cell aggregation and epithelial cell adherence. To examine the relationship between the yeast cell aggregation test and the ability of the *E. coli* to adhere to epithelial cells, the various human isolates were assayed at the same time in both systems. A high degree of correlation ($P < 0.001$) was observed between the rate of aggregation and number of *E. coli* adherent per epithelial cell (Fig. 4).

Piliation and mannose-binding activity of *E. coli* displaced from epithelial cells with αMM. The variations in the abilities to adhere to epithelial cells and in the rates of aggregation among the various human isolates may reflect either differences in the proportion of organisms in each culture possessing these

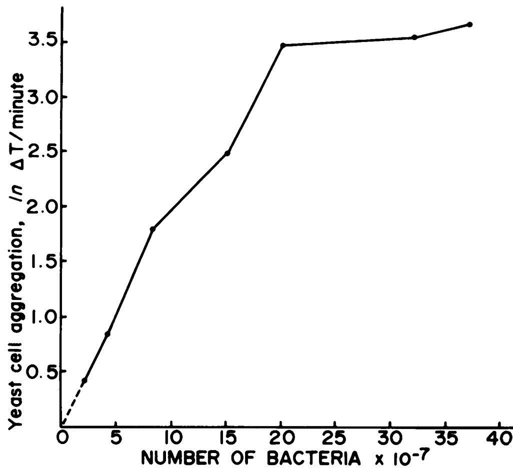

FIG. 2. *Dose-response curve of yeast cell aggregation (measured as change in percentage of transmission [ΔT] per minute) induced by E. coli.*

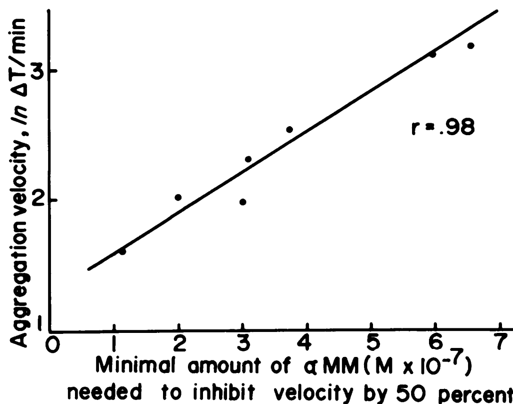

FIG. 3. *Correlation between the velocities of yeast cell aggregation (measured as change in percentage of transmission [ΔT] per minute) induced by seven E. coli isolates and the amounts of αMM needed to inhibit the rates of aggregation by 50%. Each point represents the velocity of aggregation induced by an individual strain and the amount of αMM needed to reduce the velocity by 50%.*

activities or differences in the binding activities of all the organisms from one culture to the next. To resolve this issue, we performed experiments with a strain of *E. coli* (M10) which exhibited relatively low activity in the aggregation and adherence tests and contained a low percentage of heavily piliated organisms (Fig. 5). The organisms were allowed to interact with epithelial cells at a high ratio of bacteria to epithelial cells (5,000:1) to allow maximum adherence. The epithelial cells containing adherent bacteria were separated from the nonadherent organisms by several differential centrifugations. αMM, but not the other sugars tested, displaced over 90% of the organisms from the epithelial cells within 30 min (Fig. 6). When compared with the original culture, the αMM-displaced organisms showed a much higher proportion of heavily piliated organisms (see Fig. 5), and, after washing the bacteria free of the sugar, the organisms exhibited a marked increase in their ability to aggregate yeast cells (Fig. 7). The nonadherent bacteria, collected from the supernatants of the epithelial cell-*E. coli* mixtures, were devoid of pili and agglutinating activity for yeast cells (see Fig. 7); the distribution of the numbers of pili among the three populations of bacteria is depicted in Fig. 8. A second strain of *E. coli* (M2) tested in the same way demonstrated similar results. Even though the percentage of piliated organisms and the agglutinating activity were greater in the original suspension of the second strain as compared with that of the first test strain, adsorption to epithelial cells again selected organisms that were more piliated (78% as compared with 42% in the original suspension), and were better able to agglutinate yeast cells (velocity, ln 3.8 as compared with ln 2.5 for the unadsorbed suspension). The nonadherent bacteria were similarly devoid of pili (of any length) and lacked any detectable activity in the yeast cell aggregation test. Similar results were obtained when a K-12 strain, known to contain type 1 pili (14), was used in the fractionation procedures. These results indicate that the epithelial cells selectively adsorbed piliated organisms which possess mannose-binding activity.

Comparison of surface antigens of adherent and nonadherent bacteria. The adherent organisms displaced with αMM also could be

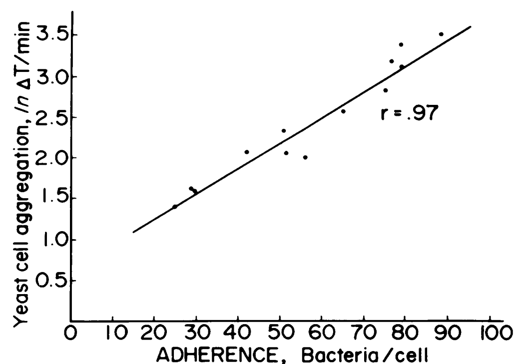

FIG. 4. *Correlation between velocities of yeast cell aggregation (measured as change in percentage of transmission [ΔT] per minute) induced by E. coli isolates and the adherences of the bacteria to human epithelial cells.*

FIG. 5. *Fractionation of E. coli by adsorption to epithelial cells and displacement with αMM. Washed epithelial cells (top left) were mixed with a suspension containing few piliated organisms (top center). Nonadherent bacteria were removed by differential centrifugation, leaving only those E. coli that were attached to epithelial cells (top right). The adherent organisms were displaced from the surfaces of the epithelial cells by the addition of αMM (bottom right). The displaced bacteria, containing many piliated organisms (bottom center), were separated from the epithelial cells (bottom left) by differential centrifugation.*

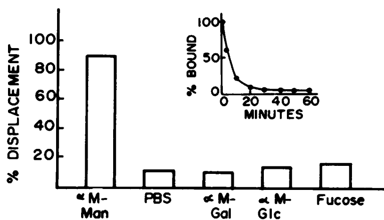

FIG. 6. *Specificity and rate of displacement of E. coli from epithelial cell surfaces by αMM. The percentages of displacement by various sugars are shown by the bars, and the rate of displacement by 25 mg of αMM per ml is shown in the inset. Abbreviations: αM-Gal, methyl α-D-galactoside; αM-Glc, methyl α-D-glucopyranoside.*

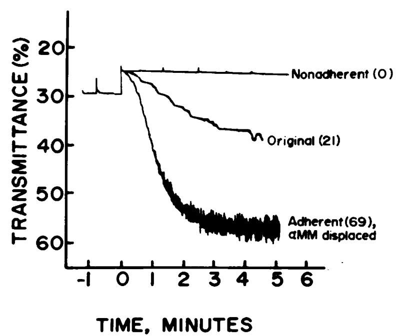

FIG. 7. *Aggregation of yeast cells induced by adherent E. coli displaced from epithelial cells with αMM (bottom curve) as compared with the original, unfractionated culture (middle curve). The organisms which were unable to adhere (nonadherent) to epithelial cells also lacked yeast cell-aggregating activity (top curve). Numbers in parentheses indicate percentages of bacteria possessing 50 or more pili.*

distinguished from the nonadherent bacteria by differences in their surface antigens, using immunofluorescent techniques. Antiserum prepared against the original suspension and absorbed with nonadherent organisms reacted

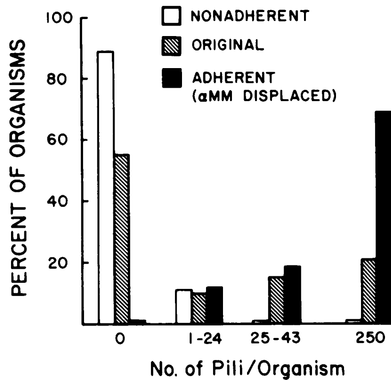

FIG. 8. *Distribution of numbers of pili among the population of E. coli fractionated by adsorption to and displacement from epithelial cells (see Fig. 5).*

strongly with all of the αMM-displaced organisms but with none of the organisms in the nonadherent fraction and with only a small percentage of the organisms in the original bacterial suspension (Table 2).

DISCUSSION

In the present study we show that the mannose-binding activity of intact bacteria can be readily and accurately monitored by aggregometry with mannan-containing yeast cells (21). The evidence that the interaction of yeast cells with *E. coli* is reversible and involves binding of intact bacteria to mannose residues on the surfaces of yeast cells is as follows: (i) the aggregation is either inhibited or reversed by the addition of D-mannose or its derivative αMM, but not by other sugars, and (ii) yeast cells pretreated with sodium metaperiodate, a reagent which cleaves vicinal hydroxyl groups of sugars, can no longer agglutinate with *E. coli*.

We previously showed that adherence of *E. coli* to human epithelial cells is mediated by a lectin-like substance specific for mannose on the surfaces of bacteria and by mannose residues on the surfaces of epithelial cells (18). In the present study we show that several human isolates of *E. coli* adhere by the same mechanism. αMM, but not methyl α-D-glucopyranoside, completely inhibits the adherence of the *E. coli* isolates to epithelial cells. The interaction of intact organisms with mannose residues, like that of lectins with sugars (20), is reversible, as shown previously (18) and in the present study. It should be stressed that the continuous presence of the sugar in the reaction mixture is essential to detect competitive inhibition, because once the sugar is washed away, the bacteria regain full adhering ability (18) or hemagglutinating activity (4). The mechanism of the interaction of *E. coli* with yeast cells is similar to that of *E. coli* with epithelial cells, since (i) there was a high correlation between the two activities in the various isolates of *E. coli,* (ii) both activities were mediated by mannose, and (iii) bacteria fractionated by epithelial cell adsorption and αMM displacement showed increased aggregating activity for mannan-containing yeast cells. Yeast cell-*E. coli* interaction, the rate of which can be accurately monitored, should prove useful for the purpose of screening large numbers of strains for their abilities to adhere to mannose residues on epithelial cells.

Our studies indicate that the ability of a culture of *E. coli* to adhere to epithelial cells is determined by the proportion of organisms which possess mannose-binding activity in that culture. Since this study used strains which were subcultured several times in the laboratory, it could not be concluded whether the differences observed in the adhering abilities of the various strains reflected constant characteristics of the strains or whether they reflected phenotypic variations which may occur during laboratory passage. Phenotypic variations in relation to adhering ability have been clearly shown for fresh isolates of *Neisseria gonorrhoeae* and their unselected laboratory derivatives (22). The method of monitoring mannose-binding activity described in the present study should enable assessment of the mannose-binding activity of *E. coli* obtained directly from clinical specimens, since only 10^7 to 10^8 bacteria per 10 μl are required for the test.

The identity of the mannose-binding substance on *E. coli* is still not known. Salit and Gotschlich (19) showed that hemagglutination induced by pili isolated from *E. coli* is specifi-

TABLE 2. *Immunofluorescence of adherent (αMM-displaced) and nonadherent E. coli treated with absorbed antiserum*[a]

E. coli suspension[b]	Fluorescent bacteria (%)[c]
Original (unfractionated)	22
Nonadherent fraction	0
Adherent fraction (αMM displaced)	100

[a] Antiserum raised in rabbits against *E. coli* (see text) was diluted 1:20 in PBS, and a 1-ml volume was absorbed with a pellet of 10^{10} nonadherent *E. coli* at 23°C for 30 min.

[b] The various *E. coli* fractions were obtained as described in Fig. 5.

[c] Numbers represent the percentages of fluorescent organisms in 200 bacteria counted by bright-field microscopy. Only bacteria which were uniformly fluorescent were scored.

cally inhibited by D-mannose. We showed in this study that the fraction of organisms which adhered to epithelial cells was richer in piliated organisms and possessed increased mannose-binding activity. Taken together, the results suggest that the mannose-binding ligand on *E. coli* cells is associated with pili.

A few comments should be made concerning the relationship between the mannose-mediated adherence of *E. coli* and infections with these organisms. Duguid (3) screened 108 strains of *E. coli* isolated from humans by hemagglutination and mannose inhibition of hemagglutination. He found that the majority (74%) of the strains possessed mannose-binding activity and that all of the mannose-sensitive strains were piliated. Mannose (or its derivatives) was found to inhibit the adherence of *E. coli* to rabbit, rat, and human intestinal cells (4; M. Thaler et al., Clin. Res., **25**:469A, 1977) as well as to human polymorphonuclear leukocytes (1; F. J. Silverblatt et al., Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 17th, no. 212, 1977) and mouse peritoneal macrophages (1). In preliminary experiments it was found that mannose prevented *E. coli* colonization of mouse urinary tracts (M. Aronson, O. Mesalia, L. Schori, D. Mirelman, N. Sharon, and I. Ofek, submitted for publication) and rat intestines (M. Hirschberger et al., Gastroenterology **72**:1069, 1977), presumably by blocking adherence to these tissues. These observations, along with many studies on concanavalin A (20), a mannose- and glucose-specific ligand, indicate that mannose residues are widely distributed among animal and human cells and probably serve as receptors for the attachment of *E. coli* which possess mannose-binding activity.

Nevertheless, it is possible that receptors other than mannose may serve for the attachment of *E. coli* to epithelial cells. First, Duguid (3) noted that hemagglutination of certain human isolates of *E. coli* could not be inhibited by D-mannose. Some of these mannose-resistant, enterotoxigenic *E. coli* have been recently shown to possess a so-called colonization factor (6). Second, it has been reported that β-galactosyl residues on epithelial cells may serve as receptors for the adherence of certain *E. coli* strains of porcine origin possessing K88 antigen (9). Finally, recent studies indicate that the adhesive properties of epithelial cells for *E. coli* may vary from tissue to tissue (5, 8, 11, 13) or from subject to subject (7). Conversely, the adhesiveness of a particular epithelial cell may vary from one clinical isolate to another (4a).

We have shown that the ability of an isolate of *E. coli* to adhere to human epithelial cells is directly related to its ability to bind mannose residues. The determination of the availability of such mannose residues on various mucosal tissues, using mannose-specific ligands, should clarify the mechanisms of adherence mediated by mannose or possibly by other receptors. In turn, the assessment of the mannose-binding activity of *E. coli* obtained from various human specimens should shed light on the ability of a particular isolate to colonize mucosal surfaces by virtue of binding to mannose residues on the membranes of epithelial cells.

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