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Relationship of Hemagglutination to Other Biological Properties of Serologically Classified Isolates of *Escherichia coli*

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The ability of 170 serologically classified strains of *Escherichia coli* to agglutinate human erythrocytes was examined. Erythrocytes of blood group A were more sensitive indicators of this property than were those of groups B or O. The predominant receptor was shown to be mannose containing; however, an additional receptor was found in two of nine strains studied. Natural mannose-like inhibitors were not found in unconcentrated urine obtained from 12 humans. Isolates from the urine or blood of patients with infections agglutinated erythrocytes significantly more frequently than did isolates from feces. Urine isolates of 10 common serogroups and isolates of less common serogroups did not differ in their ability to agglutinate erythrocytes. Among isolates from the urine of patients with infections, the ability to agglutinate erythrocytes did not correlate with either the serogroup of the strain or the clinical syndrome of the patient. Of the several other biological properties that were examined, only the production of colicins showed a significant association with the ability to agglutinate human erythrocytes.

The ability of some strains of *Escherichia coli* and of other bacterial species to agglutinate erythrocytes obtained from a variety of mammals, including humans, has been recognized for some time (3, 5, 7). This property of bacteria is not confined to erythrocytes, as certain strains of *E. coli* are capable of agglutinating leukocytes, thrombocytes, sperm, yeast cells, fungus spores, plant pollen of various kinds, and renal cells in tissue culture (9, 11). The ability to agglutinate erythrocytes generally has paralleled the presence of pili on the surface of the bacteria and has usually been inhibited by yeast mannan and closely related substances (1-3, 10). The biological significance of the possession of this property by strains of *E. coli*, presumably an indirect expression of the presence of pili, has not been defined extensively.

The present studies assessed the ability of 170 isolates of *E. coli* to agglutinate human erythrocytes. The relationships of this property to the source and serogroup of the isolates, to certain of their other biological properties, and to the clinical syndromes associated with isolates from patients with extraintestinal infections were analyzed.

MATERIALS AND METHODS

Bacteria. A total of 170 isolates of *E. coli* or late-lactose-fermenting bacteria which otherwise resembled *E. coli* were selected, generally from among those isolates included in a previous report which described the bacteriological and serological methods used for their characterization (17). A total of 87 of the isolates represented strains from O groups 1, 4, 6, 7, 16/62, 17/77, 18ab/ac, 25, 50, and 75; 79 isolates were distributed among 44 other O groups; and 4 isolates could not be serogrouped. Of the 170 isolates, 95 were recovered from fecal specimens, 63 were recovered from the urine of patients with infections of the urinary tract, and 12 were recovered from the blood of patients with bacteremia secondary to acute pyelonephritis. The isolates had been stored on paraffin-sealed, soft nutrient agar slants and were recovered by growth in Trypticase soy both.

Hemagglutination test. The test isolates were grown aerobically for 24 h at 37°C in tubes containing 10 ml of peptone water (peptone [Difco], 1.0 g; NaCl, 0.5 g; distilled water, 100 ml; pH 7.0). Growth from the third passage through peptone water was used in the hemagglutination test.

Human erythrocytes of blood groups A, B, and O were collected in Alsever solution, washed twice with a hemagglutination buffer (Difco), and made up to 3% suspensions in the same buffer. Fresh cells from the same donors were obtained on each day the tests were conducted.

The tests were performed by mixing 1 drop (disposable Pasteur pipettes) of a suspension of erythrocytes with 1 drop of a carefully mixed bacterial culture in a well of an agglutination slide which contained 12 concavities (16-mm diameter and 1.75-mm depth). The slide was rotated in a rocking motion for 3 min at

room temperature, and the presence or absence of macroscopic hemagglutination was recorded (Fig. 1). If the initial test was negative, the culture was centrifuged and 1 drop of the bacterial sediment was tested for its hemagglutinating ability as described above.

Hemagglutination inhibition test. This was performed essentially as described above for the hemagglutination test. The bacterial sediment from an overnight growth of the test strain in 140 ml of peptone water was resuspended in 1.4 ml of hemagglutination buffer. Twofold dilutions of the bacterial suspensions were tested by mixing 1 drop of the dilution of bacteria with 1 drop of a 3% suspension of type A, Rh-negative erythrocytes and 1 drop of hemagglutination buffer. One hemagglutinating unit was defined as the last dilution of the bacterial sediment causing macroscopic hemagglutination.

For the inhibition test, 1 drop of 4 hemagglutinating units of a test isolate was mixed with 1 drop of a stock inhibitor solution and incubated at room temperature for 10 min with occasional rocking of the slide. Thereafter, 1 drop of a 3% suspension of erythrocytes was added to the mixture, and the presence or absence of macroscopic hemagglutination was recorded after 3 and 10 min of additional rocking of the slide.

The inhibitors tested were D(+)-galactose, α-D(+)-glucose, sucrose, L-fucose, D-mannitol, D(+)-mannose, α-methyl-D-mannoside, and yeast mannan (Sigma Chemical Co., St. Louis, Mo.). Stock solutions of 4,000 μg/ml were prepared, which resulted in approximately 200 μg/drop.

Other biological properties. The sensitivity of the test isolates to the serum bactericidal system was measured as described previously (18). Their ability to produce colicins and to be inhibited by a standard set of colicins was determined by methods reported previously (15). Motility was assessed from a stab culture in 0.5% nutrient gelatin agar media, and the production of hemolysins was detected by growth on human blood agar plates.

Statistical analyses. Chi-square analyses were performed by using fourfold contingency tables with the Yates correction factor (4). *P* values were determined by reference to a standard table of critical values of chi square for two tails.

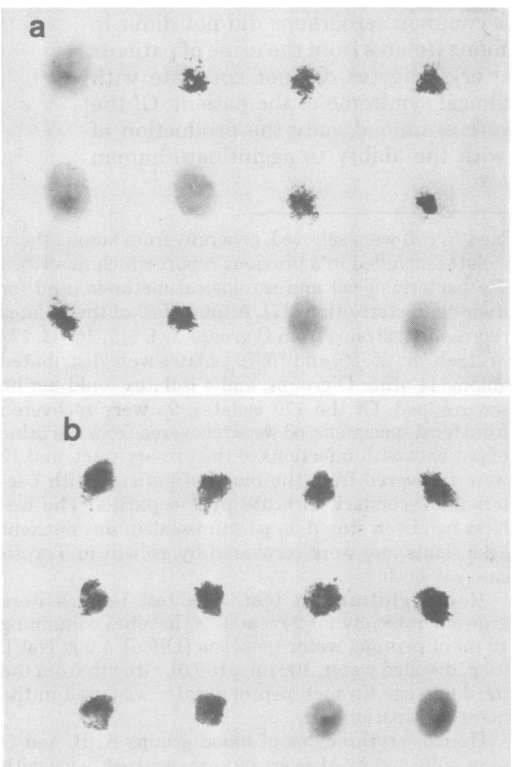

FIG. 1. *Hemagglutination inhibition test patterns. Top row (left to right): yeast mannan, L-fucose, D(+)-galactose, α-D(+)-glucose. Middle row (left to right): D(+)-mannose, α-methyl-D-mannoside, D-mannitol, sucrose. Bottom row (left to right): wells 1 and 2, positive controls of bacterial hemagglutination; wells 3 and 4, negative controls of erythrocytes. (a) Mannose-sensitive isolate. (b) Mannose-resistant isolate.*

RESULTS

Human erythrocytes. Of 47 isolates of *E. coli* which possessed the ability to agglutinate human erythrocytes, 38 (81%) reacted with A, B, and O cells, 3 (6%) reacted with both A and B but not O, and 6 (13%) reacted only with A. No isolates agglutinated B or O erythrocytes but failed to react with A, Rh-negative cells.

Characterization of the attachment site. Inhibition of the hemagglutination reaction was assessed with eight substances. Two patterns of inhibition were observed among the nine isolates that were studied. With seven isolates, the reactions were inhibited by yeast mannan, D(+)-mannose, and α-methyl-D-mannoside (Fig. 1). The minimum amounts required to inhibit hemagglutination were 12.5 μg for D(+)-mannose and α-methyl-D-mannoside and 25 to 50 μg for yeast mannan. The reactions of two isolates were inhibited by none of these three compounds nor by D(+)-galactose, α-D(+)-glucose, sucrose, D-mannitol, or L(−)-fucose at the maximum concentration used (200 μg).

Source and serogroup of isolates. Of 170 isolates obtained from specimens of feces, urine, and blood, 62 (36%) agglutinated human erythrocytes. This represented 22 of 95 (23%) fecal isolates (Table 1), 32 of 63 (51%) urinary isolates (Table 1), and 8 of 12 (75%) blood isolates (Table 2). The differences in the abilities of fecal and urinary isolates and of fecal and blood isolates to agglutinate human erythrocytes were significant ($P < 0.01$).

The proportion of isolates from urine of 10 common serogroups which agglutinated erythrocytes was not significantly different from that

of all the other serogroups (Table 1). However, the proportion of isolates of the 10 common serogroups which was positive was significantly greater ($P < 0.05$) for isolates from urine than for isolates from feces; this same relationship was found when isolates of all the other serogroups from urine were compared with isolates of all the other serogroups from feces.

Clinical syndromes. The ability of isolates obtained from patients with different clinical syndromes to agglutinate erythrocytes is presented in Table 2. When the total number of isolates was examined, a tendency toward an increasing proportion of isolates with a positive reaction was observed when isolates from patients with asymptomatic bacteriuria were compared with isolates from patients with cystitis, acute pyelonephritis, or bacteremia. However, these differences were not significant, nor was the difference observed when isolates from patients with cystitis were compared with isolates from patients with acute pyelonephritis.

The numbers of isolates were not large enough to assess the potential relationship of hemagglutination to clinical syndromes among isolates of individual serogroups. When isolates of serogroups O4 and O6 were pooled, 6 of 13 (46%), 7 of 11 (64%), and 5 of 8 (63%) isolates from patients with cystitis, acute pyelonephritis, and bacteremia, respectively, were positive. None of the pairs of clinical syndromes differed significantly from one another.

Other biological properties. The relationship of other properties to the ability to agglutinate erythrocytes was determined for 15 isolates with positive and 30 isolates with negative hemagglutination reactions. As Table 3 shows, a significant difference ($P < 0.02$) was found only in the ability to produce colicins; i.e., isolates capable of agglutinating erythrocytes were more likely to produce colicins.

Urinary hemagglutination inhibition factor. Clean catch specimens of urine were ob-

TABLE 1. *Agglutination of human erythrocytes by isolates of E. coli from urine and feces*

Serogroup of isolates	Urinary isolates		Fecal isolates	
	No. positive	Total no.	No. positive	Total no.
1	3	4	0	2
4	8	11	0	1
6	5	14	1	9
7	3	3	2	2
16/62	2	2	2	3
17/77	0	2	0	1
18 ab/ac	1	2	2	3
25	0	4	0	1
50	2	4	0	3
75	2	3	1	2
All others	6	14	14	68

TABLE 2. *Relationship of the ability to agglutinate human erythrocytes by isolates of E. coli from specimens of urine or blood obtained from patients with different clinical syndromes*

Serogroup of isolates	Clinical syndrome[a]			
	AB	Cystitis	AP	Bacteremia
1	—[b]	3/4[c]	—	—
4	0/1	4/6	4/4	1/1
6	—	2/7	3/7	4/7
7	—	3/3	—	—
16/62	—	1/1	1/1	1/1
17/77	—	0/1	0/1	0/1
18 ab/ac	—	1/2	—	—
25	0/1	0/2	0/1	—
50	—	—	2/4	1/1
75	—	1/2	1/1	—
All others	3/6	2/6	1/2	1/1
% Positive	38	50	57	75

[a] AB, Asymptomatic bacteriuria; AP, acute pyelonephritis.
[b] —, No strains studied.
[c] Number of isolates positive for agglutination/total number of isolates.

TABLE 3. *Relationship between the ability to agglutinate erythrocytes and other biological properties of 45 isolates of E. coli*

Hemagglutination reaction	Produce hemolysin	Motile	Ultrasensitive to serum bactericidal system	Sensitive to colicins	Produce colicins
Positive (15)[a]	5/15[b]	8/15	4/15	10/15	8/15
Negative (30)	13/30	24/30	8/30	15/30	4/30
Significance of difference[c]	NS	NS	NS	NS	$P < 0.02$

[a] Numbers in parentheses are numbers of strains tested.
[b] Number positive/total number.
[c] The significance of the difference between the hemagglutination reaction and each of the other variables is expressed as a P value for chi-square analysis; NS, not significant, i.e. $P > 0.05$.

tained from four males and five females without and three females with past histories of urinary tract infections to test for the presence of inhibitors of hemagglutination in human urine. The specimens were refrigerated and examined without concentration or other manipulations within 2 h of collection. None of the 12 specimens of urine inhibited the ability of a single isolate of *E. coli* to agglutinate human A, Rh-negative erythrocytes. The hemagglutination reaction with this isolate was known to be inhibited by yeast mannan, α-methyl-D-mannoside, and D(+)-mannose.

DISCUSSION

For many years some strains of *E. coli* have been known to cause direct agglutination of erythrocytes (3, 5, 7, 9). The early evidence that this property was an indirect expression of the presence of pili ("fimbriae") has been strongly supported by recent work with highly purified preparations of type I pili from *E. coli* K-12 (10). The same authors demonstrated that purified type I pili bound to monkey kidney cells in tissue culture via mannose-containing receptors that were similar to those involved with erythrocytes (11). The sharing of a mannose-containing receptor site by monkey kidney cells, erythrocytes, and bovine bladder epithelial cells (Vosti, unpublished data) suggested that erythrocytes might provide a simple indirect means of examining the virulence of isolates of *E. coli* for the urinary tract.

Because of my interest in assessing the relationship of the agglutination of erythrocytes by isolates of *E. coli* to virulence of these isolates for humans, I purposefully elected to use human erythrocytes, even though their reactivity is not as strong as that of cells from certain other species (3). In addition, their use seemed reasonable since Duguid et al. (3) found a few isolates which reacted only with human erythrocytes. The ability of our strains of *E. coli* to agglutinate human erythrocytes of different blood groups (A, B, O) was not uniform. Erythrocytes of blood group A were agglutinated by all of the test strains that gave a positive reaction. Thirteen percent of the strains reacted only with group A cells, and 19% failed to react with blood group O cells. These results varied from those of Duguid et al. (3), who reported no difference in reactivity of cells of the different blood types. In other studies the blood group of the test cells was not identified (7, 9), or only cells of a single type were used (8). The participation of a mannose-containing receptor in most reactions was confirmed; however, I also found two among the nine strains studied that possessed either another receptor in addition to the mannose-containing receptor or one or more different receptors. These findings were similar to those of Duguid and Gillies (2) but differed significantly from those of Minshew et al. (8), who in their discussion noted that in their experience the agglutination of human erythrocytes by strains of *E. coli* was resistant to inhibition by 0.5% D-mannose. Because of a potential role for this binding site in the initiation of infections of the urinary tract, unconcentrated urine was screened in the present study for the presence of a natural, mannose-like inhibitor which might participate in host defense mechanisms of the urinary tract by interfering with the attachment of *E. coli* to uroepithelial cells. None was found; thus, unless present in concentrations not detectable by the test conditions used, natural inhibitors of attachment via a mannose-containing receptor do not seem to play a role in host defenses in urinary tracts.

A comparison of my results with those of others was generally not possible since either the source of the strains was not identified (7), the results of hemagglutination were not related to the source of the strain, even though the latter was identified (3), or too few strains were studied (8). The importance of differentiating strains by source, when comparing the results of different studies, is emphasized by the striking difference in the ability of strains isolated from feces to agglutinate erythrocytes as compared with the ability of strains isolated from urine or blood. Thus, differences in the composition, by source, of a group of test strains would influence greatly the proportion of the total number of strains which would be positive. In the present study the high proportion of strains from urine and blood which agglutinated human erythrocytes was comparable to the proportion found previously with 22 strains from mixed extraintestinal sites (8). Similarly, the low proportion of fecal strains agglutinating human erythrocytes was similar to the 17% found among 70 unselected isolates from human feces (9).

Since we (17) have demonstrated previously that strains of a relatively few serogroups account for most extraintestinal infections, the possibility existed that differences in the abilities of isolates to agglutinate human erythrocytes might be related to the serogroups of the isolates rather than to their source. However, my results clearly demonstrate that this was not the case since isolates (from extraintestinal specimens) of both the common and uncommon serogroups did not differ in their ability to agglutinate human erythrocytes. Moreover, isolates of the common serogroups from urine were significantly more likely to agglutinate human eryth-

rocytes than were isolates of the common serogroups from feces. A similar relationship was found when strains of the uncommon serogroups from urine were compared with such strains from feces.

Since the isolates from extraintestinal specimens were obtained from patients with various well-defined clinical infection syndromes, differences in the abilities of isolates from patients with the various clinical syndromes to agglutinate human erythrocytes were examined. Although there was a progressive increase in the proportion of isolates from patients with asymptomatic bacteriuria, cystitis, acute pyelonephritis, and bacteremia secondary to acute pyelonephritis which agglutinated erythrocytes, these differences were not statistically significant. Again, no significant differences were found when strains of only serogroups O4 and O6 were examined in the same manner.

Studies of the relationship of the ability to agglutinate human erythrocytes to certain other biological properties of 45 strains of *E. coli* revealed only a significant association with the ability to produce colicins. Minshew et al. (8) demonstrated a high association between the ability to agglutinate human O, Rh-negative erythrocytes and virulence for chicken embryos among 22 extraintestinal isolates, including 9 from urine and 9 from blood. Unfortunately, their strains were not serogrouped, and therefore this association could have reflected differences in the serogroups of the strains rather than differences in their source.

Although strains of only a few of the more than 135 possible O groups of *E. coli* account for the majority of extraintestinal infections with these organisms (17), the biological advantage that strains of the common O groups possess is not completely understood. We and others have found that they are more likely to possess the following properties than are strains of the less common O groups: O inagglutinability (6, 14), mouse toxicity (12), skin-necrotizing capacity (12), production of hemolysins (6, 12, 14, 16), relative resistance to the serum bactericidal system (18), resistance to colicins (15), and enhanced ability to adhere to uroepithelial cells (13). Although not limited to strains of the common serogroups, the present study supports the hypothesis that the ability to adhere to a mannose-containing receptor may be advantageous to certain strains in establishing infections of the urinary tract. As with each of the other biological properties studied thus far, the findings are not absolute, and strains lacking the ability to agglutinate human erythrocytes are still capable of producing infections as serious as acute pyelonephritis with or without bacteremia. Therefore, it seems inescapable that the virulence of a given strain is complex and may be related to many and varied combinations of the previously identified biological properties and probably to some that are not yet identified.

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