INFECTION AND IMMUNITY, Dec. 1981, p. 944-948
0019-9567/81/120944-05$02.00/0

Vol. 34, No. 3

Evidence for a Bladder Cell Glycolipid Receptor for *Escherichia coli* and the Effect of Neuraminic Acid and Colominic Acid on Adherence

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Received 11 May 1981/Accepted 10 August 1981

The rat bladder epithelial cell receptors involved in mannose-sensitive adherence of *Escherichia coli* strains were studied. Sodium metaperiodate and lipase pretreatment of epithelial cells significantly reduced bacterial adherence to cells whereas trypsin and phospholipase C had a marginal or insignificant effect on adherence. Neuraminidase and colominic acid significantly increased adherence, whereas *N*-acetylneuraminic acid significantly reduced adherence. These data suggest that the rat bladder epithelial cell receptors involved in mannose-sensitive adherence are glycolipids. In addition, the data suggested that sialic acid on bladder epithelial cells acts as a nonspecific inhibitor of adherence, whereas colominic acid, a component of some *E. coli* K1 capsules, may act as a promoter of adherence.

Adherence to host tissues has been suggested by many investigators (11, 12, 16, 18, 20) to be a major pathogenic mechanism for *Escherichia coli* in urinary tract infections. Studies of *E. coli* strains isolated from human urinary tract infections have strongly suggested a relationship between the virulence and capacity of a strain to attach to human cells or agglutinate erythrocytes (16, 19, 20). Evidence from several laboratories indicates that pili or fimbriae mediate attachment and erythrocyte agglutination in several gram-negative genera (1, 4, 5, 14, 20).

Information about mammalian surface structures which interact with bacteria is sparse. Some studies done with *E. coli* suggest that mannose or closely related compounds (i.e., α-methyl-D-mannoside) block the pilus-mediated attachment of bacteria to erythrocytes and cells derived from the urinary tract (1, 4, 16). The nature of the host cell receptor that participates in mannose-sensitive adherence is not known. Other investigations suggest that some pilus-mediated bacterial adherence cannot be blocked by mannose or its derivatives (19, 20). This adherence is termed mannose resistant. Recently, Leffler and Svanborg-Edén provided evidence that a single *E. coli* strain which showed mannose-resistant adherence to human cells (mainly squamous cells and human erythrocytes) adhered to a glycosphingolipid (10).

The purpose of this study was to discern some information about the nature of the mannose-sensitive receptors on bladder epithelial cells. We present evidence that suggests that a glycolipid comprises rat bladder cell receptors and that colominic acid, a component of some *E. coli* capsules, may serve as a mechanism by which *E. coli* strains may increase adherence to host cells.

MATERIALS AND METHODS

Bacteria. The following four strains of *E. coli* were used: 07KL, K12H, K12NH, and 1974. These strains, except 1974, have been described in detail previously (1). Strain 1974 was isolated from a diabetic patient with pyelonephritis and donated by the Clinical Laboratory, John Sealy Hospital, University of Texas Medical Branch, Galveston, Tex. Bacteria were incubated statically for 24 h in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). Bacteria were harvested by centifugation, washed three times with phosphate-buffered saline (PBS) (pH 7.2, 37°C), and resuspended in PBS to a final concentration of 10^8 bacteria per ml. All strains except K12NH possessed pili as shown by transmission electron microscopy. Strains 07KL and K12H showed mannose-sensitive hemagglutination of guinea pig erythrocytes as described previously (1). Strain 1974 showed mannose-resistant hemagglutination, and strain K12NH showed no hemagglutination.

Rat bladder epithelial cell preparation. Sprague-Dawley outbred albino female rats, weighing 150 to 250 g, were obtained from ARS/Sprague-Dawley (Madison, Wis.). Animals were given water and laboratory chow ad libitum. Bladders were surgically excised immediately from rats killed by ether anesthesia. Epithelial cells were scraped from the exposed mucosal surface with a sterile glass slide. Collected epithelial cells were washed with PBS (5 ml) three times and suspended in PBS to a final concentration of 10^5/ml as determined by counts in a Petroff-Hausser chamber.

Adherence test. The details of this test have been

described previously (1). Briefly, mixtures of equal volumes (0.5 ml) of standardized epithelial cells (10^5 cells per ml) and bacteria (10^8 cells per ml) were incubated at 37°C for 60 min with agitation in a heated water bath in combination with a wrist action shaker. Unattached bacteria were removed from the incubation mixture by five washes and centrifugations (164 × *g*). Pellets were then suspended in 0.05 ml of PBS, air dried on slides, and stained with methylene blue. Bacteria adhering to 20 large epithelial cells (~3,500 μm^2) under 1,000-fold magnification were counted.

Periodate pretreatment. Epithelial cells, after the first PBS wash, were incubated with agitation in 1 ml of a 10 mM solution of sodium metaperiodate (Sigma Chemical Co., St. Louis, Mo.) in PBS (pH 7.2) for 20 min at 37°C. After two additional 5-ml PBS washes, the cells were resuspended in PBS to a concentration of 10^5 cells per ml. The adherence test was performed as described above.

Enzyme modification. Trypsin (×2 crystalline; Worthington Diagnostics, Freehold, N.J.) and phospholipase C type I (7.1 U/mg; Sigma) were dissolved in PBS (pH 7.2). Lipase type VI (24,700 U/mg; Sigma) was dissolved in PBS that contained 0.0001% $MgCl_2 \cdot 6H_2O$ and 0.001% $CaCl_2$ (pH 7.7). Neuraminidase type VI (6.5 U/mg; Sigma) was suspended in 0.067 M phosphate buffer (pH 5.0). Epithelial cells were washed two times with PBS, suspended at a concentration of 10^5 cells per ml of enzyme solution, and incubated for 10 min at 37°C with shaking. Two washes with PBS to remove the enzyme were done before the cells were used in the adherence test. In one adherence test with neuraminidase, D-mannose (12.5 mg/ml; Sigma) was added immediately after the second wash to see whether it modified adherence. Control cells for the adherence test were cells that were treated identically, except that only the various buffer suspensions without enzymes were used to ensure that the observed effects were a result of enzymatic activity and not of the buffer or the pH.

NANA and colominic acid incubation. *N*-acetylneuraminic acid (NANA) (Sigma) and colominic acid (Sigma) were added to PBS-washed epithelial cells (10^5 cells per ml) at a concentration of 25 mg/ml. Bacteria (10^8/ml) were added to the suspension to give a final concentration of either NANA or colominic acid of 12.5 mg/ml. In one experiment, D-mannose (Sigma) and colominic acid were used together, each at a final concentration of 12.5 mg/ml. The adherence test was then performed.

Statistics. The standard error of the mean for each test was calculated when the number of epithelial cells counted was 20. Statistical significance between two means was determined by Student's *t* test between experimental and control values done on the same day with cells pooled from the same epithelial cell preparation.

RESULTS

Periodate treatment. Sodium metaperiodate, a selective oxidizing agent for 1,2-diols, impaired the adherence of *E. coli* O7KL and K12H (Table 1). Impaired adherence was not significant for the weakly adherent strain K12NH.

TABLE 1. *Effect of periodate pretreatment of rat bladder epithelial cells on E. coli adherence*

E. coli isolate	No. of bacteria[a] adhering to:		% Control adherence[d]	*P* value[e]
	Control cells[b]	Periodate treated cells[c]		
07KL	28 ± 3.3	12 ± 1.5	43	<0.001
K12H	47 ± 10.3	26 ± 4.5	55	<0.05
K12NH	10 ± 2.0	11 ± 1.8	110	NS

[a] Values represent the average number of adherent bacteria per epithelial cell ± the standard error of the mean.

[b] Controls were untreated cells and bacteria incubated together.

[c] Periodate-treated cells represent a portion of cells removed from the untreated pool and pretreated for 20 min with 10 mM sodium metaperiodate, which was subsequently removed with two PBS washes before the addition of bacteria.

[d] Percent control adherence is the number of bacteria adhering to periodate-treated cells divided by the number adhering to control cells times 100.

[e] Statistical significance between control and periodate-treated cells. NS, Not significant.

Enzyme treatment. Results of periodate treatment suggested that a carbohydrate may be involved in the epithelial cell receptor; nonetheless, other components of the epithelial cell surface could also be playing a role. Consequently, enzymatic pretreatment of the epithelial cells was done to examine the possible involvement of protein, lipid, and sialic acid residues in the receptor (Table 2). Trypsin either lysed the cells or caused a minor enhancement of adherence. Conversely, lipase caused a significant dose-dependent decrease in adherence, except at the highest concentration, which resulted in cell lysis. In contrast, phospholipase C caused no significant alteration of adherence. Interestingly, neuraminidase produced a significant increase in adherence at the highest concentrations tested; the lowest concentration tested produced a slight but statistically insignificant rise.

Carbohydrate treatment. Because neuraminidase treatment resulted in an increase in bacterial attachment, we reasoned that an increase in NANA (*N*-acetylneuraminic acid or sialic acid) may result in a net decrease in adherence. The three strains of *E. coli* tested showed significant decreases in adherence when coincubated with NANA (Table 3). In contrast, colominic acid (poly-2,8-*N*-acetylneuraminic acid), which is structurally related to NANA, significantly enhanced adherence (Table 3). The increased adherence was blocked by the addition of mannose (Table 3). Because mannose was effective at blocking the increased adherence

TABLE 2. *Altered adherence of E. coli 07KL by enzymatic pretreatment of rat bladder epithelial cells*

Enzyme treatment	Dose[a]	No. of adherent bacteria per epithelial cell[b]	% Control adherence[c]	*P* value[d]
Trypsin	None	56 ± 5.4	100	
	0.0001	65 ± 6.2	116	NS
	0.001	77 ± 6.8	138	$0.05 < P < 0.1$
	0.01	—	—	—
	0.1	—	—	—
	1.0	—	—	—
Lipase	None	37 ± 4.7	100	
	0.0001	36 ± 5.1	97	NS
	0.001	29 ± 2.3	78	<0.05
	0.01	9 ± 2.3	24	<0.001
	0.1	7 ± 1.4	19	<0.001
	1.0	—	—	—
Phospholipase	None	56 ± 5.4	100	
	0.0001	59 ± 5.2	105	NS
	0.001	64 ± 6.8	114	NS
	0.01	59 ± 5.1	105	NS
	0.1	63 ± 5.4	113	NS
	1.0	58 ± 4.5	104.	NS
Neuraminidase	None	43 ± 3.1	100	
	0.0001	ND	ND	ND
	0.001	ND	ND	ND
	0.01	49 ± 4.5	114	NS
	0.1	100 ± 10.3	233	<0.001

[a] In milligrams per milliliter for trypsin, lipase, and phospholipase C or in units of neuraminidase.

[b] Values represent the average number of adherent bacteria per epithelial cell ± the standard error of the mean. —, Enzyme concentrations that destroyed epithelial cells; ND, Not determined.

[c] Percent control adherence is the average number of bacteria adhering to enzyme-treated epithelial cells divided by the average number adhering to control cells times 100.

[d] Statistical significance between control and enzyme-treated preparations. NS, Not significant.

TABLE 3. *Effect of NANA, colominic acid, and neuraminidase on adherence of E. coli to rat bladder epithelial cells*

E. coli isolate	Treatment	No. of bacteria[a] adhering to:		% Control adherence	*P* value
		Control cells	Exptl[a] Treated Cells[b]		
07KL	NANA	39 ± 2.7[b]	13 ± 1.8	33	<0.001
K12H	NANA	27 ± 2.1	11 ± 1.1	41	<0.001
1974	NANA	30 ± 3.6	16 ± 1.7	53	<0.001
07KL	Colominic acid	39 ± 2.7	71 ± 7.4	182	<0.001
K12H	Colominic acid	27 ± 2.1	35 ± 3.6	130	0.05
1974	Colominic acid	27 ± 1.8	51 ± 4.5	170	<0.001
07KL	D-Mannose	27 ± 4.6	1 ± 0.2	3	<0.01
07KL	Colominic acid	27 ± 4.6	87 ± 19.1	306	<0.05
07KL	Colominic acid and D-mannose	27 ± 4.6	2 ± 0.5	7	<0.01
07KL	Neuraminidase	79 ± 7.4	111 ± 10.6	140	<0.001
07KL	Neuraminidase followed by D-mannose	79 ± 7.4	21 ± 2.6	27	<0.005

[a] Values represent the average number of adherent bacteria per epithelial cell ± the standard error of the mean.

[b] Experimental mixtures consisted of rat bladder cells, bacterial cells, and either NANA, colominic acid, or colominic acid mixed with D-mannose or neuraminidase-treated cells (0.5 U/ml) subsequently incubated with D-mannose.

observed with colominic acid, another experiment was done to see whether the increased adherence caused by neuraminidase was also blocked by mannose. Adherence was significantly reduced compared with adherence in controls (Table 3).

DISCUSSION

Definition of the nature of the binding site between bacteria and epithelial cells is an important step in understanding the pathogenic mechanisms of bacteria and how bacteria interact with host cells. Recently, Leffler and Svanborg-Edén presented evidence that the receptor mediating mannose-resistant adherence on epithelial cells (80% squamous) exfoliated into human female urine is a glycosphingolipid (10). However, to our knowledge, no work has been published on the nature of the mannose-sensitive receptor and bladder-derived epithelial cells. In this report, we suggest that the mannose-sensitive receptor on rat bladder epithelial cells consists of periodate- and lipase-sensitive compounds, possibly glycolipids, that mediate adherence.

Periodate oxidation has been used mainly to study carbohydrates, because periodate specifically and rapidly oxidizes α-glycols (6). The conditions we used were very similar to those used by other investigators who treated mammalian cells with periodate (12, 13). Under these conditions, periodate treatment removes the two terminal carbon atoms from NANA and other carbohydrates with α-glycols and oxidizes the alcohol to an aldehyde, leaving the remainder of the NANA molecule and other α-glycol molecules unaltered (13). Other molecules probably are not altered because they are parts of more complex compounds and are not free to react with periodate (2). Consequently, it is likely that mainly carbohydrates (e.g., NANA and similar molecules) associated with the bladder cell membrane were altered in our experiments, resulting in reduced *E. coli* adherence. Others have shown that adherence of *E. coli* to buccal epithelial cells is inhibited by periodate treatment (12).

Since carbohydrates on eucaryotic cells are attached to both proteins and lipids, trypsin, lipase, and phospholipase C were used to further characterize the receptor. Our findings suggest that the *E. coli* receptor is not a protein, glycoprotein, or phospholipid, such as phosphotidylcholine, phosphotidylethanolamine, or sphingomyelin. Similarly, Salit and Gotschlich reported that neither trypsin nor protease treatment of Vero cells affects *E. coli* attachment (14). However, our results showed a dose-dependent reduction of adherence by lipase. Our data suggest that the receptor(s) has both carbohydrate and lipid moieties. Because carbohydrates are usually not free on the membrane surface, we suggest that the receptor is glycolipid in nature. This receptor is probably different from the previously described glycosphingolipid (10), because the glycosphingolipid seems to only be involved with adherence of mannose-resistant adherence factors, whereas *E. coli* 07KL showed mannose-sensitive adherence mechanisms. However, as previously suggested by several laboratories (1, 7, 21), both mannose-sensitive and mannose-resistant receptors may play a role in adherence.

Neuraminidase, which can cleave NANA from its ketosidic linkage, has been shown to increase *E. coli* pilus binding to Vero cells (14) and also to increase adherence in our system. Furthermore, an increase in NANA significantly reduced adherence to rat bladder cells of both mannose-resistant (*E. coli* 1974) and mannose-sensitive (*E. coli* 07KL) hemagglutinating strains. On eucaryotic membranes, the bulk of the negative charge is provided by sialic acid (9). Thus, removal of sialic acid from surface areas may nonspecifically remove or reduce electrostatic repulsion, thus permitting increased adherence of both strains. Also, neuraminidase-treated cells, when incubated with bacteria and mannose, showed significantly decreased adherence. Consequently, the mechanism for neuraminidase-enhanced adherence probably involves the enzymatic uncovering of mannose-sensitive receptors on epithelial cells. Recently, neuraminidase-dependent hemagglutination, which is blocked by carbohydrates, was described for *Actinomyces* spp. (3). The investigators suggested that galactosyl groups on erythrocytes, exposed by neuraminidase, are subsequently bound to actinomycete cells. Thus, the proposed neuraminidase-mediated mechanism(s) of reducing cell charge and of exposing additional sites for adherence could explain why certain bacteria that produce neuraminidase adhere better to mammalian cells than bacteria which do not produce the enzyme.

Colominic acid, which is a polymer of NANA, had the opposite effect of NANA on adherence. The mechanism for the increase in adherence may be related to increasing the availability of the mannose-sensitive receptor(s). Colominic acid is derived from the K1 capsular polysaccharide of *E. coli*, and recent studies have indicated that 80, 36, and 40% of the strains isolated from patients with neonatal meningitis, neonatal sepsis, and kidney infections, respectively, contained the K1 capsular antigen (8, 15, 17). Con-

sequently, our data suggest that Kl-containing strains present in the urinary tract may be more adhesive and thus more pathogenic than strains without Kl because they increase adherence via the mannose-sensitive receptor(s). Because colominic acid increased the adherence of three *E. coli* strains, further studies should examine the role of the Kl capsular polysaccharide in adherence to epithelial surfaces.

ACKNOWLEDGMENTS

We thank S. Gratzfeld for technical assistance.

This work was supported by Public Health Service grant AI 14508 from the National Institutes of Health. A.E.A. and R.C.F. were supported by the James W. McLaughlin Fellowship Fund.

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