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Adhesive Properties of *Vibrio cholerae*: Nature of the Interaction with Isolated Rabbit Brush Border Membranes and Human Erythrocytes

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Nonmotile vibrio mutants lacked the ability to adhere to rabbit intestinal brush border membranes and to agglutinate human group O erythrocytes, but motile revertant vibrios isolated from such strains expressed adhesiveness equivalent to that of the original parent. Two possible explanations for the relation between vibrio motility and adhesion in these assay systems are (i) that the rate of adhesion depends on the rate of chance contact brought about by motility, and (ii) that the flagellum either acts as a carrier for the bacterial adhesin or may itself be the adhesin. The present study indicates, however, that the lack of adhesion by nonmotile vibrios did not depend on motility as such and did not involve greater rates of elution. Increasing the rate of contact between nonmotile vibrio mutants and brush border membranes by compaction did not restore the adhesive properties of the defective strains. Accordingly, we speculate that the flagellum may function in some indirect way that allows the expression of the adhesive properties, such as by acting as a carrier for a specific vibrio adhesin. Adhesion to brush borders and agglutination of human group O erythrocytes was specifically inhibited by L-fucose and various glycosides of L-fucose and to a lesser extent by D-mannose. Vibrios adhered specifically to agarose beads that carried covalently linked L-fucose on their surfaces. The results suggest that L-fucose-containing structures of eukaryotic cell surfaces may function as receptors for the vibrio adhesin and may therefore be an important determinant of host susceptibility.

An accompanying paper (10) demonstrated that the intimate association of *Vibrio cholerae* with mucosal surfaces may involve the ability of the vibrio cell to penetrate intestinal mucus and to adhere to the microvilli of the epithelial cells. Further studies were initiated to ascertain the nature of the microbial and eukaryotic cell factors involved in the adhesive phenomenon. Guentzel and Berry (8) showed recently that nonmotile vibrios were less able to associate with mucosal surfaces and were relatively avirulent. Nonmotile vibrios isolated by us also possessed the surprising property of being unable to attach to brush border membranes of rabbit intestine. Consequently, we have investigated the role of the flagellum and of motility in the adhesion of vibrios to rabbit brush borders and human erythrocytes.

The attachment of vibrios to the microvilli of isolated brush border membranes (10) added some support to the idea that adhesive enteric bacteria attach to heterosaccharides of the epithelial cell surface (9). Accordingly, we investigated the ability of carbohydrates to inhibit adhesion and hemagglutination. Such carbohydrates are commonly found as components of epithelial cell heterosaccharides and may possess the ability to interact with the bacterial adhesin and prevent binding of the adhesin to the receptors on the surface of brush borders and erythrocytes.

MATERIALS AND METHODS

***V. cholerae* strains.** The streptomycin-resistant strain P employed in previous studies (4) was used. Unless stated otherwise, vibrios were cultured in Trypticase soy broth without glucose (TSB; obtained from Baltimore Biological Laboratories).

Nonmotile mutants were isolated by two methods. (i) Broth cultures were treated with *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine (10 to 30 mg/ml) at pH 5.4 for 15 min at room temperature. Three-hour broth cultures of mutagenized vibrio suspensions were prepared and plated onto agar plates. Single colonies were examined for motility in layer plates (see below). (ii) Spontaneous nonmotile forms were selected by mixing vibrio suspensions with H antiserum, prepared by absorbing antisera against live agar-grown vibrios with suspensions of heat-killed

vibrios. The antiserum was used in a concentration representing eight times its agglutinin titer. After incubation at 37 C for 2 h, mixtures were centrifuged (1,200 × *g* for 20 min) and the sedimented bacteria were gently resuspended. Agglutinated masses of bacteria were removed by centrifugation (500 × *g* for 10 min) or by filtration (Millipore filter, 0.8-μm pore size; Millipore Corp., Bedford, Mass.). Supernates or filtrates were inoculated into TSB and incubated at 37 C overnight. Layer plates were prepared with suitable dilutions of these cultures.

Examination for motility and flagella. Layer plates were prepared as follows: plates of Trypticase soy agar (TSA; Baltimore Biological Laboratories) were inoculated with suitable dilutions of a *V. cholerae* culture. The surfaces were then overlaid with TSB containing 0.5% agar (Difco) and 1 mg of streptomycin sulfate per ml. Layer plates were incubated overnight at 37 C. Compact colonies developing on these plates invariably contained nonmotile vibrios, whereas motile organisms formed "hazy" colonies. Layer plates were used for the isolation of nonmotile mutants and to estimate the percentage of cells in a culture that could produce motile daughter cells.

The presence or absence of motility was confirmed in 4-h TSB cultures by direct observation of hanging drops under the phase-contrast microscope. Flagella were detected by agglutination tests with H antisera and with Leifson's flagella stain (11).

All nonmotile cultures except one were unstable and produced clones in which occasional sluggishly motile vibrios were observed microscopically, and which on layer plates produced hazy colonies of motile vibrios at a rate of less than 10% (compared with 0% hazy colonies produced by the parental nonmotile strain and 100% by the original strain P); such partially motile strains are typified by strain NM1/R. Fully motile revertant forms (designated, for example, NM1/M2) were isolated from nonmotile mutant cultures by passage through Craigie tubes (1) containing 0.25% (wt/vol) agar (Difco) in TSB. Such revertant cultures produced 100% hazy colonies on layer plates and microscopically appeared as motile as strain P.

Adhesion of vibrios to brush borders and adhesion inhibition. Brush borders were prepared and adhesion tests were carried out as described previously (10).

Adhesion inhibition test mixtures consisted of 0.4 ml of brush border suspension, 0.05 ml of *V. cholerae*, and 0.05 ml of inhibitor in buffer at pH 7.4. Tests were incubated as usual for 15 min, and the adhesion was compared with that in control mixtures lacking added inhibitor. Control mixtures were always prepared and incubated simultaneously with mixtures containing inhibitors. The concentrations of inhibitors given in the results are the final concentrations per milliliter of the test mixture.

Hemagglutination and hemagglutination inhibition tests. Hemagglutination tests were as described in a companion paper (10). Briefly, the hemagglutination inhibition test consisted of the following steps. *V. cholerae* suspensions were titrated against erythrocytes, and the dilution of bacteria that caused complete hemagglutination (10) was determined (one hemagglutinating dose). Four hemagglutinating doses were used for the inhibition test. Serial dilutions of carbohydrate were prepared, and equal volumes of *V. cholerae* suspension (containing four hemagglutinating doses) and of erythrocyte suspension were added to each dilution of carbohydrate solution. The concentration of carbohydrate that completely inhibited hemagglutination (i.e., the erythrocytes sedimented freely to form a compact button on the bottom of the cup) was taken as the inhibitory concentration.

Carbohydrates and derivatives. Monosaccharides were purchased from Pfanstiehl Laboratories Inc., Waukegan, Ill., and Sigma Chemical Co., St. Louis, Mo. We are grateful to I. J. Goldstein, University of Michigan, for the various derivatives of L-fucose.

RESULTS

Influence of motility on the adhesion of *V. cholerae* to rabbit brush borders. Nonmotile mutants that had been isolated from strain P by selection with H antiserum or after treatment with *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine were nonadhesive (Table 1). These mutants lacked flagella with the exception of strain NM7; this strain could not be classified strictly as nonmotile because structures that may have been flagella were occasionally observed in preparations stained by Leifson's method and because an occasional sluggishly motile cell was observed in hanging-drop preparations. It is of some interest that the degree of adhesion of this strain was consistently higher than that of the nonmotile isolates. The lack of adhesiveness in cultures of nonmotile strains was independent of both the time and incubation temperature of the brush border-vibrio

TABLE 1. *Adhesion of nonmotile strains to brush borders*

Strain[a]	Treatment[b]	Motility	Flagella[c]	Adhesion index[d]
P	None	+	+	13.80
NM1	Antiserum	−	−	0.45
NM2	Antiserum	−	−	0.10
NM3	Antiserum	−	−	0.40
NM4	Antiserum	−	−	0.15
NM5	NG	−	−	0.65
NM6	NG	−	−	0.43
NM7	NG	±[e]	±	1.13

[a] P, Parent strain; NM, nonmotile mutant derived from strain P.

[b] Treatment used to obtain the nonmotile mutants; NG, *N*-methyl-*N*-nitro-*N'*-nitrosoguanidine.

[c] Determined by Leifson's stain.

[d] Number of adherent vibrios per brush border.

[e] Very few sluggishly motile cells or flagella were observed.

mixtures and, therefore, did not appear to be related to either a slow rate of adhesion to or a rapid rate of elution from the brush borders. Whereas experiments conducted at 37 C with the parental strain P showed typical adhesion and elution profiles (Fig. 3 of reference 10), the adhesion indexes of nonmotile mutants remained almost constant through the 60-min incubation period. One possible explanation for these findings would be that the reduced adhesion of nonmotile strains was due to the reduced number of chance collisions between the nonmotile vibrios and the brush border surfaces. However, attempts to increase chance contacts between nonmotile vibrios and brush borders by increasing the concentration of bacteria by a factor of 10 and/or by more vigorous agitation of the reaction mixtures failed to improve adhesion. In contrast, both procedures markedly increased the adhesion of strain P (from approximately 10 to greater than 30 vibrios per brush border).

To investigate further the possibility that reduced contact accounted for the reduced adhesion of nonmotile mutants, the vibrios were impacted onto the brush border surface by centrifugation. Mixtures of vibrios and brush borders in ratios of 1,000:1 (rather than the usual 100:1) were prepared at room temperature (to prevent premature elution possible at 37 C) and immediately centrifuged at 1,200 × *g* for 20 min at 4 C (to stabilize resulting adhesion [10]). The supernate was replaced with fresh cold KRT (10), the pellets were resuspended, and the tubes were incubated at 37 C. Microscopic observations were made over a period of 45 min and are summarized in Table 2. Despite the fact that centrifugation should provide the same amount of contact between brush borders and vibrios irrespective of whether the vibrios were motile, the adhesion of the nonmotile strains was strikingly less than that of strain P. Consequently, differences in the rates of contact between nonmotile vibrios and motile vibrios with brush borders cannot account for the difference in the adhesiveness of motile and nonmotile vibrios. For comparative purposes, partially motile strain NM1/R was included because it showed a low degree of motility (about 2% of colonies on layer plates produced hazy zones). Strain NM1/R clearly possessed some adhesive activity (Table 2).

TABLE 2. *Influence of impaction on the adhesion of nonmotile strains: subsequent elution at 37 C*[a]

Strains	Adhesion index[b] after impaction and incubation at 37 C for:		
	0 min	15 min	45 min
P	TN[c]	TN	12.44
NM1/R	13.31	11.50	3.00
NM2	3.05	0.60	1.30
NM3	3.20	1.40	0.25
NM4	5.10	1.75	0.53

[a] Vibrio-plus-brush border mixtures in a 1,000:1 ratio (at 22 C) were centrifuged in the cold and resuspended in cold KRT. The adhesion index was determined immediately after resuspension and after subsequent incubation at 37 C.

[b] Average number of adherent vibrios per brush border.

[c] TN, Too numerous to count (>30).

The possibility that all apparently adhesive vibrios in cultures of nonmotile mutants and NM1/R were motile revertants was examined as follows. Brush borders with attached vibrios were isolated from reaction mixtures by layering the reaction mixtures onto 10-cm columns of cold KRT buffer; the brush borders were sedimented at 300 × *g* for 10 min in the cold and resuspended in KRT. Free vibrios remaining in this preparation were removed by repeating the centrifugation procedure. The brush borders with attached vibrios were inoculated into TSB. After 16 h of incubation at 37 C, the cultures were used for the preparation of layer plates and for adhesion tests. After reisolation from brush borders, TSB cultures of nonmotile mutants were no more adhesive than control cultures and showed no evidence of motility (Table 3). However, increases in both adhesion index and motility of NM1/R cultures reisolated from the brush borders indicate that motile cells are more likely to be adhesive.

It should be noted that strain NM1/R reisolated from brush borders was about half as

TABLE 3. *Motility and adhesiveness of vibrios reisolated from brush borders*

Strains	Motility (%)[a]	Adhesion index[b]		
		i	ii	Avg
Reisolated				
P	100	13.70	14.70	14.20
NM1/R	6.5	13.70	10.95	12.23
NM2	<0.05	0.55	0.70	0.63
NM3	<0.05	0.40	0.20	0.30
NM4	<0.05	0.65	0.35	0.50
Control				
P	100	13.10	13.55	13.33
NM1/R	2.3	7.60	6.95	7.28
NM2	<0.05	0.45	0.55	0.50
NM3	<0.05	0.20	0.40	0.30
NM4	<0.05	0.80	1.00	0.90

[a] Determined with layer plates as the percentage of colonies that were hazy.

[b] Number of adherent bacteria per brush border, duplicate experiments.

adhesive as strain P although the percentage of motility of strain NM1/R was only 6.5%. Clearly, motility of all cells in a culture is not an essential prerequisite to adhesiveness. Indeed, partially motile strain NM1/R was as adhesive as completely motile strain NM5/M3 (Table 4); the latter was a motile revertant of NM5 showing 100% motility on layer plates. Because of the instability of the nonmotile character, it was possible to isolate totally motile revertants from all but one culture (NM6). Motile revertant strains exhibited adhesive activity equivalent to that of the parent culture with the exception of isolate NM5/M3 (Table 4). On layer plates, motile revertants produced 100% hazy colonies and microscopically appeared to be as actively motile as strain P.

Influence of motility on hemagglutinating activity of *V. cholerae*. All nonmotile mutants tested failed to adhere to brush borders and also did not cause hemagglutination. The ability to agglutinate erythrocytes reappeared with the reversion of mutant strains to a fully motile state. The hemagglutinin titer of such revertants in TSB cultures approximated that of the parent strain. Partially motile forms such as NM1/R exhibited hemagglutinating activity of a lower order (i.e., a titer of 1:64 as compared with a titer of 1:256 for the parent strain).

Characterization of the interaction between *V. cholerae* and rabbit brush borders. Monosaccharides were examined for the ability to inhibit adhesion. Some of the monosaccharides used are probably components of the brush border glycocalyx and accordingly may play some role in the adhesive process. L-Fucose inhibited adhesion; 100 μg of L-fucose per ml reduced the adhesion index from 9.0 to 3.9 (approximate reduction in adhesion of 60%). However, an increase in the concentration of L-fucose to 10 mg/ml did not increase inhibition beyond this level (Fig. 1), which suggested that an L-fucose-insensitive adhesive system was

TABLE 4. *Examples of the expression of adhesive properties by motile revertant strains*

Strain	Isolated from:	Motility (%)[a]	Adhesion index[b]		
			(i)	(ii)	Avg
P	P	100	11.15	13.30	12.23
NM1	NM1	<0.05	1.05	1.24	1.13
NM1/m2	P	100	11.20	10.30	10.75
NM5	NM5	<0.05	0.70	0.60	0.65
NM5/M3		100	5.70	5.00	5.35

[a] Determined on layer plates as the percentage of hazy colonies.

[b] Average number of adherent vibrios per brush border, duplicate experiments.

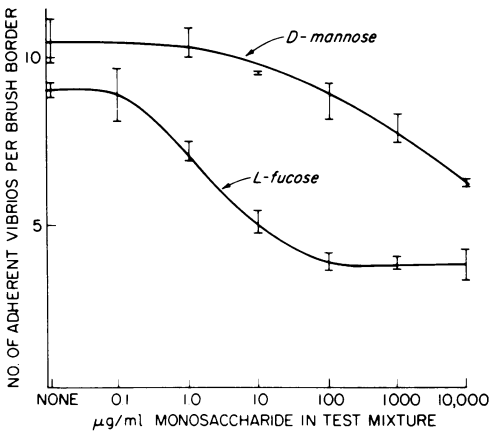

FIG. 1. *Inhibition of the adhesion of V. cholerae to brush borders by L-fucose and D-mannose. Each bar represents the average number of vibrios per brush border; averages were obtained from the microscopic counts of the total number of vibrios attached to each of 20 randomly selected brush borders.*

also present in vibrio cultures. The fucosides *p*-nitrophenyl-L-fucose and bovine serum albumin-L-fucose were, respectively, on the basis of fucose content, 5-fold and 100-fold more inhibitory than L-fucose; D-galactose linked to bovine serum albumin was not inhibitory. Of particular interest was the observation that *V. cholerae* attached to the surface of agar beads coated with covalently bound L-fucose. L-Fucose in a concentration of 10 mg/ml but not D-fucose inhibited this reaction. Nonadhesive, TSA-grown vibrios and nonmotile vibrios did not attach to L-fucose-coated agar beads.

D-Mannose also inhibited adhesion but was less inhibitory than L-fucose (Fig. 1). At 10 mg/ml, D-mannose reduced the adhesion index by less than 40%, and at 100 μg/ml and 1 μg/ml the adhesion indexes were reduced by 20 and 0%, respectively. On a weight basis D-mannose was about 1,000-fold less inhibitory than L-fucose. However, the inhibitory activities of these two sugars were not additive, because vibrio adhesion in the presence of both 10 mg of L-fucose per ml plus 10 mg of D-mannose per ml was not reduced below the level observed in the presence of 10 mg of L-fucose per ml alone. The other monosaccharides examined, D-fucose, D-glucose, *N*-acetyl-D-glucosamine, D-galactose, *N*-acetyl-D-galactosamine, and *N*-acetylneuraminic acid, were not inhibitory at final concentrations of 10 mg/ml.

Characterization of *V. cholerae* hemagglutination. Of the monosaccharides examined for the inhibition of adhesion only, L-fucose inhibited hemagglutination. As little as 150 μg of L-fucose per ml inhibited four hemagglutinat-

ing doses of vibrio TSB culture. In addition, the methyl and *p*-nitrophenyl glycosides of L-fucose, and L-fucose covalently bound to bovine serum albumin, inhibited hemagglutination to a greater extent than did L-fucose (Table 5). The absence of hemagglutination inhibition by D-mannose appeared to distinguish this activity from adhesion to brush borders. However, D-mannose was more than 100 times less efficient at inhibiting adhesion to brush borders than was L-fucose. Accordingly, it may be suspected that a concentration greater than 10 mg of D-mannose per ml (the highest concentration tested) would be necessary to inhibit hemagglutination.

The other monosaccharides examined, D-fucose, D-glucose, *N*-acetyl-D-glucosamine, D-galactose, *N*-acetyl-D-galactosamine, and *N*-acetylneuraminic acid, were not inhibitory in hemagglutinin assays.

DISCUSSION

At least some of the microbial factors that are involved in the attachment of *V. cholerae* to eukaryotic cell surfaces appear to be related to bacterial motility. This study indicates that adhesion does indeed correlate to some extent with the presence of a functional flagellum. The present data make it clear, however, that adhesion does not necessarily depend upon the motility of the vibrio cell. It seems reasonable to suspect that motile vibrios would have a greater chance of contacting the surfaces of brush borders and erythrocytes than would nonmotile mutants and that, in consequence, motile strains would appear to be more adhesive. However, this was not the reason for the marked difference in the adhesiveness of the motile and nonmotile vibrios studied by us, because normally motile phenotypes that lacked adhesive properties could be prepared by growing vibrios on TSA (10). Furthermore, compaction of nonmotile vibrio mutants upon the surface of brush borders did not improve their adhesive capacities to anywhere near the level of the parent strain.

In view of the above observations, it is tempting to speculate either that the flagellum may act as a carrier for a relatively unstable adhesin or that full expression of the adhesive properties on the vibrio surface requires the complete biosynthetic apparatus of flagella synthesis. Indeed, it is not impossible that the adhesin may be an altered form of the vibrio flagella that is produced under certain circumstances.

This study was initiated to test a working hypothesis that the adhesion of *V. cholerae* to brush borders was the result of the specific interaction between the vibrio adhesin and a receptor on the brush border surface. Furthermore, we had suspected that the receptor was probably a constituent of a glycocalyx heterosaccharide. Accordingly, some monosaccharides that are typical components of the heterosaccharides of mammalian glycoproteins and glycolipids were investigated for their ability to interact with the vibrio adhesin and to inhibit adhesion. L-Fucose and to a lesser extent D-mannose inhibited adhesion consistently. It is unlikely that the inhibition of adhesion by these monosaccharides is an artifact created by the use of formalinized brush borders because (i) *V. cholerae* adhered to freshly prepared brush borders as well as they did to formalin-preserved brush borders (L-fucose inhibited this reaction also) and (ii) brush borders preserved in 50% (vol/vol) aqueous glycerol at −20 C reacted with *V. cholerae* to the same extent as did formalinized brush borders. This reaction also was inhibited by L-fucose.

The finding that inhibition of adhesion by L-fucose was strong but incomplete suggests that the natural mucosal receptor is larger than an L-fucose residue and/or that a certain stereochemical configuration is required. Incomplete inhibition by monosaccharide constituents of receptors has been encountered in studies on other bacterial adhesins (6) and during the early investigation of the immunological determinants of blood group specificity (13). In inhibition studies of this kind, inhibition is a measure of the competition between free L-fucose and the L-fucose residues of the natural receptors. It is possible that the vibrio adhesin combines more readily with the natural receptor than it does with L-fucose and that this is the reason for the incomplete inhibition by L-fucose (within manageable concentration ranges). In-

TABLE 5. *Inhibition of V. cholerae hemagglutination by L-fucose and glycosides of L-fucose*

Inhibitor	Inhibitory concn (μg/ml)[a] as:	
	Inhibitor	Fucose content[b]
L-Fucose	160	160
Methyl-L-fucose	78	72
p-Nitrophenyl-L-fucose	39	22
BSA-L-fucose[c]	2.2	0.12

[a] Inhibitory concentrations are given as the final concentration of material that causes complete inhibition of hemagglutination by four hemagglutinating doses of *V. cholerae* culture.

[b] Calculated concentration of fucose present in the inhibitory concentration of material tested.

[c] BSA, Bovine serum albumin.

deed, the finding that L-fucosides were relatively more inhibitory than L-fucose tends to support this view. However, there was little indication that the inhibitory activity of L-fucose increased with increasing concentrations above 0.1 mg/ml. Consequently, a more likely possibility is that additional adhesive systems are operational. The possible second system does not appear to involve the D-mannose-sensitive system because D-mannose did not augment inhibition by L-fucose. Accordingly, D-mannose may constitute a part of the L-fucose receptor. Of particular interest is the finding that vibrios adhered to agar beads to which L-fucose had been covalently linked, but that vibrios did not adhere to untreated agar beads. The specificity of this reaction was demonstrated by the inhibition of adhesion by L-fucose but not by D-fucose. This observation indicates that the fucose-containing component of the adhesion system is located on the mucosa rather than on the vibrio surface.

Other bacterial hemagglutinins are known to be inhibited by monosaccharides. Hemagglutination caused by type I pili of *Escherichia coli* is inhibited by D-mannose (2), and a hemagglutinin of *Pseudomonas aeruginosa* binds to D-galactose (7). Such specific inhibitions are used to characterize adhesive materials. Accordingly, the adhesin/hemagglutinin of *V. cholerae* that is inhibited by L-fucose will be referred to as L-fucose sensitive, in the same manner as the hemagglutinins of *E. coli* or of *P. aeruginosa* may be described as D-mannose or D-galactose sensitive, respectively. Whether *V. cholerae* hemagglutinins described by others (reviewed in reference 3) are the same as the hemagglutinin described here is unknown; L-fucose sensitivity provides a convenient means of determining this point.

Recently, Guentzel and Berry (8) demonstrated that nonmotile mutants were less able to associate with the mouse mucosa as compared with motile vibrios, and in consequence were relatively avirulent in the baby mouse model. Not only would nonmotile vibrios be less able to penetrate mucus (10, 12), but they would also lack the ability to attach to the fucose-sensitive receptor on the surface of epithelial cells. Clearly, the microbial and host factors that govern the colonization of gut mucosa by vibrios are complex and require further study. The roles of the L-fucose-sensitive and other adhesins and of motility in the colonization of mucosal surfaces appear to be important and are the subject of a companion paper (5).

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