INFECTION AND IMMUNITY, July 1976, p. 246-256

Vol. 14, No. 1
Printed in U.S.A.

Adhesive Properties of *Vibrio cholerae*: Nature of the Interaction with Intact Mucosal Surfaces

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Received for publication 3 March 1976

Two companion papers in this series have characterized the interaction between *Vibrio cholerae* and the surfaces of eukaryotic cells. The present paper reports studies of the association between vibrios or *Salmonella enteritidis* and intact slices of intestinal tissue. A significant number of differences were noted in the characteristics of bacterial adhesion in these systems. The results are interpreted to indicate the presence of at least two receptors for vibrio adhesion on the mucosal surface of the rabbit small intestine. The receptor mediating the adhesion of salmonella appeared to be distinct from these. A primary role for bacterial motility in the process of adhesion of vibrios to mucosal surfaces could not be demonstrated in the assay systems studied. Rather, loss of motility in mutant vibrios appeared to be correlated with the simultaneous loss of adhesive factors (adhesins) from the bacterial surface. The inhibition of vibrio adhesion to slices of intestinal tissues by antibody to the heat-stable antigens of *V. cholerae* occurred in the absence of bacterial agglutination. Agglutination in this assay system appeared to be an artifact in that it could be observed only in experiments where extremely high concentrations of vibrios were used. We speculate that such high vibrio concentrations are not likely to be present in humans at the time of infection and that agglutination in the lumen of the intestine might therefore play only a minor role in prophylactic immunity against natural cholera and other enteric infections of humans.

The other two papers in this series (13, 14) described our studies of vibrio adhesion to structures, such as isolated brush border membranes or erythrocytes, in which the interaction obviously occurs on cell surfaces. It was therefore of interest to extend these studies to systems that might represent more completely the natural state of the mucosal surface, including the overlying mucus gel. Earlier work from this laboratory had demonstrated that bacterial adhesion and its inhibition by antibody can be assayed with slices of rabbit intestinal tissue that are kept floating for short periods of time in bacterial suspensions (8, 9). This assay system was therefore used in the studies reported below.

The effect of local antibodies in preventing the adhesion of vibrios to the mucosal surface had been demonstrated in this laboratory several years ago (8, 12). This effect was attributed to two mechanisms: (i) a direct effect of antibody on bacterial adhesion, and (ii) an antibody-dependent bactericidal reaction on the mucosal surface. The direct effect of antibody on adhesion could be obtained with antisera prepared against the heat-stable antigens of *Vibrio cholerae* and occurred in the absence of demonstrable agglutination (8). Chaicumpa and Rowley subsequently demonstrated a similar, antibody-dependent bactericidal mechanism in the infant mouse model of cholera (5). It was somewhat surprising, therefore, to learn in a more recent paper from this group that they apparently attribute the greater part of the protective activity of antibody in the infant mouse model to its agglutinating activity. In their most recent view, then, the clumping of the vibrios by antibody prevents the adherence of the bacteria to the intestinal wall of infant mice and thereby protects the animals against experimental cholera (2). An interesting recent paper by Schrank and Verwey (15) reported the clumping of cholera vibrios in the intestinal lumen of immunized rabbits. The authors interpreted these findings to show that agglutination of vibrios mechanically prevented these bacteria from penetrating the mucus layer and in this manner protected against experimental cholera. It is apparent, therefore, that a number of potentially effective antibacterial immune mechanisms have been described in different assay systems and in different experimental models of cholera. The question which, if any, of these immune mechanisms actually

operate in prophylactic immunity to natural cholera and other enteric infections of humans remains, of course, unanswered. It seems reasonable to assume, before one can even approach this question, that an extensive body of information must be gathered concerning the conditions under which one or the other of these immune mechanisms actually becomes operative in the intestinal environment. For this reason, comparative studies of the effect of antibody on bacterial adhesion in various assay systems are being carried out in this laboratory. The present paper reports results obtained with antibodies directed against the heat-stable antigens of *V. cholerae*. More elaborate studies with absorbed antisera prepared with live vibrios will be presented in a future communication.

MATERIALS AND METHODS

Bacteria. The streptomycin-resistant strain P of *V. cholerae* (Ogawa type) and the nonmotile mutants NM3 and NM6 have been described (13). The streptomycin-sensitive strain of *Salmonella enteritidis* was obtained from the stock cultures of this Department. These were propagated on Tryticase soy agar (TSA) or in Trypticase soy broth (TSB; both from Baltimore Biological Laboratories).

Antiserum. Hyperimmune rabbit antiserum to heat-stable antigens of *V. cholerae* strain P was used. Washed saline suspensions of TSA-grown vibrios were heated in flowing steam for 2 h. Eight intravenous injections were given to the rabbits at 5-day intervals. The rabbits were bled 7 days after the last injection. The sera were heated to 56 C for 30 min before use.

Hemagglutination. Human group O erythrocytes were used in the manner described in a companion paper (13).

Tests for bacterial adhesion. The assay for bacterial adhesion to isolated brush border membranes is described in a companion paper (13).

Adhesion of vibrios and salmonellae to slices of rabbit ileum was assayed as described earlier (8), except that the Krebs-Ringer solution was modified by substituting 0.01 M ADA buffer [*N*-(2-acetamido)-2-iminodiacetic acid; Sigma Chemical Co.], pH 7.0, for the classical bicarbonate-CO_2 system. A length of ileum was removed from a rabbit. The ileum was opened by a longitudinal incision, washed quickly by dipping it successively for a few seconds into each of two cylinders of saline, and cut into rectangular sections (approximately 1 by 2 cm). Two to four jars with 20 ml of Krebs-Ringer solution (one control and one to three experimental jars) each received five to six of these sections. The various substances under test were added to the experimental jars. Suspensions of *V. cholerae* and *S. enteritidis* were then added in 0.2-ml amounts. Unless otherwise indicated, the final concentration of each of these bacteria in the jars was in the order of 1×10^5 to 5×10^5/ml, and the jars were incubated at 37 C in a shaker water bath for 20 min in an atmosphere of pure O_2. Each slice was then washed by being dipped successively into two beakers containing approximately 50 ml of sterile saline. It was then transferred into a 30-ml Virtis blender jar (no. 16–117, The Virtis Co., Gardiner, N.Y.) containing 25 ml of cold TSB. One-milliliter samples of the Krebs-Ringer solution in the incubation jars were removed at the same time and transferred to separate Virtis blender jars containing 25 ml of cold TSB. The jars were transferred immediately to a water bath at 13 C and stored there until required for culture. Before culture, the washed tissue slices and the wash fluids were homogenized in the separate jars in a Virtis 45 homogenizer at full speed (45,000 rpm) for 10 s. Preliminary experiments had shown that the viable counts of vibrios in broth cultures were not affected by this method of homogenization. Viable counts of the homogenates were determined by spreading 0.1-ml amounts of suitable saline dilutions over the surface of TSA plates containing 1 mg of streptomycin per ml (to which the vibrio strains used were resistant). Homogenized intestinal slices from noninfected control rabbits rarely showed growth after plating on streptomycin agar. In those instances where a few colonies did develop, these were readily distinguishable from vibrio colonies. A parallel series of platings was carried out on SS agar (Difco). This medium suppressed the growth of *V. cholerae* and of normal intestinal bacteria from the rabbit while allowing quantitative recovery of *S. enteritidis*.

The viable counts on the washed tissue slices were related to the viable counts in 1 ml of the suspending Krebs-Ringer solution by the formula: percentage of vibrio population adhering = $[(t)/(t + k)] \times 100$, where k is the number of bacteria per milliliter of Krebs-Ringer solution present after incubation and t is the number of bacteria remaining, after rinsing, on the tissue slices incubated in the Krebs-Ringer solution.

This formula expresses the percentage of vibrios adhering to a single tissue slice in a manner suggesting that the slices had been interacting with only the vibrios suspended in 1 ml of the surrounding buffer. This is, of course, an arbitrary assumption. The data presented in this way nevertheless allow an accurate comparison of the relative degrees of adhesion in the control and experimental suspensions.

Fluorescent antibody studies. Vibrios adhering to tissue slices were determined by quick-freezing the tissue in isopentane at dry-ice temperature and sectioning with a cryostat-microtome. The sections were then stained with fluorescein isothiocyanate-labeled antibody to *V. cholerae*, as described previously (8).

Fucose determination. The L-cysteine-sulfuric acid method of Dische and Shettles was used (6).

Peptic digests of mucosal extracts. Earlier attempts at isolating from mucosal scrapings substances that inhibit the adhesion of vibrios had shown some preliminary success with the use of acetone extraction (10). This method was, however, quite unreliable because of the inherent instability of mucosal extract. As will be described, the inhibi-

tory activity of mucosal extracts could be stabilized by heat treatment. However, acetone extraction of heat-treated mucosal extract did not yield inhibitory materials. After extensive preliminary experiments, the following method was therefore adopted.

A young adult rabbit was anesthetized with pentobarbital and killed by exsanguination. The small intestine was excised and digesta were removed by flushing with approximately 100 ml of ice-cold ADA-buffered Krebs-Ringer solution at pH 6.5. The subsequent procedures were carried out in a cold room at 4 C. The gut was opened by a longitudinal incision, and the mucosal surface was scraped with the edge of a glass microscope slide. The scrapings were collected and sonicated in a Branson model S125 sonicator at 8 A for approximately 30 s. If necessary, the pH of the homogenate was adjusted to between 6.5 and 7.0. The material was sealed in glass ampoules, which were subsequently submerged in a water bath at 57 C for 10 min. (This material is referred to in the text as "crude heated scrapings.") The scrapings were then diluted with an equal volume of Krebs-Ringer solution adjusted to pH 3.5, and crystalline pepsin was added to a concentration of 2 mg/ml. The material was incubated at 37 C for 24 h with continuous slow stirring. An additional dose of pepsin (2 mg/ml) was then added, the acidity was readjusted to pH 3.5 if necessary, and incubation was continued for another day. The mixture was then centrifuged at 15,000 × *g* for 20 min. The clear supernate thus obtained is the "soluble fraction of a peptic digest of mucosal scrapings" referred to in the text below. The yield of soluble inhibitor could be improved by resuspending the sediment obtained at the final centrifugation step in the original volume of Krebs-Ringer solution and repeating the pepsin digestion in the manner described above.

Control preparations prepared by "digesting" pure Krebs-Ringer solution with pepsin in the same manner as described above for mucosal scrapings never yielded inhibitory substances, thus indicating that the soluble inhibitors obtained by this procedure did indeed originate in the mucosal scrapings.

RESULTS

In preliminary experiments, intestinal tissue slices were incubated for 1 h at 37 C in vibrio suspensions of high concentration (~1 × 10^9/ml). The slices were then washed in the usual manner, frozen, sectioned, and stained with fluorescent antibody to *V. cholerae* as described under Materials and Methods. Large numbers of *V. cholerae* could be observed in association with the mucosal surface of these sections. In contrast, vibrio association with the serosal surface was noted only very infrequently. Consequently, the test for vibrio adherence to intestinal tissue slices described under Materials and Methods appears to be an adequate system for the study of mucosal adherence by vibrios.

Further preliminary experiments showed that omission of calcium from the suspending buffer did not affect the adhesion of vibrios or salmonellae to tissue slices. This is in contrast to our earlier observations with isolated brush border membranes (13). The result is not entirely conclusive, however, because the possibility cannot be excluded that some calcium may have diffused into the buffer from the tissue slices themselves.

The experiment outlined in Table 1 was designed to test the specificity of vibrio adhesion to intestinal mucosa. Increasing concentrations of vibrios were incubated with tissue slices in different jars. Each jar also contained the same low concentration (2 × 10^5 to 4 × 10^5/ml) of salmonella. After incubation, the tissue slices were washed and homogenized. Quantitative cultures were then prepared in the standard manner. The results obtained (Table 1) indicate that at high vibrio concentrations (3.5 × 10^9 to 5 × 10^9/ml) these bacteria were competing with each other for adhesion sites, because the percentage of vibrios that succeeded in adhering to the mucosa was significantly lower (27.0 and 14.8%) than the percentage of vibrios adhering at lower initial vibrio concentrations. In contrast, adherence of the salmonellae was not affected even when large numbers of vibrios were associated with the same tissue slices. One must conclude, therefore, that at least the predominant mucosal receptors for vibrio adhesion in this system differ from those involved in the adherence of salmonella.

As reported in an accompanying paper (14), nonmotile mutants of the parent vibrio (strain P) did not adhere to isolated brush border membranes. It was therefore of interest to determine whether nonmotile mutants were also unable to adhere to an intact mucosal surface, as represented by intestinal tissue slices. The data in Table 2 demonstrate that adhesion of the nonmotile mutants in this system was indeed negligible. The percentage of "adherent" vibrios determined for the mutant strains (0.52 and 1.0%) is in the order of magnitude that one might expect to remain on the washed tissue slices even in the absence of active adhesive mechanisms.

Our companion studies of vibrio adhesion to brush border membranes (13) demonstrated two distinct features of this interaction: (i) that adhesion occurred only with vibrios grown in broth, not with those grown on agar plates; and (ii) that adherent vibrios eluted from the brush borders after 15 min of incubation at 37 C, with elution being completed at about 45 min of incubation (Fig. 3 of reference 13). However, when the same parameters were studied with the intestinal slice system (Table 3), the results were entirely different. The conditions of culture of the vibrios did not affect their adhesion

TABLE 1. *Lack of competition between vibrio and salmonella for adhesion to intestinal tissue slices*

Expt	Vibrios/ml in suspending buffer	Percentage of vibrio population adhering per slice	Mean percentage of vibrio population adhering per slice	Percentage of salmonella population adhering per slice[a]	Mean percentage of salmonella population adhering per slice
I	5×10^9	25	27.0	18	33.8
		42		35	
		15		30	
		28		46	
		25		40	
I	2×10^6	46	53.5	24	33.8
		62		36	
		63		35	
		43		50	
				24	
II	3.5×10^9	9.0	14.8	22	36.0
		17		35	
		12		38	
		21		43	
		15		42	
II	6.9×10^7	58	45.4	45	35.8
		44		33	
		57		46	
		42		28	
		26		27	

[a] The salmonella concentration in the suspending buffer was 2×10^5 to 4×10^5/ml in all experiments.

to tissue slices to any appreciable degree. Moreover, adhesion after 20 min of incubation was not significantly different from that observed at 60 min. Although the two experiments presented in Table 3 show a 5 to 10% reduction in vibrio adhesion at 60 min and a 10 to 15% lower percentage of adhesion for agar-grown over broth-grown vibrios, these differences are within the range of chance variations that must be expected to occur in this type of experiment. Even if real, these differences would be minute compared with the drastic reductions in vibrio adherence to brush border membranes observed under the same conditions (13).

To rule out the possibility that the agar-grown vibrios might have resynthesized adhesin while being incubated with tissue slices during the above-described test, the experiment was repeated in the same manner except that concentrated agar-grown vibrio suspensions, containing 1×10^8 vibrios per ml, were used rather than the standard vibrio concentration of 1×10^5 to 5×10^5/ml that had been used in the experiment shown in Table 3. After 20 min of incubation with the tissue slices, some of the vibrio suspension was removed and tested for adhesion to brush border membranes in the usual manner. These vibrios failed to adhere to brush borders, but did adhere to the tissue slices. Adhesion to tissue slices was of the same magnitude as shown in Table 3. One must conclude, therefore, that agar-grown (motile) vibrios, which were unable to adhere to brush border membranes, were not significantly impaired in their ability to associate with an intact mucosa as represented by intestinal tissue slices.

The above differences between vibrio adhesion to brush borders and intact mucosa suggested that these two processes might involve different mucosal receptors. It was of interest, therefore, to determine whether L-fucose, a specific inhibitor of vibrio adhesion to brush border membranes (14), would also affect the interaction of vibrios with intact mucosa. Surprisingly, the addition of 10 mg of L-fucose per ml to the suspending buffer failed to reduce the adhesion of vibrios or salmonellae to intestinal tissue slices in the standard test. It should be emphasized that the concentration of L-fucose used here is far in excess of that required to inhibit vibrio adhesion to brush borders (Fig. 1 of reference 14). To rule out the possibility that L-fucose might have been removed from the suspending medium by the metabolic activities of either the bacterial or mammalian cells, the concentrations of this sugar remaining in the suspending buffer after incubation of the test

TABLE 2. *Relation between motility and bacterial adhesion to intestinal tissue slices*

Bacterium	Percentage of bacterial population adhering per slice	Mean
Salmonella (motile)	42	39.0
	30	
	34	
	38	
	51	
Motile vibrio (parent strain P)	28	35.4
	50	
	34	
	33	
	32	
Nonmotile vibrio mutant MN3	1.2	1.0
	0.9	
	0.9	
	1.2	
	0.9	
Nonmotile vibrio mutant NM6	0.3	0.52
	0.4	
	0.4	
	0.6	
	0.9	

mixture were determined by the technique described under Materials and Methods. The specificity of the analytical procedure was verified by determining the concentration of free L-fucose in the following additional preparations: (i) buffer from the control mixture in which vibrios and tissue slices had been incubated in the absence of L-fucose; and (ii) buffer from the same control mixture to which L-fucose had been added after completion of the adhesion test. These analyses showed that the concentration of L-fucose did not diminish during incubation with vibrios and tissue slices under the conditions of our test. It is apparent, therefore, that L-fucose failed to affect the association of vibrios with intact mucosa at a concentration that caused a 60% reduction in vibrio adherence to brush borders (Fig. 1 of reference 14).

In addition to L-fucose, the following substances were tested at concentrations of 10 mg/ml for possible effects on vibrio adhesion to tissue slices: D-mannose, *N*-acetyl-D-glucosamine, D-galactose, D-glucose, D-galactosamine, D-fucose, and bovine submaxillary mucin. Triton X-100, Tween 80, and sodium deoxycholate were also tested at concentrations of 0.1%. None of these affected the ability of vibrios to adhere

TABLE 3. *Stability of bacterial adhesion to intestinal tissue slices and its independence of the conditions of culture*

Growth medium used for vibrio inoculum	Incubation time (min)	Percentage of vibrio population adhering per slice	Mean	Percentage of salmonella population adhering per slice[a]	Mean
Broth	20	50	55.8	19	30.2
		50		27	
		56		27	
		55		39	
		68		39	
Broth	60	37	45.4	26	29.4
		42		25	
		48		36	
		48		31	
		52		29	
Agar	20	37	40.8[b]	18	20.8
		42		24	
		45		16	
		43		24	
		37		22	
Agar	60	35	35.6	30	29.2
		44		43	
		33		29	
		28		19	
		38		25	

[a] The salmonella inoculum for all experiments was grown in broth.

[b] Agar-grown vibrios recovered from the suspending buffer after incubation with tissue slices failed to adhere to brush border membranes.

TABLE 4. *Stability and solubilization by pepsin digestion of an inhibitor of bacterial adhesion obtained from mucosal scrapings*

Expt	Inhibitor	Percentage of vibrio population adhering per slice	Mean	Percentage of salmonella population adhering per slice	Mean
I	None (control)	57	52.8	4.0	12.5
		38		20	
		59		18	
		54		9.6	
		56		11	
	Intestinal scrapings unheated, stored 24 h at 4 C	71	55.0	5.0	4.0
		36		4.0	
		51		3.2	
		61		4.2	
		56		3.8	
	Intestinal scrapings heated, stored 24 h at 4 C	1.8	6.3	0.64	2.9
		2.4		2.1	
		2.1		1.5	
		19.4		7.7	
		5.8		2.6	
II	None (control)	40	43.2	13	19.6
		45		21	
		40		18	
		41		21	
		50		25	
	Crude heated scrapings	8.6	10.3	6.5	5.8
		16		7.5	
		8.2		4.2	
		7.8		6.9	
		11		3.9	
	Pepsin-treated, soluble fraction of above scrapings	16	16.2	10	9.7
		11		4.2	
		26		12	
		15		7.5	
		13		15	
	Pepsin-treated, insoluble fraction of above scrapings	23	21.6	13	11.0
		34		14	
		12		4.1	
		21		14	
		18		10	

to intestinal tissue slices. The last three (surface active) agents listed, however, had a profound effect in that they prevented the adhesion of vibrios to glass. These three surface-active agents had no effect, however, on the adhesion of vibrios to isolated brush border membranes in the standard test.

The failure of simple carbohydrates to inhibit the adhesion of vibrios to intestinal tissue slices raised the possibility that mechanical burrowing of vibrios into the mucus gel, rather than specific adhesion to a mucosal receptor, might be an important factor in the association of these bacteria with the intestinal surface. To resolve this issue, further efforts were made to find substances that would inhibit adhesion of vibrios to tissue slices without at the same time causing conditions (such as aggregation or loss of motility) that obviously would also impair the ability of the bacteria to mechanically "burrow" into gel. A clearly promising source of such inhibitors, which might indeed represent the solubilized natural receptor(s), was scrapings from the mucosal surface of rabbit small intestine. As described below, this approach was indeed successful.

The procedure for preparing soluble inhibitor from pepsin-digested mucosal scrapings described under Materials and Methods was developed in an extensive series of preliminary experiments. The inhibitory activity in homogenized scrapings was unstable and was quickly lost even at refrigerator temperatures (Table 4, experiment I). Heating the freshly prepared scrapings to 57 C for 10 min stabilized the inhibitory activity, probably by inactivating degrading enzymes. An interesting observation was made frequently with scrapings that had lost their inhibitory activity for vibrio adhesion to intestinal slices, namely, that such material retained its inhibitory activity for salmonellae. This is also illustrated in Table 4 (experiment I). When freshly prepared, homogenized scrapings were centrifuged at 15,000 × *g* for 20 min, the larger part of the inhibitory activity remained in the sediment. Incubation at 37 C of heated homogenized scrapings with trypsin or Pronase at alkaline pH either was ineffective or resulted in inactivation of the inhibitory activity. Inactivation, however, was due to the instability of the inhibitor at alkaline pH in that control preparations of heated mucosal scrapings that were incubated under similar conditions but without added enzymes were inactivated also. In contrast, peptic digestion at pH 3.5 resulted in solubilization of half or more of the inhibitory material, which remained in the supernate after centrifugation at 15,000 × *g* for 20 min (Table 4, experiment II). These supernates were used in the studies described below. The active material passed Amicon PM30 membranes without loss of potency, which suggests a molecular weight of less than 3×10^4.

The soluble material from intestinal scrapings also inhibited the adhesion of vibrios to isolated brush border membranes (Table 5). Likewise, the adhesion of salmonellae to such membranes was inhibited by this material in the same manner and at the same concentrations as illustrated for vibrios in Table 5. Furthermore, agglutination of human group O erythrocytes was inhibited in the presence of the soluble fraction of peptic digests of mucosal scrapings. Suspensions of vibrios or salmonellae incubated with inhibitory concentrations of soluble material from intestinal scrapings did not agglutinate and showed normal motility when observed in hanging-drop preparations under the phase-contrast microscope.

In view of the fact that the studies described above had demonstrated considerable differences in the reaction of vibrios with brush border membranes and tissue slices, it was of interest to compare the inhibitory effect of antibody on adhesion in those systems. In addition, the relation between agglutination and inhibition of adhesion was investigated because such a relation had recently been reported from several laboratories. In the first type of experiment, the standard test for vibrio adhesion was modified in the following manner. The tissue slices were incubated in the vibrio-plus-salmonella suspension and washed, homogenized, and cultured in the usual manner. The bacteria remaining in the buffer, after the tissue slices had been removed, were, however, counted in a manner that was more elaborate than usual. Serial 10-fold dilutions were first prepared without homogenizing the bacterial suspension. Care was taken to use as little agitation as possible for proper mixing. The dilutions were then plated in the usual manner for the determination of viable bacterial counts, again being careful to avoid excessive agitation of the suspensions. After completion of this procedure, the original suspensions were homogenized in a Virtis blender and viable counts were again determined in the standard manner. Table 6 shows the results of two such experiments in which the experimental incubating jars contained two different dilutions of a hyperimmune rabbit antiserum prepared with heat-killed (100 C, 2 h) vibrios. The data show that in the first experiment, where the concentration of vibrios in the reaction mixture was low (i.e., in the order of 2×10^5/ml), both dilutions of antibody caused a reduction in the adhesion of vibrios to tissue slices, thus confirming earlier data reported from this laboratory (8). There was no evidence that bacterial clumping had occurred during this experiment because the viable counts of vibrios in the suspending buffer were the same before and after homogenization (Table 6). When the vibrio concentra-

TABLE 5. *Inhibition of vibrio adhesion to brush border membranes by the soluble fraction of a peptic digest of mucosal scrapings*

Reciprocal dilution of mucosal digest	Avg no. of adherent vibrios per brush border[a]	Percent inhibition
None (duplicate controls)	10.5 9.75	0
10	1.80	82
20	1.85	82
40	3.60	64
80	4.15	59
160	6.15	39
320	7.95	22

[a] Determined on 20 randomly selected brush borders.

TABLE 6. *Comparison of agglutination and inhibition of vibrio adhesion to tissue slices by antiserum to the heat-stable antigens of V. cholerae*

Antiserum dilution	Expt 1 (2×10^5 *V. cholerae*/ml)				Expt 2 (1×10^8 *V. cholerae*/ml)			
	CFU[a] ($\times 10^{-5}$)/ml remaining in suspension after incubation		Percentage of vibrio population adhering per slice	Mean	CFU ($\times 10^{-5}$)/ml remaining in suspension after incubation		Percentage of vibrio population adhering per slice	Mean
	Before blending	After blending			Before blending	After blending		
No antiserum	2.9	3.2	48 44 52 51 38 50	47.2	1,600	1,800	53 54 47 52 50	51.2
1:1,000, #7	4.4	2.0	12 24 23 20 16 13	18.0	100	3,300	7.4 7.2 8.1 3.5 5.5	6.34
1:10,000, #7	1.5	1.2	23 25 24 25 19 25	23.5	2,600	2,300	24 18 24 18	21.0

[a]CFU, Colony-forming units of *V. cholerae*, determined in quadruplicate titrations.

tion in this type of experiment was increased 500-fold (experiment 2, Table 6) there was also a decrease in the number of vibrios adhering to tissue slices. However, at the 1:1,000 dilution of the antiserum there was evidence of strong agglutination in the vibrio suspension: the viable counts obtained by making the dilution series without unnecessary agitation were approximately 20-fold below the expected value (of $\sim 1{,}000 \times 10^5$ to $\sim 3{,}000 \times 10^5$/ml, allowing for experimental error and bacterial multiplication during the experiment). In contrast, the expected viable count was obtained again after dispersion of the bacterial clumps by homogenization. Apparently, then, bacterial agglutination occurred only at high vibrio concentrations, whereas inhibition of adhesion could be observed in the absence of demonstrable bacterial clumping when the circumstances of the experiment did not favor agglutination. It is quite probable, of course, that agglutination, when it does occur, may make an additional contribution to the inhibition of bacterial association with the mucosa. This is suggested by the data in experiments 1 and 2 (Table 6), where the degree of inhibition in the presence of agglutination was considerably stronger (i.e., a reduction to 6.34% adhesion) than in the absence of clumping (to 18% adhesion).

Table 7 presents data from one representative experiment in which the same antiserum was tested for inhibition of vibrio adhesion to isolated brush border membranes and the results compared with respect to agglutination. Clumping of the bacteria was determined in the same preparations used for testing adhesion to brush borders by counting the number of bacterial clumps in 30 randomly selected microscopic fields. The limiting antiserum dilutions for agglutination were in the same order of magnitude as those for inhibition of adhesion to brush borders (Table 7). The same result was obtained in similar replicate experiments of this type. It was therefore not possible to decide conclusively whether or not the inhibition of vibrio adhesion to brush border membranes by O antibodies was simply a consequence of bacterial clumping, or whether circumstances exist under which O antibodies could inhibit bacterial adhesion in this system in the absence of agglutination. A similar situation prevailed in experiments designed to test for inhibition of hemagglutinating activity of vibrios in the presence of antiserum. Hemagglutination inhi-

bition could be observed only at serum dilutions that also caused clumping of the vibrios under the conditions of the test.

DISCUSSION

Table 8 summarizes comparative data concerning the various parameters involved in the adhesion of *V. cholerae* to brush border membranes, erythrocytes, and intestinal mucosa that have been described in this and the two companion papers of this series (13, 14). It is apparent that agglutination of erythrocytes closely resembled adhesion of vibrios to brush borders. In contrast, adhesion to intact mucosa (to the extent that intestinal slices represent intact mucosa) differed in a number of important respects and must therefore involve different mechanisms. Especially important distinguishing characteristics of the reaction of vibrios with intact mucosa appear to be the insensitivity to inhibition by L-fucose and the fact that agar-grown vibrios or vibrios incubated in buffer that had lost their ability to react with isolated brush border membranes were still able to adhere normally to tissue slices.

The present data do not support the idea that the association of vibrios with intact mucosa was predominantly a function of a mechanical "burrowing" of the bacteria into the mucus gel, a process recently invoked by Schrank and Verwey (15) to explain their experimental results. To be sure, a certain degree of invasion of mucus gel by vibrios has also been observed by us in vitro (13). Nevertheless, the finding that the adhesion of vibrios to mucosal slices could be inhibited by soluble mucosal digests suggests that vibrios bind to a specific, L-fucose-insensitive receptor on the intact mucosa. The specificity of this binding was also demonstrated by the competition experiments presented in Table 1

TABLE 7. *Comparison of agglutination and inhibition of vibrio adhesion to brush border membranes by antiserum to heat-stable V. cholerae antigens*

Determination	Controls, no antiserum		Reciprocal dilution of antiserum #7						
	#1	#2	500	1,000	2,000	4,000	8,000	16,000	32,000
Avg no. of adherent vibrios per brush border[a]	8.6	8.6	2.0	2.1	2.5	2.0	2.8	8.1	9.1
Avg no. of bacterial clumps per microscopic field[b]	0.067	0.13	8.0	7.1	ND[c]	3.8	2.3	0.8	ND

[a] Determined on 20 randomly selected brush borders.
[b] Determined in 30 randomly selected microscopic fields.
[c] ND, Not done.

TABLE 8. *Comparison of adhesive and hemagglutinating activities of V. cholerae and association of V. cholerae with mucosal surfaces*[a]

Condition	Adhesion to brush borders	Agglutination of erythrocytes	Association with slices of intestinal mucosa
Agar cultures	−	−	+
Broth cultures	+	+	+
Elution	+	+	−
Nonmotile mutants	−	−	−
Motile revertants	+	+	+
In the absence of calcium	−	−	(+)
In the presence of L-fucose	−	−	+
In the presence of mannose	±	+	+
After incubation in buffer at 37 C	−	±	+
In the presence of anti-vibrio O antibody	+ (except in the presence of agglutination)	+ (except in the presence of agglutination)	− (in absence of agglutination)
In the presence of mucosal extracts	−	−	−

[a] +, Activity present; −, activity absent or inhibited; ±, partial inhibition of activity; ( ), uncertain.

and by the frequent observation that partially degraded mucosal digests, which had lost their ability to affect vibrio adhesion, were still able to inhibit the adhesion of salmonellae to tissue slices. Furthermore, the nonspecific surface-active agents tested failed to affect the binding of vibrios to brush borders and mucosal slices, but strongly inhibited the obviously nonspecific adhesion of these bacteria to glass surfaces.

The finding that nonmotile vibrio mutants failed to adhere to intact mucosa might, at first glance, be taken as an indication that motility is important in this reaction. However, the strong evidence discussed above implicating a specific receptor in this system makes it also likely that these mutants lacked the adhesin that enabled the parent strain to bind to the L-fucose-resistant mucosal receptor. However, our data do not rule out the possibility that motility might have some (and in this assay system, a subordinate) role in the adhesion of vibrios to tissue slices. It is possible, for example, that motility may be necessary to transport the vibrios to the location of the L-fucose-resistant receptor. This is in contrast to our results with isolated brush border membranes, which ruled out motility as an important factor contributing to that type of adhesion (14).

The data presented do not permit us to determine the exact microanatomical location of the L-fucose-insensitive receptor on the mucosal surface. Our earlier observations that vibrios did not adhere to extruded mucus in vitro do not eliminate the intestinal mucus gel as a possible location of this receptor. There is evidence that the composition of intestinal heterosaccharides may differ in different regions of the intestine and in different portions of individual villi, a finding that has been explained by the action of different glycosyl transferases or glycosidases (of host or bacterial origin) in different regions of the intestine (7). Intense enzymatic activity in intestinal mucus is also indicated by our finding that the inhibitory activity of unheated intestinal scrapings was very unstable. The removal of terminal monosaccharides from an oligosaccharide chain not only may destroy the receptor activity of the molecule, but also may create a receptor of new specificity by exposing a different residue in the terminal position. The classical example of this is vibrio neuraminidase, the "receptor-destroying enzyme," which also exposes a new receptor active in T-agglutination of erythrocytes (1, 3, 4). It is therefore entirely conceivable that mucus gel in different regions of the intestine and at different positions in the intervillus spaces may differ in its receptor activity for bacterial adhesion. Obviously, then, considerably more detailed studies than those reported in the present series of papers will be necessary to determine the precise location of the L-fucose-insensitive receptor for vibrio adhesion on the mucosal surface. It should be noted that preliminary observations of Alcian blue-stained sections from tissue slices that had been incubated with vibrios in the standard manner and subsequently fixed in a variety of ways showed Alcian blue-positive material on the mucosal surface, an observation that is consistent with the presence of mucus gel in that location. These data will be presented in detail in a future publication.

Our data comparing the agglutinating activity of vibrio O antisera with their ability to inhibit bacterial adhesion may furnish a possible explanation for the divergent results reported in the literature by different investigators. It is noteworthy that in all instances reported in the literature, as well as in our experiments described above, where inhibition of adhesion or protection against experimental disease was correlated with vibrio agglutination, very high concentrations of vibrios were used (i.e., in the order of 10^7 to 10^8 bacteria, either per milliliter [Table 6], per baby mouse [2], or per intestinal loop [15]). In contrast, when the vibrio concentration was lowered to 2×10^5/ml (Table 6; 8), inhibition of adhesion occurred in the absence of demonstrable agglutination. It is well known that the rate at which bacterial agglutination occurs depends on the bacterial concentration and that very long incubation times must be used to obtain agglutination with dilute bacterial suspensions. The available data do not rule out the possibility that agglutination of bacteria may, at least under certain circumstances, prevent their adhesion to surfaces. It seems, however, quite likely that, at the moment when prophylactic immunity is important in humans, i.e., when the vibrios are first ingested, the vibrio concentration in the intestine would be considerably below 10^8/ml, i.e., below the range where agglutination would readily occur. One must therefore suspect that, in the natural disease, immune mechanisms that either are bactericidal or prevent adhesion directly (i.e., by a mechanism other than agglutination) would be more important than those based on bacterial clumping.

Unfortunately, it is at present not feasible to test the effect of antibody on the adhesion of vibrios to isolated brush borders at low vibrio concentrations, because our test is based on microscopic observations. We are presently attempting to modify the assay system to make this possible. Until this is accomplished, it will

not be possible to decide whether the mechanism by which antibody inhibits bacterial adhesion differs to some extent for the different adhesins and receptors involved or whether the correlation between different antibody activities (such as agglutination and inhibition of bacterial adhesion) is merely an accidental consequence of the circumstances under which these activities are assayed.

In summary, then, the use of two different assay systems in the present and companion (13, 14) studies has allowed a degree of insight into the complexity of microbial adhesion to mucosal surfaces that could not have been gained with either system alone. As noted previously (14), key data such as the adhesion of *V. cholerae* to brush borders and the fucose sensitivity of this reaction could be obtained with freshly prepared as well as formalin-preserved brush borders. The differences between the two assay systems, therefore, are unlikely to be a consequence of comparing preserved (brush borders) with fresh (tissue slices) material. On the contrary, the two systems most likely represent mechanisms of bacterial association with different areas of the mucosal surface. Accordingly, a working hypothesis may be formulated that is consistent with the above data as well as with those currently available in the literature: there are at least two specific mucosal receptors for vibrio adhesion. One of these is L-fucose (and mannose) sensitive and is located on the brush border surface. The second receptor is L-fucose resistant, and its exact location is unknown. The nonmotile vibrio mutants studied may lack the corresponding adhesins for both receptors, whereas agar-grown vibrios lack the adhesin for the L-fucose-sensitive receptor but still carry the adhesin for the L-fucose-resistant receptor. It is possible that motility as such may play a role in the association of vibrios with mucus gel in vivo; however, in the assay systems used in the present study such a role, if any, was of a subordinate nature. It is entirely possible that all these (and perhaps additional) interactions may occur in natural cholera, either sequentially or in different areas of the villus or of the intestine. The mechanism by which antibody inhibits bacterial association with the mucosa may differ for the different mechanisms of adhesion and may differ further according to external circumstances such as bacterial concentration and the type, specificity, and concentration of the antibody involved. It is obvious, therefore, that bacterial adherence to mucosal surfaces and the inhibition of this phenomenon by immunity are mediated by mechanisms that are considerably more complex than was anticipated when bacterial adhesion and its inhibition by local immunity were first recognized as being of importance in pathogenesis and immunity to cholera and other enteric diseases (11, 16, 17).

ACKNOWLEDGMENTS

This study was supported by contract DA-49-193-MD-2840 with the U.S. Army Medical Research and Development Command, and by Public Health Service grant AI 07631 from the National Institute of Allergy and Infectious Diseases.

The excellent technical assistance of Vivian Greenberg is gratefully acknowledged.

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