Adhesion of *Vibrios* and *Aeromonads* to Isolated Rabbit Brush Borders

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Isolated rabbit brush borders were used to investigate the adhesive properties of clinical and environmental isolates of non-O1 *Vibrio cholerae* and clinical isolates of *Vibrio parahaemolyticus* and *Aeromonas hydrophila*. A minority of the isolates were found to adhere to brush borders. The adhesion of these isolates was affected by a number of factors, including the bacteria:brush border ratio, incubation time, culture medium and growth temperature. Adhesion was inhibited by L-fucose but was independent of calcium ion concentration.

INTRODUCTION

Adhesion of *Vibrio cholerae* to the intestinal mucosa appears to play a role in the pathogenesis of cholera (Srivastava *et al.*, 1980). A number of in vitro models for the investigation of adhesion of enterotoxigenic bacteria to the mucosal surface have been described, including the use of tissue segments (Freter, 1969), isolated enterocytes (Jones, 1972) and isolated brush borders (Sellwood *et al.*, 1975).

Non-O1 *V. cholerae*, previously regarded as non-pathogenic environmental organisms, may also cause diarrhoea by a mechanism similar to that of *V. cholerae* serovar O1 (Ohashi *et al.*, 1972). A similar mechanism has been proposed in the pathogenesis of diarrhoea caused by *Aeromonas hydrophila* (Sanyal *et al.*, 1975). No studies have examined the adhesive properties of non-O1 *V. cholerae*, but the adhesion of *V. cholerae* O1 to isolated rabbit brush borders has been reported (Jones *et al.*, 1976). A recent comparison of the pathogenicity of clinical and environmental isolates of *V. cholerae* O1 did not encompass adhesive ability (Sigel *et al.*, 1980). The adhesion of *V. parahaemolyticus* to human tissue culture cells has been described: Carruthers (1977) found a correlation between adhesion to HeLa cells and production of the Kanagawa haemolysin, whereas Gingras & Howard (1980) found no significant difference in adherence of Kanagawa-positive and Kanagawa-negative strains to human epithelial cell lines.

In view of the absence of data on adhesion of non-O1 *V. cholerae* and *A. hydrophila*, the rabbit brush border assay was used to investigate the adhesion of clinical and environmental isolates of non-O1 *V. cholerae*, and clinical isolates of *A. hydrophila* and *V. parahaemolyticus*.

METHODS

Bacteria. Eighteen strains of non-O1 *Vibrio cholerae* (NCV), four strains of *Vibrio parahaemolyticus* and five strains of *Aeromonas hydrophila* were isolated from stools of patients suffering acute diarrhoeal disease. In addition, eighteen strains of *A. hydrophila* isolated from faeces were received from Miss N. Sodha of the Tooting Public Health Laboratory. Twenty-two NCV strains isolated from environmental sources were received from Dr J. V. Lee of the Maidstone Public Health Laboratory.

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Media. All the isolates studied were maintained on slopes of nutrient agar (Oxoid) containing 1% (w/v) NaCl (salt nutrient agar). Organisms to be tested were cultured statically in nutrient broth (Oxoid) containing 1% (w/v) NaCl (salt nutrient broth) for 16 h at 37 °C. After incubation, cultures were centrifuged at 2000 *g* for 25 min, washed twice in Krebs–Henseleit (KH) buffer (Sellwood *et al.*, 1975) and resuspended in KH buffer to a concentration of approximately 5×10^8 colony-forming units (c.f.u.) ml⁻¹.

Preparation of rabbit brush borders. Brush borders were prepared from the small intestine of rabbits by the method described by Sellwood *et al.* (1975). The brush borders were stored at 4 °C in 5 mM-EDTA containing 10% (v/v) formalin, and their suitability for use was determined by performing the adhesion assay using a number of control strains. If a significant variation in adhesion index occurred the brush borders were discarded and a fresh preparation was made. Brush borders were washed twice in KH buffer before use, and resuspended in KH buffer to a concentration of approximately 10^6 brush borders ml⁻¹.

Brush border adhesion assay. Equal volumes (0.1 ml) of brush border and bacterial suspensions were mixed in 0.5 ml micro-centrifuge tubes and incubated at 37 °C for 15 min with continuous gentle mixing. One drop of the test mixture was examined by phase contrast microscopy, and the number of bacteria adhering to each of 20 brush borders was counted. Each test was duplicated, and all organisms were tested three times. The adhesion index was thus determined as the mean number of bacteria adhering to each of 120 brush borders. Mean adhesion indices were compared by the Fisher–Behren’s *d*-test (Snedecor & Cochran, 1967).

RESULTS

Adhesion to rabbit brush borders

Bacteria were seen to adhere to the micro-villous surfaces of brush borders. This property was exhibited by isolates of all three species examined. The maximum number of bacteria which could be accurately counted was 30, thus the maximum value of the adhesion index was 30, although some isolates adhered in numbers greater than this. It was noted that a small proportion of the brush borders in any preparation did not support the adhesion of bacteria. Organisms with an adhesion index of 5 or less were regarded as non-adhesive; a clear distinction was observed between these isolates and those having an adhesion index of 20 or greater, which were regarded as strongly adhesive.

The adhesion indices of the 67 strains studied are summarized in Table 1. The adhesion indices of 18 clinical NCV isolates ranged from 0.2 to 29.5 bacteria per brush border. Using the above criteria, 13 clinical NCV isolates were regarded as non-adhesive. The mean adhesion index of the non-adhesive isolates was $1.1 (\pm 1.3)$, while that of the 5 adhesive NCV isolates was $20.6 (\pm 7.8)$. The difference in mean values was considered highly significant ($z = 5.56$).

The adhesion indices of the *V. parahaemolyticus* isolates ranged from 3.9 to 29.3. Three isolates were considered adhesive; the mean adhesion index of these isolates was $28.6 (\pm 1.0)$. The difference between this value and the corresponding value for the adhesive NCV isolates was significant (at the 0.05 level but not at the 0.01 level; $z = 2.26$).

The adhesion indices of 22 environmental NCV isolates ranged from 0.1 to 30. Seven isolates were considered to be adhesive, their mean adhesion index being $17.4 (\pm 9.4)$. The difference between the mean adhesion indices of adhesive environmental and clinical NCV isolates was not significant ($z = 0.64$).

Only 4 of the 23 clinical *A. hydrophila* were considered adhesive, their mean adhesion index being $9.9 (\pm 5.4)$; this was significantly different from that of the adhesive NCV isolates (at the 0.05 level; $z = 2.42$).

Effect of test and cultural conditions on adhesion index

Using selected strains, various test and cultural conditions were investigated as possible sources of the observed variation in adhesion indices.

The adhesion indices of a clinical NCV strain (154) and of a *V. parahaemolyticus* strain (40) were dependent on the bacterial concentration, and thus on the ratio of bacteria to brush borders (Fig. 1).

Table 1. Summary of adhesive properties of the 67 strains studied

Organism	No. of strains	Source	No. of strains with adhesion indices of:	
			<5	5-30
Non-O1 <i>V. cholerae</i>	18	Clinical	13	5
Non-O1 <i>V. cholerae</i>	22	Environmental	15	7
<i>V. parahaemolyticus</i>	4	Clinical	1	3
<i>A. hydrophila</i>	23	Clinical	19	4

Fig. 1. Effect of bacterial concentration on adhesion index. The bacterial concentration used in the brush border assay (5×10^8 c.f.u. ml⁻¹) gave the greatest separation between a strongly adhesive strain (*V. parahaemolyticus* strain 40; ○), and a moderately adhesive strain (NCV strain 154; ●).

Table 2. Effect of test and cultural conditions on adhesion index

Values shown are the mean adhesion indices $\pm$ s.d.				
Species (strain)	<i>V. parahaemolyticus</i> (40)	NCV (RRD)	NCV (27958)	NCV (85)
Source	Clinical	Environmental	Clinical	Clinical
Standard test	29.3 ($\pm$ 3.0)	30 (0)	27.0 ($\pm$ 6.4)	0.4 ($\pm$ 0.8)
Incubation time (min)				
5	8.7 ($\pm$ 3.6)	6.8 ($\pm$ 5.1)	6.5 ($\pm$ 5.3)	0.1 ($\pm$ 0.2)
15	29.0 ($\pm$ 4.4)	29.7 ($\pm$ 3.1)	27.9 ($\pm$ 3.1)	0.9 ($\pm$ 0.8)
30	23.5 ($\pm$ 10.2)	14.2 ($\pm$ 4.8)	21.3 ($\pm$ 9.4)	0.6 ($\pm$ 0.9)
45	6.0 ($\pm$ 5.4)	11.4 ($\pm$ 5.7)	4.7 ($\pm$ 3.6)	0.3 ($\pm$ 0.4)
60	6.5 ($\pm$ 6.2)	3.9 ($\pm$ 2.4)	2.3 ($\pm$ 2.1)	0.4 ($\pm$ 0.4)
L-Fucose concn (μ g ml ⁻¹)				
10	27.9 ($\pm$ 5.2)	30 (0)	28.6 ($\pm$ 6.7)	0.4 ($\pm$ 0.8)
100	10.4 ($\pm$ 7.2)	14.7 ($\pm$ 5.4)	13.2 ($\pm$ 3.9)	0.5 ($\pm$ 1.0)
Growth temp. ($^{\circ}$ C)				
20	0.4 ($\pm$ 0.7)	3.5 ($\pm$ 2.1)	5.2 ($\pm$ 2.9)	0.2 ($\pm$ 0.3)
25	2.8 ($\pm$ 2.3)	29.6 ($\pm$ 3.5)	16.1 ($\pm$ 8.3)	0.1 ($\pm$ 0.4)
30	21.2 ($\pm$ 10.9)	30 (0)	23.8 ($\pm$ 8.6)	0.5 ($\pm$ 0.5)
37	28.2 ($\pm$ 5.4)	30 (0)	30 (0)	1.0 ($\pm$ 1.1)
40	3.8 ($\pm$ 3.3)	0.4 ($\pm$ 1.2)	0.6 ($\pm$ 0.6)	0.2 ($\pm$ 0.5)
Culture medium*				
SNB	29.1 ($\pm$ 2.8)	30 (0)	28.3 ($\pm$ 5.4)	0.6 ($\pm$ 1.0)
SNA	0.2 ($\pm$ 0.5)	1.0 ($\pm$ 1.3)	1.1 ($\pm$ 1.4)	0.5 ($\pm$ 0.9)
BA	0.4 ($\pm$ 0.8)	0.4 ($\pm$ 1.5)	0.7 ($\pm$ 1.6)	0.4 ($\pm$ 0.8)

* SNB, Salt nutrient broth; SNA, salt nutrient agar; BA, blood agar.

Maximum adhesion of three adhesive isolates (clinical and environmental NCV isolates and a strain of *V. parahaemolyticus*) occurred after 15 min incubation, followed by a decline in adhesion index (Table 2). The adhesion indices of these strains were significantly reduced after 45 min incubation. Incubation time had no effect on the adhesion index of a non-adhesive NCV strain (85).

Adhesion of three of the strains above was inhibited by L-fucose (Sigma) at a concentration of $100 \mu\text{g ml}^{-1}$ but not $10 \mu\text{g ml}^{-1}$ (Table 2). Neither concentration affected the adhesion of the non-adhesive isolate (85).

Two NCV isolates (RRD and 27958) were strongly adhesive when grown at 25, 30 and 37 °C, but non-adhesive when grown at 20 and 40 °C (Table 2). The isolate of *V. parahaemolyticus* (40) was strongly adhesive when grown at 30 and 37 °C, but non-adhesive when grown at 20, 25 and 40 °C. Growth temperature had no effect on the adhesion of the non-adhesive NCV strain (85).

Three strains (RRD, 27958 and 40) were adhesive when cultured in salt nutrient broth but not when cultured on salt nutrient agar or blood agar (Table 2). Culture medium had no effect on the adhesion of the non-adhesive NCV strain (85).

To test the observation that adhesion of *V. cholerae* to rabbit brush borders required the presence of calcium ions (Jones *et al.*, 1976), the assay was performed using buffer containing 1.8 mM-CaCl_2 . The presence of calcium ions at this concentration had no effect on the adhesion indices of the isolates examined (thirteen clinical NCV isolates, two *V. parahaemolyticus*, one environmental NCV isolate and one *A. hydrophila* isolate).

DISCUSSION

The results presented confirm the presence of an adhesive factor (adhesin) in some isolates of non-O1 *V. cholerae*, *V. parahaemolyticus* and *A. hydrophila*, which mediates adhesion to a receptor site located on rabbit brush borders. However, the majority of the isolates examined lacked this adhesin. The proportion of clinical and environmental NCV isolates exhibiting adhesion was approximately equal. The ability to adhere has ecological benefits both for pathogens and environmental organisms. Adhesion in the natural environment enhances bacterial activity (ZoBell, 1943) while adhesion to gut mucosa facilitates colonization. Rabbit brush border membranes possess a receptor(s) which bind the adhesin(s) of environmental NCV isolates in addition to those of clinical isolates. However, the relevance of this finding to adherence in the natural environment is unknown.

Expression of the adhesive factors was markedly affected by test and cultural conditions. Similar findings were reported for *V. cholerae* O1 by Jones *et al.* (1976) and for *V. parahaemolyticus* by Carruthers (1977) and Carruthers & Anderson (1979). In addition, the adhesive factors of non-O1 *V. cholerae* were expressed only over a limited growth temperature range. An environmental NCV isolate (RRD) adhered equally strongly when grown at 25, 30 and 37 °C, while the clinical NCV isolate (27958) was considered more adhesive when grown at 30 and 37 °C than at 25 °C. Like *V. cholerae* O1 (Jones & Freter, 1976) the adhesion of NCV isolates was fucose-sensitive. At a fucose concentration of $100 \mu\text{g ml}^{-1}$ the adhesion of three strains was significantly reduced.

Adhesion of the strains studied was not calcium-dependent, in contrast to the findings of Jones *et al.* (1976) for *V. cholerae* O1. Guentzel *et al.* (1978) reported that strains of *V. cholerae* O1 exhibited a variable requirement for calcium ions. Isolates of *V. parahaemolyticus* studied by Carruthers & Anderson (1979) also required calcium ions for adhesion to occur. The mechanism by which calcium ions may or may not enhance adhesion is unknown. It has been suggested that differences in surface structure between bacterial strains may affect surface charge and account for the variations observed in adhesive ability between strains of the same species (Carruthers & Anderson, 1979). This may also explain the variable effect of cations on adhesion.

Adhesion clearly depends on the model system employed and on cultural conditions. Observations made from the study of adhesion in several models strongly suggest that multiple adhesive mechanisms may operate in *V. cholerae* O1 (Jones, 1980). A similar situation appears to occur in non-O1 *V. cholerae*.

The nature of the adhesin(s) produced by vibrios is unclear. A soluble haemagglutinin (cholera lectin) was isolated from *V. cholerae* O1 (Finkelstein *et al.*, 1978). Some *Vibrio* species bear fimbriae which mediate mannose-sensitive haemagglutination (Tweedy *et al.*, 1968). Fimbrial surface antigens mediate mannose-resistant adhesion of many enterotoxigenic *Escherichia coli* strains to intestinal mucosa. However, Nelson *et al.* (1976) could not demonstrate such fimbriae in *V. cholerae* O1 by electron microscopy. Lankford & Legsomburana (1965) proposed that adhesion of *V. cholerae* to the intestinal mucosa is mediated by the cell surface.

In view of the variation in adhesive properties detected between different model systems, it is quite probable that several adhesive structures may occur in non-O1 *V. cholerae*, their expression being dependent on a range of conditions. Further investigations are necessary to elucidate the nature of individual adhesive mechanisms.

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