ROLE OF SOMATIC ANTIGEN OF *VIBRIO CHOLERAE* IN ADHESION TO INTESTINAL MUCOSA

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SUMMARY. The in-vitro adhesion of *Vibrio cholerae* to intestinal mucous membrane was studied in isolated adult-rabbit ileal loops. Antisomatic antiserum against *V. cholerae* Inaba could inhibit adhesion of three different strains of *V. cholerae* Inaba but had no effect on the adhesion of two different strains of enterotoxigenic NAG vibrios. The antiserum's bacterial agglutinin titre was 320, its anti-Inaba lipopolysaccharide (LPS) titre was 16 000 and its anti-flagellar antibody titre was 3200. Conversely, anti-live *V. cholerae* Inaba antiserum absorbed with boiled cells of Inaba and devoid of antisomatic antibody, could not inhibit adhesion of the same three strains of *V. cholerae* Inaba. This antiserum had no anti-LPS or bacterial agglutinin activity, but its anti-flagellar antibody titre was 32 000. Thus, ability to inhibit adhesion of *V. cholerae* could be correlated only with antisomatic (anti-LPS) antibody activity. Antisomatic antiserum had no activity against 'adhesin', a *V. cholerae* surface antigen described by Freter. Conversely anti-live *V. cholerae* antiserum absorbed with boiled cells showed anti-adhesin activity even at a dilution of 1 in 200. LPS preparations from *V. cholerae* strain 569B Inaba could inhibit adhesion of two different Inaba strains to the intestinal mucous membrane. It is concluded that the somatic antigen plays a major role in the adhesion of *V. cholerae* to the intestinal mucous membrane.

INTRODUCTION

The mechanism of protection afforded by antibacterial immunity in cholera has remained obscure. In the preceding paper, we have shown that antisomatic antibodies offer protection through inhibition of adhesion of *Vibrio cholerae* to the intestinal mucosa (Chitnis, Sharma and Kamat, 1981). We have further shown that adhesion of the vibrios to the epithelium is important in the pathogenesis of cholera not only because it prevents them from being eliminated by peristalsis but also because adherent vibrios induce secretion of fluid by smaller amounts of exotoxin. The precise mechanism of bacterial adhesion is far from clear. Freter (1970), and Yancey, Willis and Berry (1979) have suggested that flagellar antigens are involved in adhesion of the vibrios to the mucous membrane. In the present paper we submit evidence to indicate that the somatic antigens of *V. cholerae* play a major role in adhesion.

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MATERIALS AND METHODS

Bacterial strains. The highly enterotoxigenic strain *V. cholerae* 569B Inaba, two enterotoxigenic wild strains of *V. cholerae* Inaba isolated from patients with cholera, and two enterotoxigenic strains of NAG vibrios, isolated from patients with diarrhoea were used and are described in the preceding paper (Chitnis *et al.*, 1982).

The antisera used to study inhibition of adhesion of *V. cholerae* included antisomatic (anti-LPS) antiserum, and anti-live cholera absorbed antiserum, i.e., anti-live strain 569B Inaba antiserum absorbed with boiled cells of strain 569B Inaba; the methods of preparation and the characteristics of these sera have been previously described (Chitnis *et al.*, 1982).

Purified soluble lipopolysaccharide (LPS) preparations from *V. cholerae* strain 569B Inaba and *Escherichia coli* were prepared and purified by the method of Westphal described by Kwapinski (1972).

In-vitro assay for bacterial adhesion to rabbit intestinal epithelium. A portion of proximal ileum was removed from an adult rabbit and loops of 5 cm long were prepared *in vitro*. The loops received the reagents under test, and then each received a challenge inoculum of 10^6 live vibrios suspended in 0.2% gelatin in Hanks's balanced salt solution (BSS). After incubation for 30 min at room temperature (28°C) they were cut open, rinsed in 10 ml of phosphate-buffered saline at pH 7.2 (PBS) and then washed gently by swirling in 100 ml of PBS in a 250-ml conical flask. After three such washings the walls were homogenised in a tissue homogeniser and a viable count was done to determine the number of colony-forming units (cfu) in the homogenate; this indicated the number of vibrios adherent to the mucous membrane.

Standardisation of the adhesion assay. To test the reproducibility of the adhesion assay, 14 consecutive 5-cm loops of proximal ileum of one rabbit were challenged with 10^6 live *V. cholerae* strain 569B Inaba suspended in 1.0 ml of 0.2% gelatin in BSS. The results are shown in table I. It can be seen that the number of adherent vibrios ranged from 3200 to 11 650. This degree of variation made the assay unsuitable for the study of inhibition of bacterial adhesion by antisera and other test reagents. However, when the number of adherent vibrios in adjacent loops (i.e., loops 1 and 2, 3 and 4, 5 and 6 etc.) were compared, the differences within each set of adjacent loops were not large. In view of the considerable variation in numbers, the data could not be analysed by parametric statistics. Hence, Wilcoxon's (nonparametric) paired rank sum test as described by Pearson and Hartley (1972) was used, and it could be concluded that the numbers of adherent vibrios in adjacent loops were not significantly different. For testing the ability of the various reagents such as antisera and LPS to inhibit the adhesion of *V. cholerae* to the mucous membrane, the reagents and their appropriate controls were tested in adjacent loops.

Adhesion of *V. cholerae* to human group-O red cells. One ml of 5% human group-O red cells suspended in Krebs's buffer at pH 7.4 (modified by Freter and Jones, 1976) was mixed with

TABLE I

In-vitro adhesion of V. cholerae strain 569B Inaba to the rabbit ileal-loop mucous membrane

Loop no.	Number of adherent <i>V. cholerae</i> (cfu)
1	8700
2	6850
3	4500
4	3800
5	7900
6	6800
7	3700
8	5000
9	5450
10	4900
11	7700
12	9200
13	11 650
14	10 200

5×10^6 live cells of *V. cholerae* Inaba (wild strain) and 1 ml of decomplexed antiserum (diluted 1 in 20) or anti-live cholera absorbed antiserum (diluted 1 in 200). Controls containing normal rabbit serum were also set up. The final volume of each suspension was made up to 5 ml with Krebs's buffer. After incubation for 30 min at 37°C the suspensions were spun at 300 *g* and the red-cell pellets were washed twice in Krebs's buffer. The final pellet was suspended in 0.1% gelatin in distilled water to lyse red cells and the lysate was subjected to viable bacterial counts to determine the number of vibrios adherent to red cells.

RESULTS

Effect of antiserum on in-vitro adhesion of vibrios to rabbit intestinal mucous membrane

Sets of alternate ileal loops received 1 ml of decomplexed normal rabbit serum or rabbit antiserum (raised against strain 569B Inaba) at a dilution of 1 in 200. An inoculum of 10^6 live vibrios was injected into each loop. After incubation for 30 min the loops were opened and adherent vibrios were enumerated. The ability of a rabbit antiserum raised against *V. cholerae* strain 569B Inaba to inhibit adhesion of the 569B Inaba strain, two other wild Inaba strains and two strains of NAG vibrios was tested in this system. Inhibition of adhesion of NAG vibrios was tested with a 1 in 20 dilution of the antiserum instead of the 1 in 200 dilution used in tests against Inaba vibrios. The data were analysed by Wilcoxon's paired rank sum test. The results (tables II and III) clearly indicate that the antiserum could inhibit adhesion of *V. cholerae* Inaba strains but did not inhibit the adhesion of NAG vibrios. Representative results of inhibition studies with a wild Inaba and a NAG vibrio are shown in tables II and III; similar results were observed with two more strains of *V. cholerae* Inaba and one more strain of NAG vibrio. The antiserum had a bacterial agglutinin titre of 320, an anti-Inaba-LPS titre of 16 000 and an anti-flagellar antibody titre of 3200 as assessed by passive haemagglutination tests (Chitnis *et al.*, 1982).

TABLE II

*In-vivo adhesion of V. cholerae wild Inaba 1 in the presence of rabbit antiserum**

Number (cfu) of adherent vibrios in	
set A in the presence of normal rabbit serum†	set B in the presence of antiserum†
15 400	4200
23 950	3750
33 500	2400
12 200	2000
19 000	6500
12 800	8750
29 000	12 000

* Rabbit anti-boiled *V. cholerae* strain 569B Inaba antiserum.

† Decomplexed sera at a dilution of 1 in 200.

[Set A is greater than Set B ($p < 0.001$) by Wilcoxon's paired rank sum test.]

TABLE III

In-vitro adhesion of NAG vibrio 1 in the presence of rabbit antisomatic antiserum*

Number (cfu) of adherent vibrios in	
set A in the presence of normal rabbit serum†	set B in the presence of antisomatic antiserum†
88 500	84 500
55 500	81 500
44 500	58 000
38 500	39 500
33 000	28 000
31 000	34 000
32 000	52 000

*† See footnotes to table II.

[Set A and B not different by Wilcoxon's paired rank sum test.]

Effect of anti-live cholera absorbed antiserum on in-vitro adhesion of vibrios to rabbit intestinal mucous membrane

Anti-live cholera absorbed antiserum free from anti-LPS activity (Chitnis *et al.*, 1982), failed to inhibit adhesion of *V. cholerae* Inaba strains to the rabbit intestinal mucous membrane. Representative results of inhibition studies with a wild Inaba vibrio are shown in table IV; similar results were observed with the other two Inaba strains. The antiserum had an antibacterial agglutinin titre of less than 2, an anti-Inaba-LPS titre of less than 2 and an anti-flagellar titre of 32 000 (Chitnis *et al.*, 1982).

TABLE IV

In-vitro adhesion of wild *V. cholerae* Inaba 1 in the presence of rabbit anti-live *V. cholerae* antiserum absorbed with boiled cells*

Number (cfu) of adherent vibrios in	
set A in the presence of normal rabbit serum†	set B in the presence of the absorbed antiserum*
43 250	40 750
51 000	34 750
73 250	38 250
71 500	53 250
145 000	94 250
125 500	78 250
130 500	141 000

* Rabbit anti-live *V. cholerae* 579B Inaba antiserum absorbed with boiled Inaba cells.

† Decomplemented sera at a dilution of 1 in 20.

[Set A and B not different by Wilcoxon's paired rank sum test.]

Effect of Inaba LPS on the in-vitro adhesion of vibrios to rabbit intestinal mucous membrane

Before testing of the effect of Inaba LPS on adhesion of *V. cholerae* to the rabbit

TABLE V

In-vitro adhesion of wild *V. cholerae* Inaba 1 in the presence of *E. coli* LPS* and Hanks's gelatin†

Number (cfu) of adherent vibrios in	
set A in the presence of <i>E. coli</i> LPS	set B in the presence of Hanks's gelatin
95 000	75 000
61 750	44 500
79 250	63 250
91 750	120 000
69 000	94 750
71 500	96 500
118 750	132 250

* 1.0 ml of *E. coli* LPS at a concentration of 2 mg/ml.

† 0.2% gelatin in Hanks's BSS.

[Set A and B not different by Wilcoxon's paired rank sum test.]

intestinal mucous membrane, it was essential to show that LPS *per se* had no non-specific effect on adhesion of *V. cholerae* to the mucous membrane. A 1-ml volume of *E. coli* LPS at a concentration of 2.0 mg/ml in BSS containing 0.2% gelatin was injected into *in-vitro* rabbit intestinal loops. Alternate loops received 1.0 ml of LPS or 1.0 of 0.2% gelatin in BSS. Each loop then received 10^6 live cells of a wild *V. cholerae* Inaba strain. After incubation for 30 min, the loops were opened and washed and the adherent vibrios were enumerated. The numbers of adherent vibrios in the two sets of loops, i.e., those with and without *E. coli* LPS, were not significantly different (table V). This indicates that LPS *per se* has no non-specific effect on bacterial adhesion to the mucous membrane.

The effect of homologous Inaba LPS at a concentration of 2.0 mg/ml was then tested against *V. cholerae* strain 569B Inaba and one wild Inaba strain. Representative results of inhibition studies with one wild Inaba strain are shown in table VI; similar results were observed with the 569B Inaba strain. It

TABLE VI

In-vitro adhesion of wild *V. cholerae* Inaba 1 in the presence of Inaba LPS* and Hanks's gelatin†

Number (cfu) of adherent vibrios in	
set A in the presence of Hanks's gelatin	set B in the presence of Inaba LPS
206 750	44 000
101 250	63 250
157 500	74 500
148 500	38 500
110 500	28 000
122 750	13 750
187 500	39 000

*† See footnotes to table V.

[Set A is greater than Set B ($p < 0.001$) by Wilcoxon's paired rank sum test.]

TABLE VII

Adhesion of V. cholerae Inaba to human group-O red cells in the presence of antiserum

Number of vibrios adherent* to red cells in the presence of normal rabbit serum diluted 1 in 20	Mean ($\pm 90\%$ confidence limit)	Number of vibrios adherent* to red cells in the presence of antiserum diluted 1 in 20	Mean ($\pm 90\%$ confidence limit)
4.1×10^5 4.4×10^5 3.7×10^5 3.4×10^5 3.0×10^5 3.8×10^5	3.7×10^5 ($\pm 0.64 \times 10^5$)†	3.8×10^5 2.7×10^5 4.3×10^5 3.4×10^5 3.3×10^5 2.6×10^5	3.35×10^5 ($\pm 0.67 \times 10^5$)†

* Number of vibrios retained by red-cell pellet from 1 ml of 5% suspension of red blood cells.

† Difference not significant.

is clear that Inaba LPS specifically inhibited adhesion of the *V. cholerae* Inaba strains.

Anti-adhesion activity of the antisera

This was assayed by testing the ability of the two antisera to inhibit adhesion of *V. cholerae* to human group-O red cells. The results given in table VII show that the antiserum at a 1 in 20 dilution did not inhibit the adhesion, whereas the anti-live cholera absorbed antiserum, devoid of anti-LPS activity, inhibited adhesion of *V. cholerae* to human group-O red cells even at a dilution of 1 in 200 (table VIII). This antiserum had an anti-flagellar antibody titre of 32 000.

Studies on possible clumping effects

It could be argued that the antiserum used in our experiments may cause clumping of the bacteria by the agglutination reaction, and that the

TABLE VIII

Adhesion of V. cholerae Inaba to human group-O red cells in the presence of anti-live V. cholerae antiserum absorbed with boiled Inaba cells

Number of vibrios* adherent to red cells in the presence of normal rabbit serum†	Mean ($\pm 99\%$ confidence limits)	Number of vibrios* adherent to red cells in the presence of anti- live <i>V. cholerae</i> antiserum absorbed with boiled cells†	Mean ($\pm 99\%$ confidence limits)	Percentage inhibition
6.8×10^5 8.65×10^5 9.4×10^5 9.8×10^5 1.2×10^6 1.2×10^6	9.777×10^5 ($\pm 2.416 \times 10^5$)	2.8×10^4 1.2×10^4 1.0×10^4 3.8×10^4 5.4×10^4 7.16×10^4	3.56×10^4 ($\pm 4.03 \times 10^4$)	96.4

* Number of vibrios retained by red cell pellet of 1 ml of 5% suspension of red blood cells.

† Both sera tested at a dilution of 1 in 200.

reduction in the number of adherent vibrios attributed to the antiserum was due to clumping of the bacteria and not to inhibition of adhesion. Indeed if this were true, the observed in-vitro effects of the antisomatic antiserum may have no in-vivo relevance, because the local antibody in the gastro-intestinal tract is IgA and is a poor agglutinin. To resolve the issue of bacterial clumping, 10^6 live *V. cholerae* wild Inaba-I, were incubated in 1.0 ml of different dilutions of the antisomatic antiserum at 37°C for 45 min. The bacterial suspensions were then diluted and plated out to determine the number of cfu in each suspension. No reduction in the cfu count was observed even at a 1 in 10 dilution of the antiserum.

DISCUSSION

Many investigators (Freter, 1970; Schrank and Verwey, 1976; Holmgren and Svennerholm, 1977) have reported that antisomatic *V. cholerae* antibodies offer passive protection, thus implying a major role for the somatic antigens in bacterial adhesion to the intestinal mucous membrane. However the relative importance of the various bacterial surface molecules in the adhesion process is still in doubt. Holmgren and Svennerholm (1977) have indicated a major role for the somatic antigen, whereas Yancey *et al.* (1979) presented evidence in favour of the flagellar antigens. Freter and Jones (1976) demonstrated that a flagellar protein, 'adhesin', is important in the adhesion of *V. cholerae* to the intestinal epithelial brush border. Because most of these conclusions are based on studies with different experimental systems, comparison of the data is very difficult.

In the preceding paper we reported that an antisomatic antiserum with predominant anti-Inaba LPS antibodies passively protected adult-rabbit ileal loops against challenge with live *V. cholerae* Inaba strains (Chitnis *et al.*, 1982). However, the anti-live cholera absorbed antiserum free from anti-LPS antibodies failed to protect. The remarkable point is that the absorbed antiserum retained most of the anti-flagellar activity (passive haemagglutination titre, 32 000).

Our present findings indicate that antisomatic antiserum against *V. cholerae* Inaba was effective at a dilution of 1 in 200 in preventing the in-vitro adhesion of the three different strains of *V. cholerae* Inaba to rabbit intestinal mucous membrane but had no effect on the adhesion of two test strains of NAG vibrios. The antiserum had an anti-LPS titre of 16 000 and an anti-flagellar antibody titre of 32 000 as assessed by passive haemagglutination tests. However, the anti-live cholera absorbed antiserum even at a dilution of 1 in 20 could not block the adhesion of the same three strains of *V. cholerae* Inaba to the intestinal epithelium, despite the fact that its potent anti-flagellar activity was retained. Thus, the inhibition of in-vitro adhesion of *V. cholerae* to rabbit ileal mucous membrane correlated with the antisomatic (anti-LPS) antibody activity of the antiserum and not with its anti-flagellar activity. The studies on possible clumping effects clearly indicate that the observed in-vitro reduction in the number of vibrios adherent to the mucous membrane brought about by the antisomatic antiserum is not due to bacterial clumping.

It is also possible that the observed in-vitro effects of the antisomatic antiserum on *V. cholerae* adhesion may be due to inhibition of motility, because the non-motile and the feebly motile strains have been shown to adhere to the mucous membrane poorly (Bhattacharjee and Srivastava, 1979). This can be ruled out because the anti-live cholera absorbed antiserum having an anti-LPS titre of less than 2 and anti-flagellar titre of 32 000 could not inhibit bacterial adhesion even at a dilution of 1 in 20.

The two antisera were also checked for antibody activity against the flagellar protein 'adhesin' reported by Freter and Jones (1976) to be important in the adhesion of *V. cholerae* to the intestinal epithelial brush borders. The antisomatic antiserum, which could block the adhesion of *V. cholerae* to the ileal mucous membrane at 1 in 200 dilution, did not have any anti-adhesin activity even at a dilution of 1 in 20. Conversely, the anti-live cholera absorbed antiserum showed anti-adhesin activity at a dilution of 1 in 200, but it could not block the adhesion of *V. cholerae* to the mucous membrane even at a dilution of 1 in 20. Thus, it can be concluded that in our experimental system flagellar antigens did not play a major role in the adhesion of *V. cholerae* to the adult rabbit ileal mucous membrane.

It could be argued that the observed blockade by the antisomatic antiserum was due to steric hindrance after interaction between the epithelial cell surface and a hypothetical bacterial surface molecule other than LPS. Because Inaba LPS effectively blocked the adhesion of *V. cholerae* Inaba, we conclude that the somatic antigens play an important role in the adhesion of *V. cholerae* to the mucous membrane.

We do not wish to imply that bacterial surface entities other than the somatic antigen have no role in the adhesion of *V. cholerae*. The reported evidence and our data together indicate that more than one vibrio surface molecule may participate in the adhesion to the mucous membrane. However, all need not be equally important. Because passive protection could be induced by antisomatic antibodies and not by anti-flagellar antibodies (Chitnis *et al.*, 1982), it is proposed that the somatic antigens play a major role in the interaction between the mucous membrane and the vibrios. We feel that the flagellar antigens probably play a secondary role in stabilising the adhesion primarily brought about through the somatic antigens.

In the light of the evidence of this and the preceding paper (Chitnis *et al.*, 1981), the limitations of the conventional formalin-killed cholera vaccine (Joo', 1974) still need to be explained. We have clearly demonstrated that antisomatic antibodies, if present in intestinal secretions, could be capable of offering excellent protection (Chitnis *et al.*, 1981). The conventional cholera vaccine given by the subcutaneous route probably cannot induce sustained production of effective secretory antibodies in the gastro-intestinal tract. If this is true, any other form of vaccine given by the subcutaneous route is also likely to be unsatisfactory. This would include toxoid vaccines; indeed, the results of a recent field trial with a toxoid vaccine are as unsatisfactory as those obtained with conventional cholera vaccines (Curlin *et al.*, 1975).

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