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Hemagglutination and Intestinal Adherence Properties of Clinical and Environmental Isolates of Non-O1 *Vibrio cholerae*

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Hemagglutination and intestinal adherence properties of non-O1 *Vibrio cholerae* were studied in vitro. No definite correlation between the cell-associated hemagglutinin titers and the intestinal adhesion indices was noted. Sugar- and glycoprotein-mediated inhibition data also indicated differences between the hemagglutination and adherence processes in respect to the receptor structures. Intestinal adherence of most *V. cholerae* strains could be inhibited to various extents by *N*-acetyl D-glucosamine. This observation provides a likely explanation for the ecological behavior of these organisms, which are known to associate themselves with chitinous (chitin:homopolymer of *N*-acetyl D-glucosamine) surfaces of zooplankton. The absence of any significant difference between the intestinal adherence indices of clinical and environmental isolates suggests that intestinal adhesion may be an essential but not sufficient prerequisite for colonization by and subsequent expression of pathogenicity of these microorganisms.

Non-O1 *Vibrio cholerae* strains are abundant in the natural aquatic environment and appear to be autochthonous estuarine bacterial species (2). During the last two decades or so, a large volume of literature from different parts of the world has implicated these organisms in sporadic cases and localized outbreaks of diarrhea (21). Pathophysiologic mechanisms underlying non-O1 *V. cholerae*-induced diarrhea remain largely unknown since most clinical and environmental isolates do not express cholera toxin (CT) (5, 9, 16, 28) or do not even possess the structural gene for CT (17).

In an earlier study (5), we were able to demonstrate that the enteropathogenic potential of clinical isolates of non-O1 *V. cholerae* was significantly higher than that of environmental isolates. However, characteristics responsible for enteropathogenicity remained to be identified. Mucosal adherence properties of certain bacterial species are believed to play a key role in the expression of their pathogenicity (7). Furthermore, the colonization potential of certain types of bacteria is known to be mediated by their cell-associated hemagglutinin (CAHA) (6, 8). In the present communication, we have studied the CAHA and intestinal adherence properties of some of the clinical and environmental isolates of non-O1 *V. cholerae* with a view to find out the interrelationship between the two properties, if any. We have also tried to evaluate the role of the intestinal adherence capacity of these organisms in the expression of their enteropathogenicity.

MATERIALS AND METHODS

Bacterial strains. The clinical isolates of non-O1 *V. cholerae* were isolated as pure cultures from the stool samples of diarrhea patients in Calcutta (5). A few clinical isolates were kindly supplied to us by R. Sakazaki (The National Institute of Health, Tokyo, Japan) and H. L. Smith (Jefferson Medical College, Philadelphia, Pa.). Environmental isolates of non-O1 *V. cholerae* were obtained from the water and sediments of local (Calcutta) ponds and the Hooghly river.

Determination of CAHA titer. *V. cholerae* strains of non-O1 serovars were grown in tryptic soy broth (TSB) (Difco Laboratories, Detroit, Mich.) without glucose for 4 h at 37°C. Cells were harvested by washing three times with normal saline and finally suspended in 0.01 M Krebs-Ringer-Tris (KRT) buffer, pH 7.6. The concentration of the bacterial suspension was adjusted to 600 Klett units. The CAHA titer of the bacterial suspension was determined by using a rabbit erythrocyte suspension (2.5% in KRT buffer) as described by Dasgupta et al. (4).

Inhibition of CAHA activity by simple sugars and glycoproteins. Inhibition of the CAHA activity of different non-O1 *V. cholerae* strains by simple sugars and glycoproteins was studied by following the methodology of Osawa and Matsumoto (20). For this, bacteria were grown in TSB (without any sugar) for 4 h at 37°C, harvested, and washed and suspended in KRT buffer. The concentration of the bacterial suspension was chosen so as to give 4 hemagglutinin (HA) doses in the inhibition assay mixture that contained bacteria, inhibitor (sugar or glycoprotein), and erythrocytes. All sugar and glycoproteins used were purchased from Sigma Chemical Co., St. Louis, Mo. Serially twofold-diluted solutions of sugar (starting from an initial concentration of 0.2 M) and glycoprotein (initial concentration of 1 mg/ml) were used in these inhibition experiments. Hemagglutination inhibition reactions were carried out in microdilution plates, and the results were expressed as the MIC of the sugar or glycoprotein that could completely inhibit the HA reaction.

Determination of intestinal adherence activity in vitro. Adherence activities of non-O1 *V. cholerae* to isolated intestine slices from rabbits were determined in vitro by using an assay system using ^{14}C-labeled vibrios (C. Dasgupta, Ph.D. thesis, University of Calcutta, 1986). For this, bacteria were grown for 4 h at 37°C in TSB (glucose-free) containing D-[U-^{14}C]glucose (Bhaba Atomic Research Centre, Trombay, India; specific activity = 53 mCi/mmol) at a concentration of 1 μCi/ml of culture. Radiolabeled bacteria were washed and resuspended in KRT buffer, pH 7.6, to a density of 70 to 80 Klett units. To 5 ml of this bacterial suspension, two pieces of intestine (ileum) segments (1-cm diameter each) from a rabbit were added, and the mixture

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was incubated at 37°C with gentle shaking. After 10 or 30 min of incubation, each tissue slice was removed separately, washed thoroughly with cold KRT buffer, transferred to scintillation vials, and digested (19), and the radioactivity was counted. Results were expressed as follows: adhesion index = $(X/Y) \times 100$, where X is the total counts per minute of adhering bacteria per slice and Y is the counts per minute per milliliter of the interacting bacterial suspension.

Inhibition of intestinal adherence by simple sugars and glycoproteins. Intestinal adherence activities of the ^{14}C-labeled non-O1 *V. cholerae* strains were inhibited in the presence of simple sugars or glycoproteins. For this, radiolabeled bacteria were grown as described above, harvested, washed and suspended in KRT buffer, and incubated for 30 min at 37°C with appropriate sugars (0.2 M) or glycoproteins (1 mg/ml). Next, rabbit intestine slices were added and the mixture was incubated for 10 min at 37°C. Finally, tissues were washed and digested and radioactivity was measured as described above. The control flask contained radiolabeled bacteria and tissue only. Results were expressed as the percent inhibition of intestinal adherence caused by simple sugars or glycoproteins by comparing the adhesion indices of the experimental set with those of the control set (without any inhibitor).

RESULTS

Comparison of CAHA activities of clinical and environmental isolates of non-O1 *V. cholerae*. CAHA activities of clinical and environmental isolates were determined against rabbit erythrocytes. No marked difference in the CAHA titers could be noted between the clinical and the environmental isolates; the titers ranged between 4 and 256 for the clinical groups and between 16 and 512 for the environmental groups.

Inhibition of CAHA activities of non-O1 *V. cholerae* by simple sugars and glycoproteins. Simple-sugar-mediated inhibition of CAHA activities of some of the non-O1 *V. cholerae* strains were tested against rabbit erythrocytes; the results are presented as the MICs in Table 1. Of five clinical and three environmental isolates tested, four (two clinical and two environmental) were not inhibitable by any of the simple saccharides tested. However, other strains showed variable degrees of inhibition by one or more of the sugars, such as D-mannose, D-glucose, D-galactose, D-glucosamine, D-galactosamine, *N*-acetyl D-glucosamine (GlcNAc), and *N*-acetyl D-galactosamine (GalNAc).

Of three glycoproteins used for inhibition studies, only fetuin and, to a lesser extent, normal rabbit immunoglobulin G (IgG) showed definite inhibition of the CAHA activities of some of the non-O1 strains (Table 1).

Comparison of adherence activities of clinical and environmental isolates of non-O1 *V. cholerae*. The same clinical and environmental isolates used for the CAHA study were examined to determine their adherence properties to rabbit intestine slices in vitro. It may be seen (Fig. 1 and 2) that the time-dependent adherence profiles of clinical and environmental isolates were somewhat variable in nature. Most isolates showed a rise in their adhesion index values mainly during the first 10 min of incubation. Thereafter, the values either continued to increase or remained essentially unchanged. For two environmental isolates (N75 and 58N), the value decreased during the 10- to 30-min incubation period. It is rather difficult to provide an explanation for these data, although the observed decrease may arise because of altered metabolic status of the radiolabeled bacteria N75 and 58N in their tissue-adherent state. Interestingly, no significant difference could be noted between the clinical and environmental isolates with respect to the mean of their adherence indices determined after either 10 or 30 min of incubation (Table 2).

TABLE 1. MICs of different sugars and glycoproteins inhibiting CAHA activities of some non-O1 *V. cholerae* strains[a] as determined against rabbit erythrocytes

Inhibitor	MIC[b] for non-O1 *V. cholerae* strain:							
	Clinical					Environmental		
	V_2	10259	10357	10325	9802	N70	N75	N5
Sugars								
D-Glucose	—	0.025	—	0.1	0.2	—	—	—
D-Galactose	—	0.1	—	—	—	—	—	—
D-Mannose	—	0.025	—	0.05	0.1	—	—	—
L-Fucose	—	—	—	—	—	—	—	—
D-Glucosamine	—	0.1	—	0.2	0.2	—	—	—
D-Galactosamine	—	0.2	—	—	—	—	—	—
GlcNAc	—	0.025	—	0.05	0.05	—	—	—
GalNAc	—	0.025	—	—	—	0.1	—	—
Glycoproteins								
Fetuin	62.5	500	—	250	500	250	250	—
Mucin	—	—	—	—	—	—	—	—
Normal rabbit IgG	500	500	—	500	500	500	500	—

[a] Strains were cultured for 4 h in glucose-free TSB at 37°C.
[b] Expressed as the molar concentration (for sugars) or in micrograms per milliliter (for glycoproteins). —, No inhibition occurred at up to 0.2 M sugar or a 500-μg/ml concentration of glycoproteins.

No correlation between adherence activities and CAHA titers. CAHA titers of seven clinical and seven environmental isolates were compared against their corresponding ad-

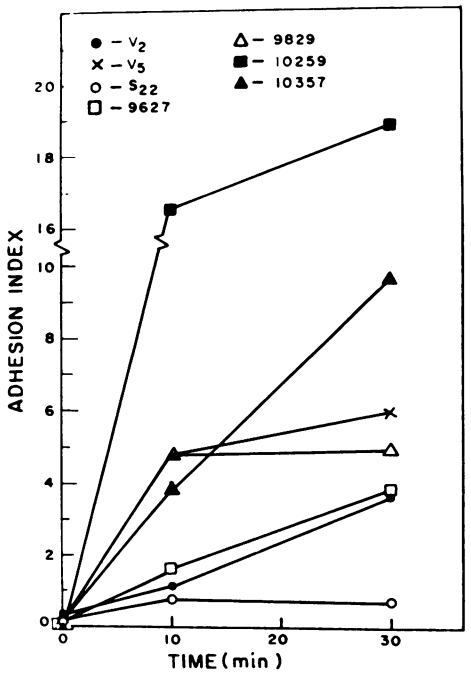

FIG. 1. Adherence profiles of different clinical isolates of non-O1 *V. cholerae* at various incubation times.

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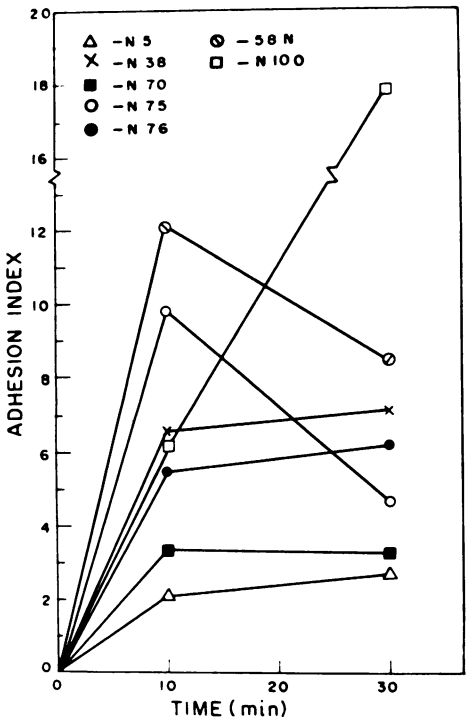

FIG. 2. Adherence profiles of different environmental isolates of non-O1 *V. cholerae* at various incubation times.

hesion indices obtained after 30 min of incubation. Statistical analysis of the data failed to establish a significant ($P > 0.05$) correlation between the adherence and the CAHA activity, as the correlation coefficient (r_p) for the clinical isolates was +0.13 and that for the environmental isolates was +0.18.

Inhibition of adherence activities by simple sugars and glycoproteins. One clinical (10259) and one environmental isolate (N75) of non-O1 *V. cholerae* with high adherence indices were chosen for this study. Results showed that the intestinal adhesion of the clinical isolate 10259 could be significantly inhibited by GlcNAc and GalNAc at a 0.2 M sugar concentration, the percent inhibition being 77 and 45, respectively. Other simple sugars, such as D-glucose, D-galactose, D-mannose, L-fucose, D-glucosamine, and D-galactosamine, had no such inhibitory effect. Adherence of the environmental isolate N75 was inhibitable by 0.2 M concentrations of D-glucose, D-galactose, D-glucosamine, GlcNAc, and GalNAc, and the percent inhibition was 55, 26, 27, 87, and 14, respectively. No inhibition of adherence of either 10259 or N75 could be noted for the glycoproteins fetuin, mucin, and normal rabbit IgG at 1 mg/ml.

TABLE 2. Comparison of adhesion indices of clinical and environmental isolates of non-O1 *V. cholerae*

Isolate (no. studied)	Adhesion index[a] at:			
	10 min		30 min	
	Range	Mean ± SD	Range	Mean ± SD
Clinical (7)	2.1–12.1	6.5 ± 3.5	2.7–17.8	7.2 ± 5.1
Environmental (7)	0.83–16.6	4.8 ± 5.2	0.73–18.9	6.5 ± 6.1

[a] Determined after a 10- or 30-min incubation period. There was no significant difference between the clinical and environmental isolates (calculated on the basis of mean ± standard deviation [SD] values) after either 10 or 30 min of incubation ($P > 0.05$).

DISCUSSION

In certain gram-negative bacteria, particularly in enterotoxigenic *Escherichia coli*, a close correlation between the HA properties and intestinal colonization was observed (6). This observation led to the belief that HA can be used as a reliable index of bacterial adherence to and subsequent colonization of the intestinal epithelium. However, this generalization had important exceptions, particularly for *V. cholerae* O1 strains (4, 13, 27). The present study also confirmed a lack of correlation between CAHA titers and intestinal adherence indices of non-O1 *V. cholerae*. Furthermore, sugar-mediated inhibition data indicate differences between the HA and intestinal adherence processes with respect to their receptor structures. This difference is also substantiated by the observation that normal rabbit IgG and fetuin could inhibit the CAHA but not the adherence activity of the non-O1 organisms. It may be noted here that Spira and Daniel (25), in a preliminary study, failed to establish a correlation between the CAHA activity and the brush border adherence properties of *V. cholerae* non-O1 strains. It is possible that the non-O1 strains express a variety of HAs, all of which are not relevant to the intestinal adherence process. Expression of multiple varieties of HAs in *V. cholerae* O1 strains had already been reported by various workers (1, 10, 11, 15). Recently, two groups of workers (3, 24) independently reported the presence of mannose-sensitive HAs in most of the non-O1 strains. However, it may be noted that these workers used chicken and human erythrocytes, while rabbit erythrocytes were used in our present study. The presence of multiple HAs in non-O1 strains is suggested on the basis of the sugar inhibition data (Table 1). Our results also indicate that the ligand-receptor binding in hemagglutination by non-O1 *V. cholerae* is mediated by complex carbohydrate structures such as substituted *N*-acetyl lactosamine units present in both the fetuin and the rabbit IgG (23).

Apart from the complex nature of HA reactions, the present study also indicates the complexity of the tissue adhesion process (Fig. 1 and 2) by the clinical and environmental non-O1 isolates. Interestingly, intestinal adhesion of two of the isolates (one clinical and another environmental) used in this study could be significantly inhibited by GlcNAc. Similar results were also obtained with most *V. cholerae* O1 strains (Dasgupta, Ph.D. thesis). These findings may have some relevance to the ecological behavior of *V. cholerae* organisms, as these were shown to remain intimately associated with the external surfaces of a group of chitinous (chitin:homopolymer of GlcNAc) zooplanktonic copepods (12).

Results presented here are in apparent disagreement with the earlier reports that L-fucose could inhibit the adhesion of both O1 and non-O1 *V. cholerae* (14, 18) while D-mannose was responsible for the inhibition of O1 strains (1). Such differences in the results may arise because of differences in the strains as well as in the assay systems used by various workers.

In an earlier study, we showed that the enteropathogenic potential of the clinical isolates of non-O1 *V. cholerae* was, in general, higher than that of the environmental ones (5). However, no significant difference between the intestinal adhesion capacities of clinical and environmental isolates could be noted in the present study (Table 2). Thus, the relevance of the in vitro adherence property of *V. cholerae* to the expression of their enteropathogenic potential in vivo remains to be established. Lack of any significant difference

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between the brush border adhesion indices of the clinical and environmental isolates was also observed by Levett and Daniel (18). On the other hand, Spira et al. (26) found clinical isolates to be far more capable of colonization than the environmental ones in the RITARD model. All these findings suggest that intestinal adhesion, although an essential prerequisite for the colonization process, may not be the only parameter to determine the enteropathogenic potential in vivo. Thus, colonization seems to be a multifactorial process involving intestinal adhesion, multiplication, and other metabolic steps leading to disease production. On the other hand, antibodies that are capable of inhibition of intestinal attachment of vibrios are likely to induce protection against vibrio challenge (22).

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