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Characterization and Distribution of the Hemagglutinins Produced by *Vibrio cholerae*

LARRY F. HANNE† AND RICHARD A. FINKELSTEIN*

Department of Microbiology, School of Medicine, University of Missouri, Columbia, Missouri 65212

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Examination of the distribution of cell-associated and soluble hemagglutinins (HA) produced by *Vibrio cholerae* revealed the existence of four different HAs. A cell-associated mannose-sensitive HA (MSHA) was produced only by the El Tor biotype. This was evident with all El Tor strains examined. It appears to be responsible for the HA biotyping differentiation of El Tor from classical biotype *V. cholerae*. The MSHA had no apparent divalent ion requirement; it was inhibited by D-mannose and D-fructose; and it was active on all human (A, B, O) and all chicken erythrocytes tested. Spontaneous $MSHA^-$ mutants of El Tor strains were selected by cosedimentation of $MSHA^+$ parent bacteria with erythrocytes. An L-fucose-sensitive HA was detected transiently in early log-phase growth with two of the four classical strains examined and with $MSHA^-$ mutants of El Tor biotype strains 3083, 26-3, and 17. $MSHA^-$ mutants also expressed another cell-associated HA in late log-phase cultures. A "soluble" HA was detected in late log-phase cultures of all strains tested. This HA was not inhibitable by any sugars tested; it required CA^{2+} ions for maximum activity; and it was active on some chicken erythrocytes but not others.

Mucus secretion and the continual peristaltic movement of contents through the lumen of the small bowel pose impediments to colonization by pathogenic and normal flora organisms (1, 7). Pathogens of the small bowel thus have had to evolve strategies to circumvent the normal clearance mechanisms in order to cause disease. In several instances, with enterotoxigenic strains of *Escherichia coli*, surface organelles (pili or fimbriae) have been shown to mediate attachment of the bacteria to the small bowel (4, 19, 20). These pilus-like adhesins usually also manifest hemagglutination activity against one or more species of erythrocytes (RBCs) (4, 8, 19), although this is not always the case (20).

Although *Vibrio cholerae* is the prototype of those organisms which produce disease by colonizing the epithelial surface of the small intestine and elaborating a potent enterotoxin (9), relatively little is known about its mechanism of attachment. In 1961, Bales and Lankford (Bacteriol. Proc., p. 118, 1961) observed that cholera vibrios attach to erythrocytes and suggested that this phenomenon may represent the same interaction as occurs between the vibrios and host intestinal epithelium. Subsequently, Finkelstein and Mukerjee (12) reported that El Tor, but not classical, biotype vibrios, when grown on solid media, agglutinated chicken RBCs. Other investigators have since described a variety of hemagglutinins (HAs) produced by vibrios, which have been studied as possible mediators of attachment (2, 5, 6, 10, 14, 17, 18). Suggestive evidence that *V. cholerae* have fimbriae (pili) was presented in 1968 (23), but this isolated report has not been substantiated by subsequent investigations. Nelson et al. (21, 22), in extensive studies of colonization in experimental rabbit models, could find no evidence of the participation of surface organelles (fimbriae or pili) in the attachment of *V. cholerae* to intestinal epithelium. Rather, the evidence suggested that adherence was the result of a more direct interaction(s) between the surface coat of the vibrios and the tips of the microvilli of the host intestinal epithelial cells. Subsequently, Finkelstein et al. (10) demonstrated that a partially purified "soluble" HA (which they called "cholera lectin"), isolated from culture supernatants of a classical biotype strain, inhibited attachment of an El Tor biotype and thus was a likely candidate for an adhesive factor.

None of the previous studies has addressed the distribution of the various HAs among cholera vibrios. This communication describes four distinct HAs and their distribution within the species *V. cholerae*.

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† Present address: Department of Microbiology and Immunology, University of Oregon, Health Science Center, Portland, OR 97201.

versity of Texas Health Sciences Center at Dallas, of which the majority was performed in absentia at the University of Missouri-Columbia.)

MATERIALS AND METHODS

Bacterial strains. The *V. cholerae* strains used and their sources are described in Table 1. All were preserved by lyophilization in 10% skim milk or at −70°C in syncase broth (11) with 20% glycerol. For convenience, strains were maintained at 4°C on meat extract agar. These stocks were transferred every 2 weeks and discarded after the third passage.

Media and buffers. Meat extract agar, tryptic soy broth (TSB; Difco Laboratories), and Krebs-Ringer buffer (KRT) (14) were used routinely. Tris-EDTA-azide-NaCl buffer was as described previously (11).

Growth conditions. Bacteria were inoculated to approximately 10^6/ml in 50 ml of TSB in 1,000-ml Erlenmeyer flasks. Cultures were shaken at 60 longitudinal oscillations per min for 20 h in a 30°C water bath. Samples were removed periodically, and bacterial cells were sedimented at 4°C for 10 min at 4,500 × *g*.

Supernatants were collected, and cells were resuspended to a concentration of approximately 10^9/ml. Supernatants and cells were then tested separately for HA activity by the microtiter technique (see below) or by a slide test (12).

Source and preparation of RBCs. Chicken RBCs were obtained from an inbred line of white leghorns from the University of Missouri Department of Poultry Husbandry. Human RBCs were provided by "volunteers" in our laboratory. RBCs were also obtained from goats, BALB/c mice, and New Zealand white rabbits. Blood was drawn and transferred to heparin-coated tubes (Pan heparin; Abbott Laboratories). RBCs were washed three times in 0.85% saline and maintained at 4°C as a 10% packed cell volume in saline. For use, cells were removed from these stocks and diluted to 1.5% RBCs in KRT buffer. Stocks were discarded after 1 week.

Hemagglutination assay. Techniques for quantitation of HA and HA inhibition with sugars were adapted from Jones et al. (17). HA preparations were diluted in twofold series in round-bottomed microtiter plates (no. 1-221-24, Dynatech Laboratories, Inc.) in 25 μl of KRT. RBCs (1.5%) were added in 25 μl, the plates were tapped to mix the interactants, and RBCs were allowed to settle at 25°C for 30 min. The titer is defined as the reciprocal of the highest dilution in which HA was visible to the naked eye. For determination of cell-associated HA, vibrios were washed and suspended in saline to a concentration of 1×10^9 to 2×10^9 vibrios per ml before assay.

To test whether the HA reaction was inhibitable by specific monosaccharides and other compounds, the substances (10 mg/ml in saline) were serially diluted in microtiter plates in 25 μl of KRT. Portions, 25 μl, of HA, diluted to a titer of 16, were added to each well and allowed to interact for 15 min at 25°C; RBCs were then added and HA reactions were examined after 30 min. Sugars and other potential inhibitors tested included D-mannose, L-fucose, D-galactose, D-mannitol, D-glucosamine, D-fructose, D-ribose, D-glucose, *N*-acetyl galactosamine, dextran, mixed gangliosides, phosphatidyl choline, and dithiothreitol.

Enrichment and selection of MSHA-negative mutants of El Tor biotype vibrios. Spontaneous variants which had lost the ability to produce mannose-sensitive HA (MSHA) were selected from three El Tor biotype strains (3083, 26-3, and 17). Single-colony isolates of each strain were inoculated into syncase broth and propagated overnight at 30°C in stationary culture. The culture was diluted 1:10 in saline, and 1 ml was added to 10^8 chicken RBCs. After incubation for 15 min at 25°C to allow the vibrios to adhere to the RBCs, the RBCs were sedimented at 100 × *g* for 2 min, in effect removing HA^+ vibrios from the population. The HA^--enriched supernatant was adsorbed three more times and then inoculated into fresh syncase medium and the cycle was repeated. Part of the enriched supernatants were diluted and plated on meat extract agar. After overnight incubation at 37°C, isolated colonies were screened with a rapid slide agglutination test (12) to detect $MSHA^-$ mutants. At least three such selective passages were required to permit isolation of $MSHA^-$ mutants. Spontaneous mutants were not detected during passage without the enrichment protocol.

Enzyme treatment of RBCs. A total of 40-μl of packed chicken RBCs (responder and nonresponder) and human O RBCs were obtained by centrifugation and resuspended in 10 mg of mixed glycosidase (Miles Laboratories, Inc.) or receptor-destroying enzyme (Behring Diagnostics) per ml in KRT buffer, pH 5.3. Control cells were resuspended in the same buffer without enzymes. Cells were incubated for 30 min at 37°C and then washed three times with KRT, pH 7.4. Enzyme-treated and control cells were then used to assay HA activity. Neither enzyme-treated nor control cells autoagglutinated.

RESULTS

Previous work with *V. cholerae* has suggested that there are three HAs. Of the two HAs which are clearly cell associated, one was reported to be inhibited by L-fucose (17), and one was inhibited by D-mannose (2). An HA which was found in culture supernatants and which was evidently not cell associated (10) was not inhibited by any sugars tested.

Four classical and six El Tor biotype strains (Table 1) were studied for the production of HA. When vibrios were grown on solid medium (meat extract agar), they responded as reported previously (12). All El Tor biotype vibrios were HA^+ and all classical biotypes were HA^- by the slide and microtiter HA assays. Sugar inhibition studies revealed that the HA which dictates the biotype is sensitive to D-mannose at 4 μg/ml. It is also blocked by D-fructose. Identical results were obtained when vibrios were grown in TSB for 20 h at 30°C, washed, and then tested. However, under this set of conditions, a soluble HA was elaborated by all strains examined.

Further characterization of the El Tor cell-associated HA and the soluble HA from both biotypes revealed several other differences (Table 2). Dialysis of soluble preparations against

TABLE 1. *V. cholerae* strains, characteristics, and origins

Biotype	Strain	Serotype	Source
Classical	CA401[a]	Inaba	Calcutta, 1953
	NIH35A3[b]	Inaba	India, 1941
	569B[c]	Inaba	India, ca. 1945
	CA411[a]	Ogawa	Calcutta, 1953
El Tor	HP30[d]	Inaba	Thailand, 1966
	64890[d]	Inaba	Vietnam, 1964
	26-3[d]	Ogawa	Manila, 1961
	3083[d]	Ogawa	Vietnam, 1964
	17	Ogawa	Watanabe and Verwey (24)
	1-86-O[e]	Ogawa	Teheran, 1965

[a] Isolated by C. E. Lankford and received from R. J. Yancey and C. D. Parker, respectively. Described in reference 16.
[b] From the Walter Reed Army Institute of Research; collection of R. A. Finkelstein.
[c] Originally from N. K. Dutta. Obtained from the collection of R. A. Finkelstein (13).
[d] Isolated by and from the collection of R. A. Finkelstein.
[e] From A. S. Benenson; collection of R. A. Finkelstein.

TABLE 3. Activity spectrum for soluble HA from *V. cholerae* strain CA401

Source of RBCs[a]	HA titer[b]
Chicken (responder)	128
Chicken (nonresponder)	4
Rabbit	8
Human (type O)	8
Human (type A)	8
Mouse BALB/c	128
Goat	32

[a] Fresh blood was withdrawn from different species into heparin-coated syringes. Erythrocytes were washed three times in saline (0.85%) and resuspended to 1.5% (vol/vol) in KRT.
[b] A 25-μl portion of crude soluble HA from strain CA401 was diluted in a microtiter plate. A 25-μl amount of 1.5% RBCs was added to each well, mixed, and allowed to sediment, and titers were determined as the reciprocal of the last dilution exhibiting complete hemagglutination.

Tris-EDTA-azide-NaCl buffer to remove divalent cations revealed that Ca^{2+} had to be included in the assay buffer (KRT) to obtain maximum HA titers. No such Ca^{2+} requirement was demonstrable with the El Tor biotype cell-associated MSHA, as washed cells resuspended in Tris-EDTA-azide-NaCl buffer were still active.

These HAs differed in the spectrum of RBCs upon which each was active. The cell-associated HA was active on all human (A, B, O) and chicken RBCs tested, exhibiting equivalent titers. However, the soluble HA was markedly more active on RBCs from certain species than from others (Table 3). It gave maximum titers with some chicken and mouse RBCs and was minimally active on rabbit and human RBCs. The soluble HA was active on only 10 of 18 chickens tested. There was not a continuum of responsiveness. Rather, chickens fell into one of three classes. Soluble HA activity with RBCs from individual chickens was either extremely weak or undetectable, high (e.g., 1:128 to 1:512), or at one intermediate titer (approximately 1:32).

When nonresponder chicken RBCs were treated with mixed glycosidases at 10 mg/ml (Table 4), they became responsive to the soluble HA. Glycosidase treatment had no effect on responder chicken RBCs, but it eliminated the responsiveness of human RBCs. Receptor-destroying enzyme treatment of responder chicken RBCs increased titers significantly, but had no effect on nonresponder or human RBCs.

Jones et al. (17) had previously demonstrated an L-fucose-inhibitable HA(FSHA) on one classical biotype strain of *V. cholerae*. It was interesting that none of the 10 strains tested exhibited an FSHA when assayed after overnight growth. The four classical strains were grown at 30°C on a platform shaker and monitored at intervals for soluble or cell-associated HA. Figure 1 represents the expression of HA by classical biotype

TABLE 2. Characterization of soluble and cell-associated HAs of *V. cholerae*

Hemagglutination	Sugar inhibition[a]	Ca^{2+} requirement[b]	No. of positive responses/no. of RBC prepn	
			Human RBC[c]	Chicken RBC
Cell associated[d]	D-Mannose	−	6/6	18/18
Soluble[e]		+	—[f]	10/18

[a] Sugars tested = D-mannose, L-fucose, D-galactose, D-mannitol, D-glucose, and D-ribose at 10 mg/ml.
[b] Demonstrable only after dialysis against EDTA-containing buffer.
[c] A, B, and O blood types were included.
[d] From El Tor biotype vibrios grown at 37°C overnight on meat extract agar.
[e] From cell-free supernatant of all strains grown for 20 h, aerated in TSB.
[f] Very low titers were observed for soluble HA when assayed with human RBCs.

TABLE 4. Effect of glycosidase and receptor-destroying enzyme treatment of human and chicken RBCs on their response to soluble HA[a]

Treatment	Titer of HA activity		
	Responder chicken RBCs	Nonresponder chicken RBCs	Type O RBCs
Buffer[b]	128	4	16
Glycosidases (10 mg/ml)[c]	128	512	0
RDE[d] (10 mg/ml)	1,024	8	16

[a] RBCs were treated at 37°C for 30 min with the enzymes in KRT, pH 5.3, and then washed twice in KRT, pH 7.4. Cells were resuspended to 1.5% in KRT, pH 7.4, and used to assay HA of crude soluble HA from *V. cholerae* strain CA401.
[b] RBCs were incubated for 30 min in KRT buffer, pH 5.3, without enzyme.
[c] Glycosidase alone did not cause autoagglutination.
[d] RDE, *V. cholerae* receptor-destroying enzyme (Behring Diagnostics).

strain CA401. Very early in log-phase growth a cell-associated HA was expressed, but only transiently, as it was not detectable beyond mid-log phase. This cell-associated HA was inhibited by L-fucose and was found only in strains CA401 and NIH35A3.

In contrast, the soluble HA was detected in the media during mid-log phase and remained for as long as it was monitored (36 h). Kinetic studies were performed at 30°C rather than at 37°C, because the soluble HA titers dropped dramatically in late log, possibly due to enzymatic degradation.

It was felt that isogenic mutants of El Tor strains which were MSHA defective would be a useful tool for later attachment studies as well as for characterization studies. MSHA$^-$ mutants of strains 3083, 26-3, and 17 were selected according to the protocol in Materials and Methods.

The mutants were grown in TSB at 30°C in shaken culture, and soluble and cell-associated HAs were monitored at intervals as with the classical biotypes. Figure 2 illustrates the kinetics of production of HA by the MSHA$^-$ mutant of strain 3083. The mutant produces a cell-associated HA, transiently in early log phase, which is inhibited by L-fucose. This was also found for mutants of strains 26-3 and 17. Later in the log and stationary phases another cell-associated HA was expressed which was not inhibitable by sugars. Since this late-appearing cell-associated HA was active on nonresponder, as well as responder, chicken RBCs, it is unlikely that it is a cell-associated form of the soluble HA, which was also expressed by these mutants late in the culture cycle. The soluble HA produced by these MSHA$^-$ mutants was active on responder chicken RBCs, only. Thus, it is likely that it is the same soluble HA as is produced by the parent strain. L-Fucose-sensitive HA has not previously been detected in El Tor strains, apparently because it is transient and is masked by the MSHA, which was present under all growth conditions tested.

DISCUSSION

Previous reports have described HAs produced by *V. cholerae* and suggested that they may serve as possible mediators of attachment to host epithelial cell surface receptors (2, 10, 18). In general, the earlier papers dealt with one

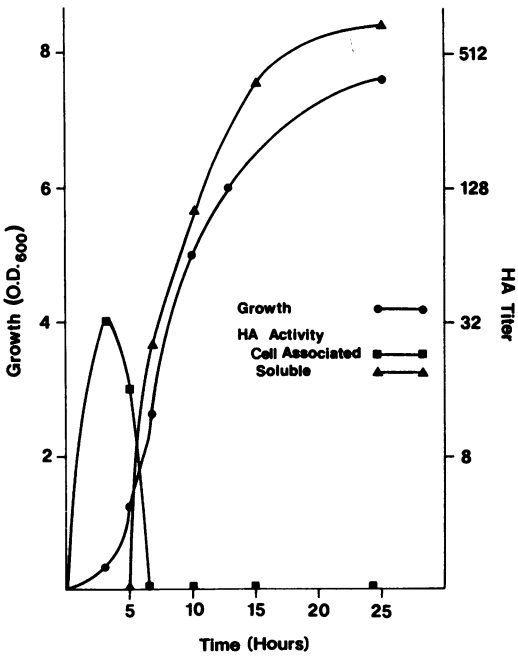

FIG. 1. HA production by *V. cholerae* strain CA401 (Inaba serotype, classical biotype). A total of 10^6 vibrios per ml were inoculated in 50 ml of TSB (in a 1,000-ml Erlenmeyer flask) and incubated at 30°C on a longitudinal shaker. Samples were removed periodically. After centrifugation to remove bacterial cells, soluble HA was assayed against responder chicken RBCs. Vibrio cells were resuspended so that a 1:100 dilution of the suspension gave an optical density at 600 nm ($O.D._{600}$) of 0.2. The cell-associated HA, determined with non-responder RBCs, was inhibited by L-fucose.

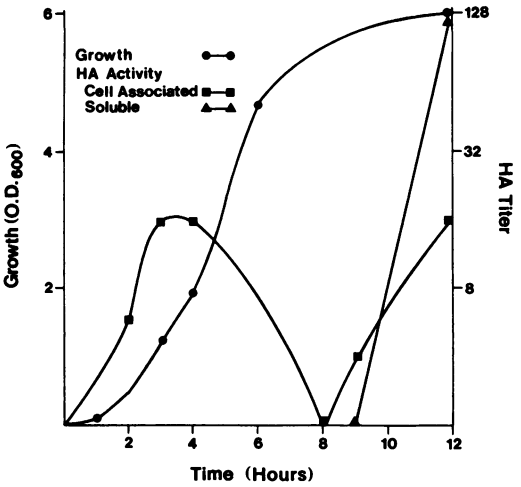

FIG. 2. HA production by the MSHA$^-$ mutant of *V. cholerae* strain 3083 (Ogawa serotype, El Tor biotype). A total of 10^7 bacteria per ml were inoculated in 50 ml of TSB (in a 1,000-ml Erlenmeyer flask) and incubated at 30°C on a longitudinal shaker. Samples were removed periodically. After centrifugation to remove bacterial cells, soluble HA was assayed against responder chicken RBCs. Vibrio cells were resuspended so that a 1:100 dilution of the suspension gave an optical density at 600 nm ($O.D._{600}$) of 0.2. The first appearing cell-associated HA, determined with nonresponder RBCs, was inhibited by L-fucose. The second cell-associated HA was not inhibited by either D-mannose or L-fucose.

or another HA and the results did not allow generalization regarding HA production within the species *V. cholerae*.

The present work establishes that the three *V. cholerae* HAs reported earlier are indeed distinct and characterizes their distribution. The major cell-associated HA of El Tor biotype strains grown on solid media, which aids in biotyping, is an MSHA. El Tor strains also produce MSHA when grown in broth. The classical strains examined produced either no cell-associated HA or a cell-associated FSHA transiently.

Since the El Tor biotype produced MSHA, it was felt that MSHA-negative mutants would be valuable in evaluating the possible role of the MSHA as an adhesin. Mutants of strains 3083, 26-3, and 17 defective in production of MSHA were selected by the protocol outlined in Materials and Methods. The MSHA$^-$ El Tor mutants, grown in TSB and monitored throughout the growth cycle, were found to express a cell associated FSHA transiently during early log-phase growth. It is likely that the parent strains also express FSHA transiently during early log phase, but it has not previously been detected, because it is masked by the more potent MSHA. Some classical strains of *V. cholerae* also produced an FSHA transiently during log-phase growth in TSB. The MSHA$^-$ mutants also expressed another cell-associated HA in late log- to stationary-phase cultures which was not inhibited by either mannose or fucose and was active on both responder and nonresponder chicken RBCs (thus differentiating this HA from the soluble HA which was also produced late).

Jones and Freter and Jones et al. (14, 17, 18) previously reported that an L-fucose-inhibitable cell-associated HA was produced by a classical vibrio strain. They found human RBCs to be optimal for detection of the FSHA and observed weak or negative HA reactions with rabbit, guinea pig, horse, chicken, sheep, or bovine RBCs. In contrast to the transient expression of the FSHA observed here, they detected activity at 18 h. It is possible that their strain either expressed the FSHA continually or failed to produce a factor (protease?) which could be degrading the FSHA after it is expressed in our cultures.

Jones and Freter did not describe the growth conditions, but indicated that they did not detect HA activity in cell-free supernatants. A soluble HA was produced by all strains tested herein. There are several possible explanations for Jones and Freter's inability to detect the soluble HA. Since the soluble HA is only observed late in the growth cycle, if they assayed for soluble HA too early during growth, it would not have been detected. If they incubated their cultures at 37°C and looked too late, it could also be missed. There is also a possibility that they used less responsive RBCs and therefore may have overlooked lower titers of soluble HA. In the present work, maximum soluble HA titers were observed only with RBCs from 10 of 18 chickens tested. Variation in responsiveness among individual chickens indicates differences in RBC surface components. Numerous blood group systems have been identified in chicken populations (3, 15). Briles (3) reported that the polymorphic state (i.e., preservation of multiple alleles within each blood group) occurs even in long established inbred lines. Fifty-two different blood group genes from 11 blood group systems were identified in three lines which had been inbred for 15 to 20 generations. It was therefore not surprising that variations in responsiveness to the soluble HA exist in the inbred line of chickens used here. The present observations confirm earlier results with chicken RBCs tested in Japan and Texas (10). Interestingly, treatment with mixed glycosidase apparently exposed the receptor in nonresponder RBCs and neuraminidase increased the responsiveness of the responder RBCs. These observations may be applicable to future studies to define the receptor.

From the data, it can be concluded that El Tor vibrios produce MSHA constitutively under all growth conditions examined. Functionally underlying the MSHA of the El Tor strains tested, and on the surface of some classical strains, there is a cell-associated FSHA which is expressed transiently during early log-phase growth in broth. The common denominator among all strains tested of both serotypes and biotypes of *V. cholerae*, as revealed in this study, is the production of a sugar-insensitive soluble HA which is similar to the cholera lectin described previously (10). A subsequent paper (Finkelstein and Hanne, Infect. Immun., in press) describes the purification and characterization of this factor and provides further evidence of its role in adherence of cholera vibrios.

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