Weak Serum and Intestinal Antibody Responses to Vibrio cholerae Soluble Hemagglutinin in Cholera Patients

ANN-MARI SVENNERHOLM,* MYRON M. LEVINE,3 AND JAN HOLMGREN1,2

Department of Medical Microbiology, University of Göteborg, S-41346 Göteborg, Sweden1; The International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh2; and University of Maryland, School of Medicine, Baltimore, Maryland 21201

Received 24 January 1984/Accepted 1 June 1984

Vibrio cholerae O1 bacteria have been found to agglutinate certain species of erythrocytes both by cell-bound hemagglutinins (HAs) and by an extracellular, soluble HA (5, 6, 10). Although differences in cell-associated HAs have been noted between classical and eltor V. cholerae (6), all V. cholerae O1 strains hitherto tested have been found to produce an indistinguishable, soluble HA, although in very different amounts (10). The soluble HA, originally referred to by Finkelstein et al. as “cholera lectin” (2), manifests a number of diverse biological activities which may all be ascribed to its protease activity (3). It has been suggested that soluble HA may be an important virulence factor and a protective antigen of V. cholerae (3, 5).

An enzyme-linked immunosorbent assay (ELISA) method which allows determination of specific antibodies against soluble HA (10) was used to study whether recovery from clinical cholera infection is accompanied by significant rises in intestinal or systemic antibody to soluble HA. Since clinical cholera has been shown to give substantial immunity against new cholera attacks (4, 7), we assumed that intestinal and serum antibodies to soluble HA would arise in response to infection, if these antibodies are important in protection. Two other antibodies believed to play a role in protection, antitoxin and antiligopolysaccharide, have been found to arise in both intestinal fluid and serum in most individuals during the convalescent stage of cholera (9; A.-M. Svennerholm, M. Jertborn, L. Gothefor, A. M. M. M. Karim, D. A. Sack, and J. Holmgren, J. Infect. Dis., in press). We have also compared the serum antibody levels to soluble HA in healthy individuals living in a cholera-endemic and in two nonendemic areas.

Acute and convalescent serum samples were collected from Bangladeshi patients with moderate to severe diarrhea due to naturally acquired, culture-proven cholera and from North American volunteers who were experimentally infected with V. cholerae O1. The acute sera were obtained a few days before (North American) or 1 to 2 days after (Bangladeshi) onset of disease, and the convalescent sera were obtained after 14 to 28 days. The Bangladeshi patients were 2 to 45 years old and were recruited from persons treated for cholera at the Cholera Hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh, in Dhaka. The North American volunteers were college students and other adults, 20 to 45 years old, who had been infected with 105 to 108 V. cholerae eltor bacteria (strain P27459 Inaba, N16961 Inaba, or N1677 Ogawa) after neutralization of gastric acidity (7, 8); infection resulted in moderate or severe cholera in all of the volunteers. In addition, a number of sera from healthy Bangladeshis and Swedes, 20 to 45 years old and not cholera vaccinated, were studied. We also collected intestinal lavage specimens (9; Svennerholm et al., in press) from 20- to 45-year-old Bangladeshi cholera convalescents 9 and 28 days after onset of moderate to severe cholera disease, and jejunal aspirates (8) from adult North American volunteers who had been infected with V. cholerae eltor strain N16961 or E7946 (Ogawa) on the day before infection and 28 and 56 days after onset of diarrhea. The sera, prepared from blood drawn by venous puncture, were frozen at −20°C, and the intestinal lavages and jejunal fluids were lyophilized and stored at 4°C until used.

The ELISA tests of anti-HA antibodies were performed essentially as previously described (10), using as solid-phase antigen, soluble HA purified by a sequence of ammonium sulfate precipitation, gel filtration, and agarose electrophoresis (10). In short, wells of polystyrene microtiter plates were coated with 0.1 ml of a 0.3-μg/ml solution of soluble HA in phosphate-buffered saline (PBS) at 4°C for 18 h. After the plates were washed twice with PBS, they were incubated with a 1% human serum albumin-PBS solution at 37°C for 30 min to block any unoccupied binding sites on the plastic. The test samples, serum or intestinal fluid, were then fivefold serially diluted in the plates with PBS as diluent supplemented with Tween 20 to a final concentration of 0.05%, and the various dilutions were then incubated in the plates at room temperature for 18 h. After being washed 3 times in PBS-Tween, the plates were incubated at room temperature for 2 to 4 h with an anti-human immunoglobulin preparation conjugated with alkaline phosphatase. For the titrations of serum samples, a pool of anti-human immunoglobulin G (IgG), IgA, and IgM alkaline phosphatase conjugates (Orion, Helsinki, Finland), each in 1:100 dilution in PBS-Tween, was used, whereas the anti-human IgA conjugate, diluted 1:100, was used alone for the assays of intestinal specimens. The plates were then washed 3 times again in PBS-Tween, p-nitrophenyl phosphate enzyme substrate was added, and color changes were recorded by automated spectrophotometry at 405 nm with a Titertek Multiscan (Flow Laboratories, Inc., Helsinki, Finland). The titer was determined as

* Corresponding author.
the reciprocal interpolated dilution of specimen giving an absorbance of 0.3 above background when the enzyme was reacted with substrate for 100 min. The precision of these titer determinations when adjusted in relation to an antisem-um standard included in each experiment was ±16%, which meant that a twofold titer difference or greater was well outside the methodological variation and thus statistically significant. Titors of ≥40 were considered as evidence for the presence of specific antibody because they exceeded the unspecific reactions seen in uncoated, albumin-blocked mi-crotiter plates.

Since soluble HA is a potent protease (3), control experi-ments were performed to evaluate whether the solid-phase attached HA may have a proteolytic effect on immunoglobulin in the test specimens during incubation in the plates. Serial dilutions of human sera containing significant anti-HA titers and rabbit hyperimmune serum against soluble HA (10) were preincubated in parallel in HA-coated and in noncoat-ed, but albumin-blocked, ELISA plates, respectively, at room temperature overnight and then transferred to new HA-coated plates for ELISA tests as described above. No significant difference in anti-HA titer after preincubation in HA-coated and noncoated plates was observed in any case.

Production of soluble HA by different V. cholerae strains during in vitro shake culture at 37°C for 18 h in tryptic soy broth without glucose was assayed as described previously (10), by an ELISA inhibition method. Total IgA concentra-tions were determined by means of a capture antibody ELISA technique which has been described elsewhere (Svennerholm et al., in press).

The anti-HA antibody levels in preinfection sera from the North American volunteers were compared with the anti-HA levels in serum samples obtained from age-matched healthy Bangladeshi and Swedes. Sera from either group of persons had detectable levels of anti-HA (titers ≥40), but the anti-HA titers among Swedes were significantly lower than in the other two groups which had very similar levels of anti-HA antibodies (Table 1).

The serum anti-HA antibody response to clinical choler-a infection was studied both in experimentally infected North American volunteers and in naturally infected Bangladeshi cholera patients. In neither group did the geometric mean anti-HA titer rise significantly between the acute and conval-scent serum sampling (Fig. 1). Despite the fact that all of the V. cholerae strains used in the studies with the North American volunteers produced moderate to high levels of soluble HA (10) when grown in vitro (0.10 to 1.1 μg/ml in nonconcentrated culture medium), only 1 of the 17 volunteers responded with a twofold or greater anti-HA titer increase to infection. Among the 10 Bangladeshi cholera patients, 2 showed a twofold or greater titer increase, and 1 showed a titer decrease in anti-HA antibody between the acute and convalescent serum samples (Fig. 1).

Jejunal aspirates collected from 10 American volunteers immediately before and 28 and 56 days after onset of experimentally induced cholera in no instance showed any specific antisoluble HA activity, i.e., they did not react in higher titer in plates coated with soluble HA than they did in noncoated albumin-blocked plates.

Intestinal lavage specimens collected from Bangladeshi cholera patients 9 and 28 days after onset of disease did, however, in most cases show detectable but low-titer antisol-uble HA ELISA activity. As a mean, there was a 1.4-fold increase in specific anti-HA IgA titer per total IgA between the two occasions, but this increase was not statistically significant. Of eight patients studied, two had a significant (twofold or greater) increase in specific IgA antisoluble HA activity between days 9 and 28, whereas two had a significant decrease. Titration of serum specimens from the same patients revealed a slight, but not statistically significant, mean titer increase (1.5-fold) against soluble HA from days 9 to 28 when pooled anti-IgG, anti-IgA, and anti-IgM conjugates were used in the ELISA tests; of eight specimens, four had a significant titer increase and one had a significant decrease. No difference in serum titer was seen between these dates when an IgA-specific conjugate was used.

These results suggest that whereas most adults in both cholera endemic and nonendemic areas have low levels of serum antibodies to soluble HA or V. cholerae, the response of these antibodies after experimental or natural clinical cholera infection is minimal with the ELISA method described herein. For comparison, 88% of the Bangladeshi cholera patients responded with serum antitoxin antibodies, 100% responded with serum vibrioidal antibodies, 88% responded with intestinal antibodies to cholera toxin, and 88% with intestinal titers against V. cholerae lipopolysacch-aride (Svennerholm et al., in press). Among the infected North American volunteers, the corresponding figures were 90, 90, 60, and 50% (7).

It is interesting to note that both the sera from the healthy Swedes and those taken before infection from the North American volunteers had measurable, albeit generally low, levels of antibodies binding to soluble HA. Since none of

---

**TABLE 1. Antibody titers to V. cholerae soluble HA in serum from healthy Bangladeshi, North Americans, and Swedes (20 to 45 years of age)**

<table>
<thead>
<tr>
<th>Subject (no.)</th>
<th>Antibody titera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladeshi (11)</td>
<td>235 (205-270)</td>
</tr>
<tr>
<td>North Americans (16)</td>
<td>180 (155-215)</td>
</tr>
<tr>
<td>Swedes (14)</td>
<td>80 (65-100)</td>
</tr>
</tbody>
</table>

a Reciprocal geometric mean ±1 standard error of the mean) titers of anti-HA antibodies.

b P < 0.01 versus titers of both Bangladeshi and North American sera.
these persons had visited a cholera endemic area or been vaccinated against cholera, the findings suggest that there might exist antigens that cross-react immunologically with soluble HA from *V. cholerae* O1 bacteria, especially as the titers differed significantly between these two geographical areas. The antibodies in the North Americans are difficult to explain but might be the consequence of other vibrios reported to occur in waters along the North American eastern coast (1).

Although the HA antigen used for coating was not absolutely pure (10), it is unlikely that antibodies other than those directed against the soluble HA were assayed by means of the ELISA method. Rabbit sera against cholera toxin and *V. cholerae* lipopolysaccharide lacked significant ELISA titers against the solid-phase-coupled HA in spite of having very high titers against the respective homologous antigen (10). Furthermore, very low ELISA anti-HA titers (<100) were observed in hyperimmune sera against washed whole *V. cholerae* organisms, although these sera contained very high titers against, for instance, lipopolysaccharide (>1,000,000).

Our previous experimental studies in animals showed that injection of purified soluble HA into rabbits regularly gives rise to high antibody titers and, furthermore, that all *V. cholerae* O1 strains hitherto studied produce soluble HA when cultivated in vitro, although in very different amounts (10). An explanation for the poor local as well as systemic immune responses to soluble HA observed in both the experimentally cholera-infected North American volunteers and the Bangladeshi cholera patients might be that during the clinical infection insufficient amounts of soluble HA were produced by the infecting *V. cholerae* strains to evoke more than a marginal antibody response. The production and release of soluble HA vary considerably with the culture conditions. Thus, soluble HA is produced only in the late stationary phase (5, 10), is more effectively produced in shaken than in still cultures (10), and is produced in higher concentrations in tryptic soy broth than in, for instance, synecase medium, brain heart infusion medium, or meat extract media (Svennerholm et al., unpublished data). Although the present results would not rule out a possible role for soluble HA in the pathogenesis of cholera infection, they suggest that in the majority of cholera cases too little soluble HA is produced in vivo to effectively stimulate antibody formation to this per se immunogenic protein. Hence our results do not lend support to antibodies to soluble HA having an important role in the termination of cholera infection or being responsible for the long-lasting protective immunity known to result from such infection.

In conclusion, after clinical infection with *V. cholerae* O1 bacteria, an antibody response to soluble HA seems to occur less frequently and to be of a lesser magnitude than the responses to other putative protective antigens, such as cholera toxin and lipopolysaccharide.

The skillful technical assistance of G. Jonson Strömberg, B.-M. Apelgren, and G. Wiklund is gratefully acknowledged.

Financial support was obtained from the Swedish Medical Research Council (grant no. 16X-3382) and Public Health Service research contract no. IAI 12666 from the National Institute of Allergy and Infectious Diseases.

**LITERATURE CITED**


