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Purification of *Vibrio cholerae* Soluble Hemagglutinin and Development of Enzyme-Linked Immunosorbent Assays for Antigen and Antibody Quantitations

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Soluble hemagglutinin (HA) from an El Tor *Vibrio cholerae* strain (serotype Ogawa) was purified by means of a sequence of salt precipitation, gel filtration, and agarose electrophoresis. The purified material, which gave a single precipitation line in immunodiffusion tests with homologous antiserum, showed immunological identity reactions in double diffusion-in-gel with soluble HA produced by various classical and El Tor strains of different serotypes. Purified HA was used for development of an enzyme-linked immunosorbent assay for titration of specific antibodies against soluble HA and for quantitation of this antigen. Rabbit anti-HA serum reacted in high titer with the soluble HA coated on polystyrene microtiter plates, whereas antiserum against cholera toxin, lipopolysaccharide, or whole washed *V. cholerae* showed little or no reactivity. In inhibition tests as little as 2.5 ng of soluble HA could be detected with the enzyme-linked immunosorbent assay. Culture supernatants of different El Tor as well as classical *V. cholerae* strains all completely inhibited the binding of anti-HA antibody to solid-phase-bound homologous antigen, but the amounts of HA produced by individual strains varied at least 1,000-fold. Only 2 of 10 paired acute- and convalescent-phase sera from Bangladeshi cholera patients showed significant titer increases against soluble HA in parallel titrations.

Vibrio cholerae agglutinates certain species of erythrocytes both by expressing cell-bound hemagglutinins (HAs) and by secreting soluble HA. At least three different types of cell surface HAs have been identified on *V. cholerae*: one that is associated with the classical biotype and inhibited by L-fucose, one that is associated with the El Tor biotype and preferably inhibited by D-mannose, and one that is found in strains of either biotype and not inhibited by either of these sugars (3, 6). In addition, both classical and El Tor vibrios produce a soluble HA which is resistant to mannose as well as to fucose (2). A possible role has been suggested for all of these HAs in the attachment of the vibrios to intestinal cells (2), but little is yet known about their relative importance, if any, in cholera pathogenesis. To assess whether these structures are expressed during clinical infection and whether they give rise to an immune response, suitable assay methods have to be developed. In this study we purified the soluble HA from a *V. cholerae* El Tor strain and developed an enzyme-linked immunosorbent assay (ELISA) that allows sensitive, specific quantitation of soluble HA produced by various *V. cholerae* strains of different bio- and serotypes and that also permits determination of specific antibodies against this antigen.

MATERIALS AND METHODS

Bacteria. *V. cholerae* O17SR Ogawa, biotype El Tor, which was used for purification of HA, was kindly provided by Wanpen Chaicumpa, Bangkok, Thailand. In addition, the classical *V. cholerae* strains 1451 (Ogawa) and 35A3 (Inaba) and the El Tor *V. cholerae* strains 1836 (Inaba) and Phil 6973 (Inaba) were used. All strains were kept freeze-dried until passage on meat extract agar slants (pH 8.5) immediately before use.

Erythrocytes. Erythrocytes were obtained from one of three chickens which had been pretested to have erythrocytes which were readily agglutinated by soluble HA. The erythrocytes were collected in sodium citrate and washed three times in modified Krebs-Ringer solution (KRT buffer; pH 7.4 [8]) before use.

LPS. Purified lipopolysaccharides (LPS) from *V. cholerae* strains 34 (Ogawa) and 569B (Inaba) were prepared by hot phenol-water extraction followed by repeated ultracentrifugation (11). The purified Ogawa and Inaba LPS contained 1.3 and 0.9% (wt/wt) protein, respectively, as determined by a sensitive assay (Bio-Rad Laboratories, Munich, Federal Republic of Germany). A portion of purified Ogawa LPS was treated at 37°C for 72 h with pronase (Sigma Chemical Co., St.

Louis, Mo.) added to a concentration of 0.1 mg of enzyme per mg of LPS and was then boiled for 1 h.

Purification of soluble HA. *V. cholerae* O17SR was grown in four 200-ml stationary flasks, each containing 50 ml of tryptic soy broth (Difco Laboratories, Detroit, Mich.), for 17 h at 37°C. The pooled culture was inoculated in equal volumes into 16 1-liter flasks, each containing 0.5 liter of tryptic soy broth, and incubated on a shaker (type R020; Gerhardt, Bonn, Federal Republic of Germany) at 200 rpm for 17 h at 37°C. The cells were removed by centrifugation twice in the cold at 8,000 × *g* for 30 min, and the supernatant was treated with 50% saturated ammonium sulfate (added as dry powder) at 4°C for 20 h with stirring. The precipitate, sedimented by centrifugation at 11,300 × *g* for 30 min, was dissolved in 20 to 50 ml of KRT buffer and dialyzed against KRT buffer for 2 days. After centrifugation at 10,000 × *g* for 5 min, the soluble fraction was filtered through a 0.45-μm Millipore membrane and then concentrated on a PM10 membrane (American Corp., Lexington, Mass.) to ~25 ml. This "SAS-precipitated HA" was then subjected to gel filtration on a Sephacryl S-300 column (100 by 2.6 cm) (Pharmacia Fine Chemicals, Uppsala, Sweden), using KRT buffer for elution (flow rate, 25 ml/h; fraction volume, 6 ml). The fractions with peak HA activity were pooled, concentrated on a PM10 filter to ~20 ml (to give "S-300 HA"), and lyophilized. The lyophilized material was then dissolved in 2 ml of sterile distilled water, and after centrifugation at 3,000 × *g* for 5 min, 1 ml of the supernatant (5 mg of protein) was subjected to agarose gel electrophoresis essentially as described by Johansson (7). Briefly, 1 ml of the lyophilized, redissolved S-300 HA (about 5 mg of protein) was applied in a 3-mm-wide and 130-mm-long basin cut in a 3-mm-thick 1% agarose gel (110 by 160 mm) (type HSB; Litex, Glostrup, Denmark) in 0.075 M barbitol buffer containing 2 mM calcium lactate (pH 8.6). The electrophoretic separation was carried out at 6 V/cm for 3 h. After the gel was cut into 3-mm-wide strips it was frozen, and each section, after thawing, was eluted by addition of 5 volumes of KRT buffer, homogenization in a glass homogenizer, and centrifugation twice at 10,000 × *g* for 5 min. The supernatants were analyzed for HA activity. The eluates from the two gel strips that contained the highest HA activity were pooled and designated "purified HA."

Preparation of rabbit antisera. Antisera were produced in adult New Zealand white rabbits given three to four subcutaneous injections, 2 weeks apart, with either purified HA (100 μg of protein per injection), purified *V. cholerae* Ogawa LPS (1.25 mg per injection), or purified cholera toxin (Schwarz/Mann, Orangeburg, N.Y.) (30 μg per injection); the initial two injections were given together with Freund complete adjuvant. Antisera to whole *V. cholerae* bacteria were similarly produced by giving rabbits four to six subcutaneous injections with 5×10^9 live, washed, classical (strain 1451) or El Tor (strain 1836) vibrios in each; the bacteria had been grown for 7 h with shaking in tryptic soy broth without glucose. All animals were bled 2 weeks after the last injection, and the sera were stored at −30°C.

Absorption of antisera. Rabbit antiserum against purified HA was extensively absorbed with either untreated or pronase-digested and boiled Ogawa LPS. Absorptions were performed twice by incubating the antiserum at 37°C for 1 h with LPS added to a concentration of 10 mg/ml. The supernatants obtained after centrifugation at 6,000 × *g* for 10 min were used.

Human sera. Acute- and convalescent-phase serum samples were collected from Bangladeshis (1 to 70 years of age) with moderate to severe bacteriologically confirmed clinical cholera. The initial serum specimen was collected at the day of onset of disease, and the second specimen was collected 14 to 28 days later.

Hemagglutination test. The preparations that were tested for HA activity were twofold serially diluted in KRT buffer in polyvinyl microtiter trays (Dynatech Laboratories, Inc., Alexandria, Va.) at 25 μl/well. To each well was then added 25 μl of a 1% chicken erythrocyte suspension prepared as previously described (5). After incubation at room temperature for 1 h, the erythrocyte sedimentation pattern was recorded as negative (no agglutination), weakly positive (partial agglutination), or strongly positive (complete agglutination). Slide agglutination was performed by mixing 15 μl of soluble HA with 15 μl of 3% chicken erythrocytes and then slowly rotating the slide for 3 min. HA inhibition experiments were performed by incubating serial dilutions of HA with a certain concentration of either D-mannose or L-fucose (Sigma) at room temperature for 15 min and then at 4°C for 1 h before addition of the erythrocytes. The inhibitory effect of each sugar was determined by comparing the HA titers obtained in the presence and absence of the sugar.

Immunodiffusion. Immunodiffusion analyses according to the method of Ouchterlony were performed with the microplate modification devised by Wadsworth (13).

SDS-polyacrylamide gel electrophoresis. The different HA preparations were analyzed by means of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The lyophilized preparations were dissolved in duplicate tubes in 0.05 M Tris buffer containing 3% SDS, 0.04 M iodoacetamid, and 8 M urea (pH 8.2) to a final concentration of 2 mg of protein per ml; dithiothreitol was added to one of the tubes to a concentration of 0.05 M. After being heated at 80°C for 30 min and at 100°C for 5 min, the samples were subjected to electrophoresis (9) with 0.1 M Tris (pH 8.2) containing 0.1% SDS as the electrophoretic buffer. Cholera toxin (in the presence and absence of dithiothreitol) and a mixture of other reference proteins (electrophoresis calibration kit, Pharmacia) with known molecular weights were included in each plate.

ELISA. The ELISA procedure previously described for cholera toxin antigen (4) was used, with some modifications. Coating with HA was performed by incubating polystyrene microtiter plates (Dynatech, Plochingen, Federal Republic of Germany) with different concentrations of purified HA dissolved in KRT buffer (0.1 ml per well); different times and temperatures were tested. Coated plates could be stored at 4°C for at least 3 weeks without loss of antigen activity. After the antigen-coated plates were washed twice in phosphate-buffered saline (PBS) (pH 7.2), they were incubated with a 1% bovine serum albumin (BSA)-PBS solution at 37°C for 30 min and then washed twice with PBS containing 0.05% Tween 20.

For titration of antibodies, antisera were fivefold serially diluted in PBS-Tween containing 1% BSA in the antigen-coated plates (0.1 ml per well). After incubation at room temperature for 4 h, the plates

were washed three times in PBS-Tween and then incubated with anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (4) diluted in PBS-Tween-BSA (0.1 ml per well) at room temperature for 18 h. After the plates were washed three times in PBS-Tween, the enzyme substrate nitrophenyl phosphate, diluted in 1 M ethanolamine buffer (pH 9.8) to a final concentration of 1 mg/ml, was added in 0.1-ml volumes to each well. The enzyme-substrate reactions were read spectrophotometrically with a Titertek Multiscan (Flow Laboratories, Helsinki, Finland), at 405 nm; titers were determined as the reciprocal of the interpolated dilutions resulting in an optical density of 0.3 above background when the enzyme was reacted with its substrate for 100 min.

For quantitation of purified HA, the preparations to be assayed for HA were fivefold serially diluted in PBS in uncoated plates. To each dilution was added an equal volume of antiserum against purified HA diluted 1:5,000 (corresponding to 25 times the highest dilution giving a specific ELISA reaction) in PBS. After incubation at 37°C for 1 h, 0.1 ml of each mixture was transferred to washed, HA-coated, BSA-blocked plates, and the incubation was continued at room temperature for another 2 h. After washing in PBS-Tween, the anti-rabbit immunoglobulin G conjugate was added, and the procedure was continued as described above for titration of antibodies.

RESULTS

Purification and properties of HA. Soluble HA was purified by means of a sequence of ammonium sulfate precipitation, gel filtration, and agarose electrophoresis. About four-fifths of the HA activity was lost by the ammonium sulfate precipitation step (Table 1); in other experiments in which the culture supernatant had first been concentrated against polyethylene glycol, the loss in this step could be decreased to ~50% (data not shown). Separation of the precipitated material on a Sephacryl S-300 column resulted in two major protein peaks, the second of which was associated with the HA activity (Fig. 1). The fractions of this HA-containing protein peak were pooled, concentrated on a PM10 membrane, and lyophilized.

Separation by means of agaorse electrophoresis of this material dissolved in a small volume of distilled water resulted in the recovery of most of the applied HA activity in a distinct band close to the basin, referred to as purified HA (Fig. 2). The molecular weight of HA as estimated from the gel filtration rate of the peak HA activity on Sephacryl S-300 appeared to be around 60,000. SDS electrophoresis of the purified HA in the presence as well as the absence of dithiothreitol, on the other hand, revealed one major protein band at a position corresponding to a molecular weight of ~45,000; in addition, a weaker protein band was seen at a position corresponding to a molecular weight of ~15,000 (data not shown).

The purified HA gave a strong single precipitation line in immunodiffusion with rabbit antiserum raised against it but did not react with antiserum against either *V. cholerae* LPS or cholera toxin. Rabbit antiserum against purified HA, however, reacted with boiled, pronase-treated Ogawa LPS in immunodiffusion; this reaction could be eliminated by absorbing the antiserum with Ogawa LPS, without affecting the precipitin titer against the purified HA (Fig. 3).

Whereas the crude or purified HA effectively agglutinated chicken erythrocytes in microtiter trays (~2,000 HA units per mg of protein for the purified HA), no agglutinating activity could be detected in either crude or purified HA preparations (containing up to 1.5 mg of HA per ml) by means of conventional slide agglutination.

The HA activity of the purified HA was fully stable on incubation of the material at 4°C for several months and was also stable for at least 1 week at room temperature or at 37°C. Incubation at 56°C, on the other hand, resulted in the complete loss of activity within 3 h.

The HA activity of purified HA was not affected by incubation at 4°C for 1 h in KRT buffer adjusted to pH 3, 5, or 8. HA incubated in buffers of lower pH (2 to 1) lysed the erythro-

TABLE 1. Purification of soluble HA from *V. cholerae* El Tor

Prepn	HA content		Vol (ml)	Total HA (U)	% Recovery	Protein (mg/ml)	HA (U/mg of protein)
	HA (U/ml)[a]	ELISA (U/ml)[b]					
Culture supernatant	50	625	7,840	392,000	100	0.07	715
SAS-precipitated HA	2,430	40,000	25	60,750	15.5	1.7	1,429
S-300 HA, lyophilized and redissolved	7,290	NT[c]	2	14,580	3.7	5.1	1,429
Agarose electrophoresis-purified HA	270	3,780	24	6,480	1.7	0.14	1,929

[a] Defined as the highest reciprocal dilution giving rise to strong agglutination of chicken erythrocytes.
[b] Defined as the reciprocal dilution resulting in 50% inhibition of binding of anti-HA serum (diluted 1:5,000) to solid-phase-bound HA in the ELISA.
[c] NT, Not tested.

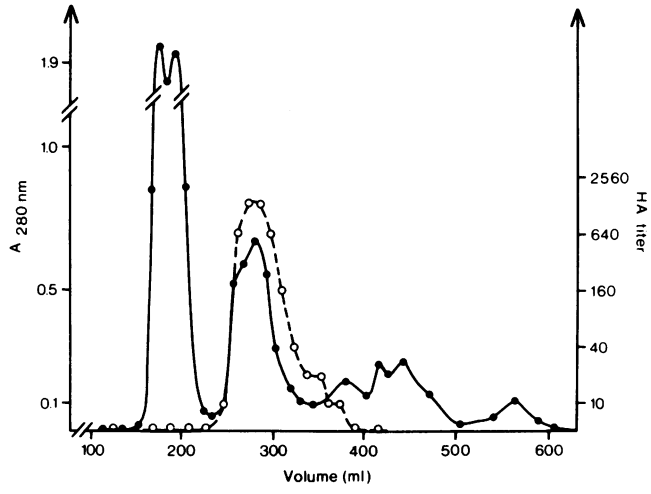

FIG. 1. Gel filtration of SAS-precipitated HA (ammonium sulfate precipitate of HA from strain O17SR) on a Sephacryl S-300 column (100 by 2.6 cm). A 20-ml portion of the sample was applied and chromatographed in KRT buffer (flow rate, 25 ml/h; fraction volume, 6 ml), and fractions were analyzed for protein content (●) and HA activity (○).

cytes, and incubation at higher pH (9 to 10) resulted in two- to fourfold-higher agglutinating activity than obtained for the HA stored in KRT buffer (pH 7.4).

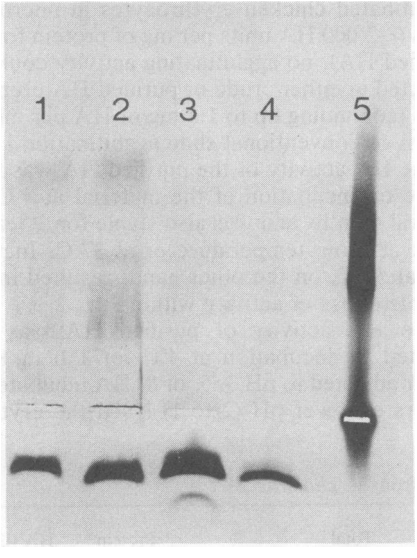

FIG. 2. Qualitative agarose electrophoresis of concentrated culture supernatant from *V. cholerae* O17SR (lane 1), SAS-precipitated HA (ammonium sulfate-precipitated and lyophilized culture supernatant) (lane 2), S-300 HA (pooled HA containing fractions obtained by gel filtration and concentrated on a PM10 membrane) (lane 3), purified HA obtained by preparative agarose electrophoresis of S-300 HA (lane 4), and normal human serum diluted 1/3 (lane 5). Samples were applied in 10-μl volumes; lanes 1 through 4 contained 4 to 7 mg of protein per ml. Electrophoresis was done with 0.075 M barbital buffer (pH 8.6) at 13.5 V/cm for 90 min.

Neither D-mannose nor L-fucose in final concentrations of 0.01 to 1% was capable of inhibiting the HA activity of purified HA in microtiter plates.

ELISA. We developed an ELISA for the determination of antibodies against *V. cholerae* soluble HA which also allowed quantitation of specific HA antigen. The optimal concentration for coating of the microtiter plates was about 0.3 μg of soluble HA per ml (Fig. 4). Coating with 0.01 to 50 μg of HA per ml did not result in binding of hyperimmune serum against Ogawa LPS, anti-cholera toxin, or normal rabbit serum (Fig. 4). Plates coated with HA at room temperature (~22°C) overnight gave higher titers than plates coated at 4°C or at 37°C for 18 h. However, due to a tendency for more nonspecific reactions with normal rabbit serum with coating at room temperature, we chose coating at 4°C overnight.

The specificity of the HA-ELISA was tested

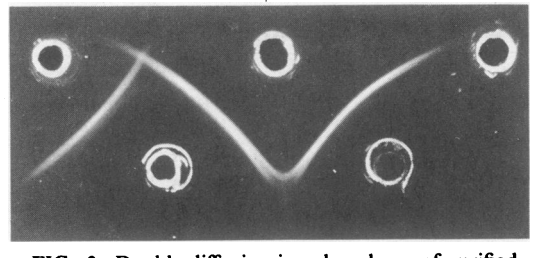

FIG. 3. Double diffusion-in-gel analyses of purified HA from strain O17SR (upper central well) and pronase- and heat-treated purified anti-*V. cholerae* Ogawa LPS (upper left and right wells), using antiserum to purified HA from strain O17SR (lower left well) and the same antiserum absorbed with pronase- and heat-treated specific Ogawa LPS (lower right well).

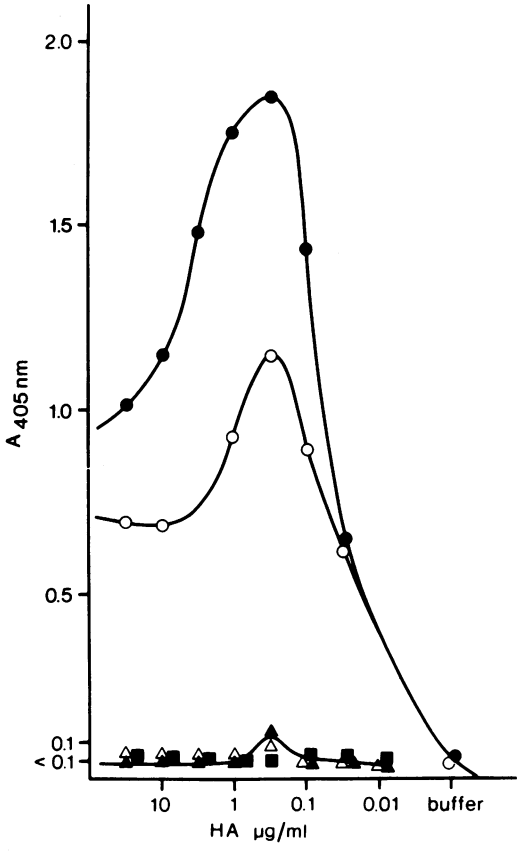

FIG. 4. Determination of optimal concentration for coating with purified HA from strain O17SR. Coating was performed at 4°C for 18 h. Rabbit antiserum against purified HA from strain O17SR (diluted 1/100 [●] and 1/10,000 [○]), purified Ogawa LPS (▲), purified cholera toxin (■) and normal rabbit serum (△) diluted 1/100 were assayed with anti-rabbit immunoglobulin G-alkaline phosphatase conjugate.

by titrating rabbit antisera of different specificities in plates coated according to the standard procedure (Fig. 5). Whereas the antiserum against soluble HA reacted in high titer (1:125,000) with the solid-phase HA antigen, antiserum against purified *V. cholerae* Ogawa LPS, cholera toxin, or normal rabbit serum had no or very low titer (≥30) against soluble HA. Very low anti-HA titers were also observed in hyperimmune serum against washed classical or El Tor vibrios, although these sera contained very high anti-LPS titers (≥1:1,000,000).

For quantitation of soluble HA, we developed an ELISA inhibition test. By this method the capacity to inhibit the binding of HA antibodies to solid-phase-bound HA was tested by incubating anti-HA serum with different concentrations of the sample before addition to the plates (Fig. 6). When using the anti-HA serum diluted 1:5,000, corresponding to 25 times the lowest concentration giving a specific reaction in ELISA, as little as 2.5 ng of soluble HA (25 ng/ml) inhibited antibody binding by 50% (Fig. 6). The use of lower serum concentrations resulted in a somewhat higher sensitivity but gave inhibition curves too flat to permit reproducible determination of 50% inhibitory concentration values. No inhibition of the anti-HA serum was obtained when testing concentrations of *V. cholerae* LPS or cholera toxin that were 20,000-fold higher than those of HA (Fig. 6). A purified preparation of soluble HA from an El Tor Inaba strain (T19479) had equally good inhibitory activity per milligram of protein as did HA purified from the El Tor Ogawa strain O17SR.

Significant inhibition was also obtained when the anti-HA serum was mixed with concentrated supernatants of 18-h cultures of other El Tor, as well as classical, *V. cholerae* strains of both Inaba and Ogawa serotypes (Table 2). All culture supernatants tested were capable of completely inhibiting the ELISA reactivity of the anti-HA serum dilution used (1:5,000), but their inhibitory capacity varied more than 1,000-fold (Table 2).

Comparative immunodiffusion analyses of concentrated supernatants from *V. cholerae* strains of different serotypes and biotypes re-

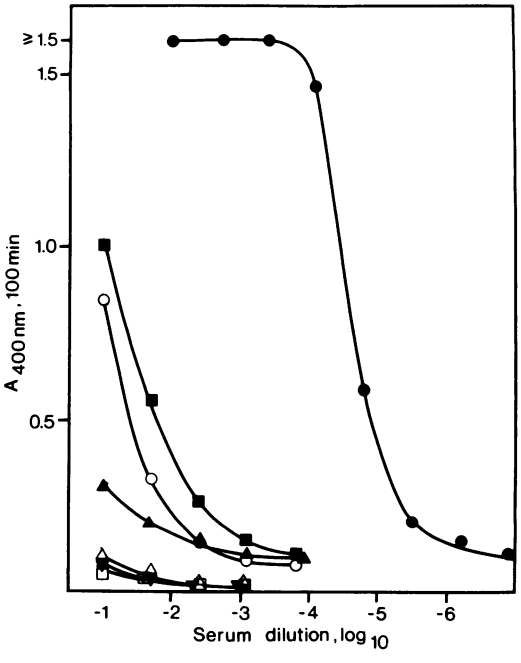

FIG. 5. ELISA titration curves of rabbit antiserum against soluble HA from strain O17SR (●), live classical vibrios (■), live El Tor vibrios (▲), purified *V. cholerae* LPS (○), cholera toxin (△), formalin-treated El Tor vibrios (▼), and normal rabbit serum (□). Titers were determined as the interpolated dilutions giving an absorbance of 0.3 above background.

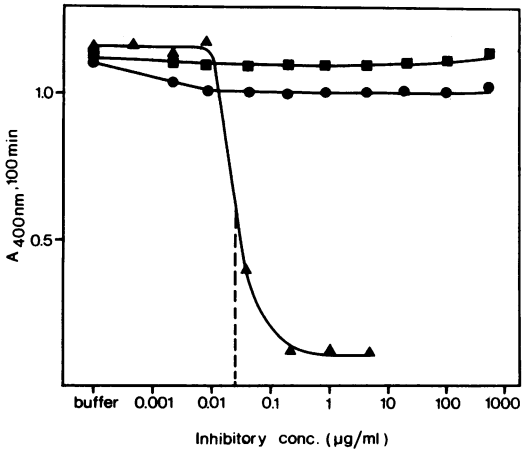

FIG. 6. Quantitative ELISA inhibition of a rabbit antiserum against soluble HA (diluted 1:5,000) with purified soluble HA (▲), purified Ogawa LPS (■), and cholera toxin (●) with soluble HA as solid-phase antigen.

vealed that the various strains produced immunologically closely related or identical soluble HA. Thus, there were identity reactions between the purified HA from strain O17SR (El Tor Ogawa) and soluble HA in the supernatants of strains Phil 6973, T19479 (El Tor Inaba), and 35A3 (classical Inaba) with LPS-absorbed anti-HA serum.

Titration of 10 paired acute- and convalescent-phase sera collected from Bangladeshi cholera patients at the day of onset of disease and 14 to 28 days later revealed a significant (>twofold) titer increase to *V. cholerae* soluble HA in only 2 of the patients.

DISCUSSION

Soluble HA from *V. cholerae* was purified, which enabled the development of an ELISA for specific quantitation of soluble HA as well as for titration of specific antibodies against this antigen.

The purified HA gave a single precipitation line in immunodiffusion with homologous hyperimmune sera and migrated as a single protein band in agarose electrophoresis. However, the material was not absolutely pure, since antiserum raised against it contained antibodies which precipitated with homologous LPS in immunodiffusion and reacted with LPS in the ELISA (4). These reactions were not due to low levels of soluble HA in the LPS preparations, because the antiserum precipitated boiled and pronase-treated LPS equally as well as it did the native LPS, and there was no sign of immunological relatedness (10) between the precipitates formed in double diffusion-in-gel of soluble HA and purified LPS, using the anti-HA serum.

The soluble HA purified from *V. cholerae* El Tor (strain O17SR) has similarities with reported properties of the soluble HA purified from classical vibrios by Finkelstein and Hanne (2) and by Chaicumpa et al. (*in Enteric Infections in Man and Animals*, in press). Thus, all of these preparations seem to be proteins with molecular weights between 30,000 and 60,000 which are precipitable with ammonium sulfate and mediate hemagglutination reactions that are not inhibited by either mannose or fucose (2; Chaicumpa et al., in press). However, our material was more resistant to incubation at room temperature or at 37°C, and the yield of active material was higher than described for the soluble HA purified from classical vibrios (2).

The finding in comparative immunodiffusion analyses that the soluble HA purified from strain 017SR and soluble HA in supernatants from different classical and El Tor strains showed identity reactions suggests that classical and El Tor vibrios may produce immunologically identical or closely related soluble HAs.

Although the purified HA gave strong hemagglutination in microtiter plates, it failed to agglutinate chicken erythrocytes on glass slides. The reason for this discrepancy might be that relatively weak interactions are formed between the purified HA and the chicken erythrocytes; these interactions may be broken by the rotation movement of the slides, but they remain when the reactants are left still in the plates.

The purification procedure, which included salt precipitation, gel filtration, and agarose electrophoresis, resulted in a HA preparation of sufficient purity to allow development of an ELISA. A low concentration of HA, 0.3 µg/ml, was optimal for coating. The decrease in antibody binding with higher antigen concentrations

TABLE 2. HA inhibitory activity of different classical and El Tor *V. cholerae* culture supernatants[a]

Strain	Biotype	Serotype	Inhibitory titer[b]
T19479	El Tor	Inaba	1:12,500
Phil 6973	El Tor	Inaba	1:160
1836	El Tor	Inaba	1:5
O17SR	El Tor	Ogawa	1:18,000
35	Classical	Inaba	1:80
1451	Classical	Ogawa	1:8

[a] Overnight cultures were in tryptic soy broth without glucose; the supernatants obtained by centrifugation at 8,000 × *g* for 30 min and concentrated 50- to 100-fold in dialysis bags against polyethylene glycol were tested.

[b] The dilution resulting in 50% inhibition of binding of anti-HA serum (diluted 1:5,000) to solid-phase-bound HA in the ELISA.

for coating was probably due to release of solid-phase-bound antigen during incubation with antibodies. The HA-coated ELISA plates were blocked with a high concentration of BSA before use to avoid nonspecific binding to possible remaining binding sites on the plastic surface (12); exclusion of this blocking step had no effect on the binding of specific antibodies but gave rise to a higher background staining due to some nonspecific binding of unrelated antisera and of the conjugate.

The ELISA method allows accurate, sensitive quantitation of soluble HA. In titration experiments as little as 2.5 ng of soluble HA was demonstrated, and the specificity of the determinations was high since neither *V. cholerae* LPS nor cholera toxin nor cell-bound HAs had any inhibitory activity in this system.

ELISA tests showed that soluble HA from a number of different classical and El Tor *V. cholerae* strains could completely inhibit the binding of anti-HA antibody to solid-phase-bound homologous HA, supporting the strong or complete immunological cross-reactivity between HA from various strains. Although all *V. cholerae* strains hitherto analyzed seem to produce a common, soluble HA, there are marked differences in the amount produced by individual strains. Thus, comparable overnight cultures of various classical and El Tor strains contained between 1 and 3,000 ng of soluble HA per ml. In contrast, a certain strain varied only little in its production of soluble HA on repeated cultivations.

The soluble HA appeared to be immunologically unrelated to the cell-bound HAs described for *V. cholerae* (1, 3, 6). Thus, several bacterial strains that after repeated washing retained strong hemagglutinating capacity could not inhibit the anti-soluble HA serum from binding to solid-phase-bound soluble HA. This was seen with classical *V. cholerae* strains that were effective in agglutinating erythrocytes in a fucose-sensitive manner and with El Tor strains that showed mannose-sensitive or mannose- and fucose-resistant hemagglutination patterns (3, 6). Furthermore, antisera raised against whole washed El Tor or classical vibrios with high cell-bound HA activity did not react significantly with soluble HA in the ELISA.

The HA ELISA permitted sensitive demonstration of antibodies to soluble HA, with high specificity. Thus, whereas preimmune sera only reacted in titers of $\geq$1:30, the anti-HA serum had a titer of <1:100,000. The HA ELISA developed might also be used for analyses of local and systemic responses to soluble HA in humans, e.g. after clinical or asymptomatic cholera infection. A study is now being carried out (Svennerholm et al., manuscript in preparation) to determine anti-HA antibody levels in intestinal lavage and serum specimens collected from healthy individuals living in an area (Bangladesh) endemic for cholera and from convalescents from clinical cholera at different intervals after onset of disease. The preliminary results from that trial suggest that clinical cholera in an endemic area rarely induces significant titer rises against *V. cholerae* soluble HA in serum. Whether the poor anti-HA response observed in the Bangladeshi cholera convalescents was due to infection with strains producing low levels of soluble HA, to poor release of soluble HA in vivo, or to poor systemic immunogenicity of soluble HA in the intestine remains to be clarified in more extensive studies.

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