AN ERYTHROCYTE-SENSITISING ANTIGEN FROM *VIBRIO CHOLERAE*

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THE ABILITY of crude suspensions of *Vibrio cholerae* to sensitise sheep erythrocytes has been reported (Felsenfeld, Freeman and Mooring, 1955; Barua and Sack, 1964). Soluble cell-wall suspensions and intracellular substances have likewise been utilised for indirect haemagglutination (IHA) tests with tanned RBC (Burrows and Schlub, 1958). A phenol-extracted polysaccharide fraction from vibrios has been used to sensitise RBC but with variable results (Gallut, 1960). More recently, a suspension of vibrios of Ogawa and Inaba serotypes that had been disintegrated ultrasonically has been used to detect coproantibodies and serum antibodies in immunised rabbits by means of IHA tests (Agarwal and Ganguly, 1972). This communication reports the successful use of an alkali extract (AE) from strains of *V. cholerae*, probably a polysaccharide complex, in which the active principle acts as a sensitising agent in IHA tests. The simplicity of the method of extraction makes it an attractive tool for use in future investigations of the serology of *V. cholerae*.

MATERIALS AND METHODS

Strains. The *V. cholerae* cultures, of Inaba (no. 9459), Ogawa (no. 9458), and Hikojima (no. 11,558) serotypes, and of the El-Tor biotype (no. 14,033), were obtained from the American Type Culture Collection. The *Escherichia coli* and *Salmonella enteritidis* strains were locally isolated.

Preparation of the antigenic extracts

Alkali extract (AE). The organisms were grown in Roux bottles containing 130 ml of nutrient agar, pH 8.0–8.2, for 18–24 h at 37°C and then harvested and washed three times with physiological saline. After the last washing the sediment from each Roux bottle was suspended in 20 ml of physiological saline and then digested with an equal volume of 0.5N NaOH in a water bath at 56°C for 5–6 h. It was then centrifuged at 2500 r.p.m. for 5 min., and the pH of the supernate was adjusted to 7.0–7.2 by the addition of 1.0N HCl. The soluble bacterial substance was then precipitated by the addition of 5 volumes of absolute alcohol and stored overnight at 4°C. The precipitate was collected by centrifugation, washed three times with acetone, and vacuum dried. In other experiments, the collected precipitate was dissolved in a minimum volume of distilled water and dialysed against distilled water for 18–24 h in the cold. The dialysed material was then centrifuged at 100,000 g in an analytic (Model L, Spinco) ultracentrifuge for 1 h. The supernate (100S60) and the sediment (100P60) were dialysed again and lyophilised separately.

The AE was also fractionated by column chromatography on Sephadex G-200 (Pharmacia, Sweden) and eluted with phosphate buffer (0.01M, pH 7.2). The fractions were

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dialysed against distilled water in the cold for 48 h with frequent changes of water, and lyophilised.

Enzyme-treated extract. To study the effect of enzymes on the antigen, portions of the Inaba AE (2 mg per ml) were treated separately with trypsin (0.2 mg per ml, pH 8.2) for 5 h at 37°C and with ribonuclease (RNAase) (0.2 mg per ml, pH 7.4) for 3 hr at 37°C. The enzymatically-treated antigens were then dialysed against distilled water for 18 h in the cold and lyophilised. These antigens together with control AE were tested by IHA against homotypic and heterotypic immune sera.

Preparation of antisera

Twenty-four-hour-old cultures of *V. cholerae* on agar slants were washed twice with physiological saline and the suspension was boiled for 2 h in a water bath to destroy the H (flagellar) antigen. The bacterial suspension from each *V. cholerae* strain was diluted with physiological saline to a density of 10^{10} cells per ml.

For immunisation of rabbits, a 3-week schedule was followed. A first dose of 1.5 ml of vaccine was injected into the foot-pads and the nuchal areas of the animals. Thereafter, at weekly intervals, the animals were given 1-ml booster doses of vaccine subcutaneously in the nuchal areas. A week after the last injection, the animals showing titres of 640 or over in agglutination tests with homotypic antigen were bled to death and the sera collected, pooled and stored at -20°C.

Antisera against *E. coli* and *S. enteritidis* were obtained from Difco laboratories. Polyvalent cholera antiserum was obtained from the Wellcome Research Laboratories.

Serological tests

Antigen for agglutination tests was prepared by washing 24-h-old agar cultures of *V. cholerae* with physiological saline. The suspension was heated for 2 h in a water bath at 100°C and standardised to contain $10^{9.2}$ organisms per ml. Agglutination tests were performed with serial dilutions of serum (volume 0.5 ml) in small tubes and equal volumes of antigen. The tubes were then placed in a water bath at 56°C for 2 h, followed by incubation at 37°C for 18–24 h. The readings were taken by means of an agglutinoscope.

For the IHA test, fresh human group-O blood, 20 ml, was mixed with 20 ml of modified Alsever's solution (Bukantz, Rein and Kent, 1946). This was stored at 4°C and was used within 9 days. A portion of the blood was centrifuged and the RBC were washed with 10 ml of physiological saline. This was repeated three times. Sensitisation of RBC was carried out by mixing nine parts of antigen (w/v), containing 2 mg per ml of bacterial extract in 1 ml

TABLE I
Titration of the Vibrio cholerae (Ogawa) AE against polyvalent V. cholerae rabbit antiserum in indirect haemagglutination tests*

Concentrations of the AE used for sensitisation of erythrocytes (mg per ml)	Titres of haemagglutination by <i>V. cholerae</i> polyvalent rabbit antiserum
6	640
4	640
2	640
1	320
0.5	320
0.25	160
0.125	10

* Material extracted with alkali and precipitated with alcohol (see text).

of physiological saline, with one part of packed RBC. The mixture was incubated in a water bath at 37°C for 2 h with occasional shaking. The RBC were then centrifuged and washed once with physiological saline and a 0.5% suspension prepared in the same solution.

The IHA tests were performed in round-bottomed test tubes (100×13 mm). Serum diluted 1 in 10 in physiological saline was placed in a water bath at 58–60°C for 20 min., and then used to prepare doubling dilutions of 0.25-ml volume in physiological saline. Next, 0.25 ml of the sensitised erythrocytes was added to each of the tubes. Control tubes of sensitised and of normal RBC, but without serum, were also prepared. The tubes were shaken and left at room temperature for 1 h, after which readings were taken according to the pattern of settled RBC. In positive tests, the RBC formed clumps and settled in an irregular pattern at the bottom of the tube. In negative tests they settled evenly assuming a button-like appearance.

RESULTS

The concentrations of the AE from the *V. cholerae* capable of sensitising erythrocytes are shown in table I. An antigen concentration of 2 mg per ml was found to be the smallest amount giving maximum titres in the test, and therefore this concentration was used in subsequent determinations.

The results of bacterial agglutination and of IHA tests are presented in table II. High titres were obtained when the *V. cholerae* serotypes (Ogawa, Inaba, Hikojima) and *E. coli*, and *S. enteritidis* were tested against their homotypic antisera. Contrariwise, low titres or titres of less than 10 were obtained when they were tested with heterotypic immune sera. The titres of the IHA tests were in a number of cases somewhat higher and more specific than those of bacterial agglutination tests. It was apparent from these results that the El-Tor biotype used in these tests belonged to the Inaba serotype.

Table III shows a comparison of the sensitivity and the specificity of the AE, 100P60 and 100S60 antigens in IHA tests. The El-Tor vibrios, *E. coli*

TABLE II
Titres of *Vibrio cholerae*, El-Tor vibrio, *Escherichia coli* and *Salmonella enteritidis* rabbit antisera in agglutination and indirect haemagglutination tests

Bacterial strain	Titre of antiserum prepared against											
	<i>V. cholerae</i> (Ogawa)		<i>V. cholerae</i> (Inaba)		<i>V. cholerae</i> (Hikojima)		El-Tor vibrio		<i>E. coli</i> †		<i>S. enteritidis</i> ‡	
	Aggl.*	IHA†	Aggl.	IHA	Aggl.	IHA	Aggl.	IHA	Aggl.	IHA	Aggl.	IHA
<i>V. cholerae</i> (Ogawa)	320	640	320	20	160	640	320	20	<10	<10	<10	<10
<i>V. cholerae</i> (Inaba)	40	40	640	1280	160	40	640	1280	<10	<10	<10	<10
<i>V. cholerae</i> (Hikojima)	160	20	80	<10	640	1280	160	<10	<10	<10	<10	<10
El-Tor vibrio	80	40	640	1280	80	20	640	1280	<10	<10	<10	<10
<i>E. coli</i>	<10	<10	<10	<10	<10	<10	<10	<10	640	320	<10	<10
<i>S. enteritidis</i>	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	320	1280

* Obtained in bacterial agglutination test.

† Obtained in indirect haemagglutination test with AE (see footnote to Table I)

‡ Polyvalent O antisera.

TABLE III
Titres in indirect haemagglutination (IHA) tests with various antigenic preparations of Vibrio cholerae

Antiserum	Titre of IHA by the stated antiserum of RBC sensitised with extract								
	AE* of strain			100P60† of strain			100S60‡ of strain		
	Ogawa	Inaba	Hikojima	Ogawa	Inaba	Hikojima	Ogawa	Inaba	Hikojima
Ogawa	640	40	20	80	20	40	640	40	40
Inaba	20	1280	<10	<10	640	<10	<10	2560	<10
Hikojima	640	40	1280	320	<10	640	1280	<10	2560
El-Tor	20	1280	<10	<10	640	<10	<10	2560	<10
<i>E. coli</i>	<10	<10	<10	<10	<10	<10	<10	<10	<10
<i>S. enteritidis</i>	<10	<10	<10	<10	<10	<10	<10	<10	<10

* See footnote to Table I.

† Pellet after centrifuging AE at 100,000 g.

‡ Supernatant after centrifuging AE at 100,000 g.

and *S. enteritidis* are also included in the test. The 100S60 fraction gave a slightly higher titre with the homotypic antiserum than did the AE or the 100P60 antigen. Likewise the cross reactivities between various serotypes were at their minimum when the 100S60 fraction was used as antigen in IHA tests. The non-reactivity of *E. coli* and *S. enteritidis* antisera with *V. cholera* extracts and the high titres of El-Tor antiserum obtained in reactions with Inaba antigens are to be noted.

Fig 1 presents the elution pattern of the crude AE of *V. cholerae* Inaba serotype on Sephadex G-200 with phosphate buffer, pH 7.2, as eluant. Three fractions were obtained. The sensitivity and specificity of the three fractions were determined in IHA tests (table IV). Fraction II gave a titre of 640 both with homotypic and the El-Tor antisera and a titre of less than 10 with other

FIG. 1.—Elution profile of the AE of *Vibrio cholerae* (Inaba) on Sephadex G-200 at 280 nm (—) and 260 nm (---).

TABLE IV

Titres in indirect haemagglutination (IHA) tests of Vibrio cholerae (Inaba) antigenic fractions (I, II and III) obtained by column chromatography on Sephadex G-200

Antiserum	Titre of IHA by the stated antiserum of RBC sensitised with			
	AE*	Fraction I	Fraction II	Fraction III
Ogawa	40	<10	<10	<10
Inaba	1280	40	640	<10
Hikojima	40	<10	<10	<10
El-Tor	1280	320	640	<10

* See footnote to Table I.

cholera antisera. Fraction I gave a titre of 40 with its homotypic antiserum and 1 : 320 with the El-Tor antiserum. The titres with fraction III were less than 10 with all sera tested.

A comparison of spectrophotometric absorption patterns of the AE, 100P60 and 100S60 fractions of *V. cholerae* Inaba serotype, is presented in fig. 2. Absorption at 260 nm shows a hump that may be indicative of the presence of nucleic acid. The Inaba AE antigen treated with RNAase showed a marked decrease in its nucleic-acid content as shown in fig. 3A, without losing to any significant extent its sensitivity and specificity in IHA tests (table V). The AE, 100P60 and 100S60 fractions were Molisch-positive.

DISCUSSION

It is apparent from the results that *V. cholerae* extracts used in the present investigations were not free from non-specific components, yet they generally yielded somewhat more specific results in IHA tests than did bacterial suspensions in agglutination tests (table II). The extracts probably contained more than one antigen and this may explain the relative cross reactivities between the various preparations. The results of IHA tests indicate that each sensitising

TABLE V

Effect of ribonuclease and trypsin treatment on AE antigen of Vibrio cholerae (Inaba)*

Antiserum	Titre of indirect haemagglutination by the stated antiserum of RBC sensitised with AE antigen (2 mg per ml)			
	Untreated	Ribonuclease-treated (0.2 mg per ml)	Untreated	Trypsin-treated (0.2 mg per ml)
Ogawa	<10	<10	<10	80
Inaba	640	320	640	1280
Hikojima	<10	<10	<10	160
El-Tor	640	1280	320	1280

* See footnote to Table I.

FIG. 2.—Absorption spectra of the AE ($\blacktriangle$ — $\blacktriangle$), and of fractions 100S60 ($\bullet$ — $\bullet$) and 100P60 ($\circ$ — $\circ$), obtained from *Vibrio cholerae* (Inaba).

antigen extracted from a particular serotype of *V. cholerae* contained a major antigenic component for the homotypic strain and a minor component for the heterotypic one. Nevertheless, the *V. cholerae* extracts showed no cross-reactivity with *E. coli* and *S. enteritidis* extracts. The results of IHA were uniform and reproducible usually within one tube difference. Erythrocytes of both human and sheep origin could be used.

The results in table III indicate that dialysis and ultracentrifugation of the AE somewhat improved its specificity. Dialysis was used to eliminate from the extract soluble salts, breakdown products and low-molecular-weight antigenic components. The presence of such substances in the sensitising antigen might have influenced adversely the results of the IHA tests.

Of the three AE fractions obtained by Sephadex chromatography (table IV and fig. 2), fraction II gave titres closest to those obtained with the crude AE antigen, indicating that most of the serologically active substance was found in this fraction. The trypsinised Inaba AE antigen showed a significant increase in titres in IHA tests with Ogawa and Hikojima antisera (table V). This may be important, in that trypsin was probably uncoating some of the surface layers of the antigen and thus exposing the deeper antigens which apparently were less specific in their reactivity. However, when the same antigen was treated with RNAase, no significant change in IHA tests were noted despite a noticeable loss in nucleic acid content (fig. 3, A). The inability of trypsin to remove protein from the extracts, the maintenance of the titres in IHA after RNAase treatment, the ability of the extracts to sensitise untanned erythrocytes, and the positive reactions in Molisch tests of the AE, 100P60 and 100S60 fractions, strongly suggest that the antigenic determinant in IHA tests was a carbohydrate complex.

We suggest that the AE is an active antigenic component of *V. cholerae*, perhaps more specific than the whole-cell antigen. It remains to be seen if this antigen could be used also for immunisation purposes.

FIG. 3.—Absorption spectra of RNAase- and trypsin-treated AE of *Vibrio cholerae* (Inaba): A (left), ●—● = AE; ○—○ = RNAase-treated AE; B (right), ●—● = AE, ○—○ = trypsin-treated AE, ▲—▲ = control protein, △—△ = trypsin-treated control protein.

SUMMARY

Erythrocyte-sensitising antigens (AE) were prepared from *Vibrio cholerae* serotypes, from El-Tor vibrio, *Escherichia coli* and *Salmonella enteritidis* by digesting the organisms with NaOH followed by precipitation with alcohol. When AE was used in indirect haemagglutination (IHA) tests, the results in a number of cases were somewhat more sensitive and more specific than those obtained in classical agglutination tests. No cross reactions occurred between *V. cholerae* serotypes and *E. coli* and *S. enteritidis*. Much of the reactive part of the AE was not sedimentable at 100,000 *g* for 1 h. The eluant from the *V. cholerae* AE on Sephadex G-200 yielded three fractions, one of which was the most active in IHA tests. Treatment of the AE with trypsin resulted in an appreciable increase in the heterotypic serum titres in IHA tests. The spectrophotometric absorption of the AE at 260 nm showed a hump that may have been indicative of the presence of nucleic acid. Treatment of the antigen with ribonuclease reduced its nucleic acid content but did not change to any significant extent the reactivity of the preparation. The AE antigen of *V. cholerae* was Molisch-positive and was capable of sensitising untanned erythrocytes in IHA tests. It is suggested that the reactive part of the AE antigen is a carbohydrate complex.

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