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Corresponding type-specificity of vibriocidal and agglutinating activities of *Vibrio cholerae* antisera: relevance to vaccine immunogenicity

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SUMMARY

Cholera vibrios can be allocated to one of three biotypes (classical, intermediate and El Tor), each of which can be sub-divided into two serotypes (Ogawa and Inaba). Vibriocidal tests with absorbed antisera have shown no evidence of biotype specificity in the killing of bacteria, but they have confirmed the role of the two serotype-specific antigens in immunity to cholera. The same presence of serotype specificity, and absence of biotype specificity, has been found by bacterial agglutination, an easier and quicker serological test. The use of this simpler test in ensuring a balanced serotype response to cholera vaccine is discussed, together with evidence that may lead to the production of more effective vaccine and better immunity.

INTRODUCTION

Interest in cholera has been intensified recently by the occurrence of epidemics in Latin America constituting 72% of the world's reported cases [1]. Whereas earlier pandemics have originated in Bengal and have been caused by the classical (CS) biotype of cholera vibrio, the current (seventh) pandemic began in south-east Asia in 1961 and spread gradually during the next decade across India and the Middle East into Mediterranean countries and most parts of Africa [2]. Isolates from this pandemic have usually been reported as of El Tor (ET) biotype – differing from the CS biotype in sensitivity to polymyxin B, in susceptibility to a specific CS phage, and in the Voges–Proskauer (VP) test for acetylmethylcarbinol. However, they can be distinguished from strains of the ET biotype by pyrolysis gas–liquid chromatography [3] and by their inability to produce the persistent soluble haemolysin demonstrable in the Greig test, so that they constitute an intermediate (IM) biotype [4]. Indeed, this haemolysin-negative property is a useful marker by which strains of the current pandemic (IM biotype) can be distinguished readily from those of the ET biotype [4, 5].

Regardless of biotype, strains of cholera vibrio can be typed with antiserum into one of two serotypes, Ogawa and Inaba; and such sera have been shown to produce serotype-specific killing of the bacteria in a vibriocidal test [6]. It has been known for many years that vibriocidal titres of human sera correlate with immunity in family contacts of cholera patients [7], and that such immunity is

serotype-specific [8]. Accordingly, parenteral vaccine has contained equal numbers of killed Ogawa and Inaba vibrios, of CS biotype; but it has given rather poor protection (*c.* 50% efficacy) lasting only *c.* 6 months. However, the individual serotype-specific antibody responses have rarely been studied. Rather, the current pandemic has been met, empirically and unsuccessfully, by incorporation of either an additional ET biotype component in the vaccine, or cholera toxoid, but with a continuing lack of investigation of the serotype-specific responses [9–11].

The aim of the present study was to investigate the possible existence of biotype-specific immunity, by use of the vibriocidal test, and to compare the vibriocidal test with simple bacterial agglutination as indicators of serotype- and biotype-specific immunity.

MATERIALS AND METHODS

Strains of Vibrio cholerae

Six strains of curved Gram-negative rod were vigorously motile, oxidase-positive, arginine-negative, non-halophilic and anaerogenic; they fermented sucrose and mannose but not arabinose, they gave a positive result in the cholera-red test, and they were agglutinated by *V. cholerae* O1 antiserum. Two were of CS biotype (sensitive to polymyxin B and phage IV, VP-negative, haemolysin-negative): Ogawa serotype from a patient in Calcutta, Inaba serotype from Glaxo Laboratories. Two were of ET biotype (resistant to polymyxin B and phage IV, VP-positive, haemolysin-positive): Ogawa from a fish in Calcutta, Inaba from the Manchester University Collection of Bacteria. Two were of IM biotype, from the current pandemic (resistant to polymyxin B and phage IV, VP-positive, haemolysin-negative): Ogawa from Nigeria, Inaba from Tehran.

Antisera

Unabsorbed antiserum against each of the six strains of vibrio was available from previous investigations. The sera had been raised in rabbits against suspensions of bacteria (8×10^9/ml) inactivated with merthiolate 0·01%, by a procedure described in detail for the preparation of pertussis antisera [12].

Each serum was absorbed with a dense suspension of merthiolate-killed heterologous organisms, as indicated below, until the latter no longer reacted with the absorbed serum. Details of the absorption procedure have been published previously [12].

Vibriocidal test

Suspensions of live bacteria were tested, in the presence of complement, against serial dilutions of antiserum, and with serum- and complement-controls. The tests were performed in plastic microtitration plates with U cups, as described in detail previously [6].

Bacterial agglutination

Serial dilutions of antiserum were shaken mechanically with bacteria in rows of cups (16 mm diameter) in a plastic tray, before microscopic examination for bacterial clumps. Full details of this rapid test, which gave a result in *c.* 1 h, have

Table 1. *Vibriocidal titres of Ogawa antisera (each absorbed with a strain of heterologous serotype and heterologous biotype) when tested against Ogawa strains of homologous and heterologous biotype*

Serotype and biotype of strain of vibrio			Vibriocidal titre* against Ogawa strain of		
Against which antiserum had been raised	With which the serum was absorbed	Intended specificity of absorbed serum	CS† biotype	IM biotype	ET biotype
Og CS	In ET	Og CS	1600	2300	800
Og IM	In ET	Og IM	570	1600	140
Og ET	In IM	Og ET	4500	9100	400

* Geometric mean of results from duplicate tests, in which each pair of results showed no more than twofold variation.

† Og, Ogawa; In, Inaba serotype; CS, classical; IM, intermediate; ET, El Tor biotype.

been published previously [12]. The end-points were easy to read and the titres were reproducible, with no more than twofold variation, though they were somewhat lower than those obtained with overnight methods. Bacterial suspensions were inactivated with merthiolate 0·01%, preliminary tests having shown that such vibrios gave slightly more obvious agglutination than those killed with formalin, azide, or heat and phenol.

RESULTS

Vibriocidal activity of antisera absorbed with heterologous organisms

Antisera to Ogawa organisms of CS, IM or ET biotype were absorbed with Inaba organisms of heterologous biotype, so that they would have Ogawa serotype specificity together with the possibility of a superimposed biotype specificity. The vibriocidal titres of the absorbed sera against Ogawa organisms of homologous and heterologous biotype (Table 1) showed no evidence of increased activity against organisms of homologous biotype – no evidence of biotype specificity in killing. Rather, with the three strains that were used, the IM biotype happened to show the greatest susceptibility to killing and the ET biotype the least, regardless of the biotype against which the antiserum had been raised.

In order to provide a possible opportunity for unimpeded expression of biotype specificity, further samples of the same three antisera were absorbed with Ogawa organisms of heterologous biotype, so that they would retain little or no serotype-specific antibody but may contain biotype-specific antibody. The titres (Table 2) show that almost all of the vibriocidal antibody in the original sera had been absorbed by a strain of homologous serotype; they provide no evidence of any residual antibody that could kill the homologous biotype preferentially. The titres of the positive vibriocidal control, with Ogawa specificity, confirmed the susceptibility of the bacterial suspensions and, thereby, the validity of the experiment.

With this lack of evidence of biotype specificity, it seemed prudent to use the

Table 2. *Vibriocidal titres of Ogawa antisera (each absorbed with a strain of homologous serotype and heterologous biotype) when tested against Ogawa strains of homologous and heterologous biotype*

Serotype and biotype of strain of vibrio			Vibriocidal titre* against Ogawa strain of		
Against which antiserum had been raised	With which the serum was absorbed	Intended specificity of absorbed serum	CS biotype	IM biotype	ET biotype
Og CS	Og IM	CS	5	5	14
Og IM	Og ET	IM	7	10	10
Og ET	Og IM	ET	7	5	14
Positive vibriocidal control					
Og ET	In ET	Og	3200	3200	400

See footnotes to Table 1.

The lowest dilution of serum tested was 1 in 10; in cases where the result was negative at this dilution, an arbitrary titre of 5 was recorded.

Table 3. *Vibriocidal titres of Ogawa and Inaba antisera (each absorbed with a strain of heterologous serotype and homologous biotype) when tested against Ogawa and Inaba strains*

Serotype and biotype of strain of vibrio			Vibriocidal titre* against a CS biotype of	
Against which antiserum had been raised	With which the serum was absorbed	Intended specificity of absorbed serum	Og serotype	In serotype
Experiment 1				
Og IM	In IM	Og	6400	< 100
In ET	Og ET	In	< 100	230
Experiment 2				
Og CS	In CS	Og	1500	< 40
In-specific serum (Wellcome)			< 40	3600

See footnotes to Table 1.

available sera in an attempt to re-confirm the existence of serotype specificity in vibriocidal activity [6]. In the first such experiment (Table 3), a pair of recently absorbed sera did indeed show marked serotype specificity, though the results also revealed a weaker activity of the Inaba serum (titre 230) than the Ogawa (titre 6400), and this Inaba serum was actually the best of the three available. In a second experiment, a commercial Inaba serum (Wellcome) gave a higher specific titre (3600), similar to those of the Ogawa sera.

Bacterial agglutination by antisera absorbed with heterologous organisms

All six unabsorbed antisera had agglutinin titres of 320 or 640 against suspensions of all six strains of vibrio, regardless of serotype or biotype. confirming the presence of a common antigen [6]. After absorption with various

Table 4. *Agglutinin titres of Ogawa antisera (each absorbed with a strain of heterologous serotype and heterologous biotype) when tested against Ogawa and Inaba strains of homologous and heterologous biotype*

Serotype and biotype of strain of vibrio			Agglutinin titre against					
			Ogawa strain of			Inaba strain of		
Against which antiserum had been raised	With which the serum was absorbed	Intended specificity of absorbed serum	CS biotype	IM biotype	ET biotype	CS biotype	IM biotype	ET biotype
Og CS	In ET	Og CS	80	110	80	< 10	< 10	< 10
Og CS	In IM	Og CS	40	40	40	< 10	< 10	< 10
Og IM	In CS	Og IM	40	40	40	< 10	< 10	< 10
Og IM	In ET	Og IM	57	80	40	< 10	< 10	< 10
Og ET	In CS	Og ET	40	80	40	< 10	< 10	< 10
Og ET	In IM	Og ET	40	80	80	< 10	< 10	< 10

See footnotes to Table 1.

Table 5. *Agglutinin titres of Ogawa antisera (each absorbed with a strain of homologous serotype and heterologous biotype) when tested against Ogawa strains of homologous and heterologous biotype*

Serotype and biotype of strain of vibrio			Agglutinin titre					
			(After one absorption) against Ogawa strain of			(After two or three absorptions) against Ogawa strain of		
Against which antiserum had been raised	With which the serum was absorbed	Intended specificity of absorbed serum	CS biotype	IM biotype	ET biotype	CS biotype	IM biotype	ET biotype
Og CS	Og IM	CS	40	57	40	< 5	< 5	< 5
Og CS	Og ET	CS	40	40	40	< 5	< 5	< 5
Og IM	Og ET	IM	20	28	20	< 5	< 5	< 5
Og IM	Og CS	IM	20	20	20	< 5	< 5	< 5
Og ET	Og IM	ET	10	7	10	< 5	< 5	< 5
Og ET	Og CS	ET	20	20	20	< 5	< 5	< 5

See footnotes to Table 1.

Table 6. *Agglutinin titres of Ogawa and Inaba antisera (each absorbed with a strain of heterologous serotype and homologous biotype) when tested against Ogawa and Inaba strains of homologous and heterologous biotype*

Serotype and biotype of strain of vibrio			Agglutinin titre against					
			Ogawa strain of			Inaba strain of		
Against which antiserum had been raised	With which the serum was absorbed	Intended specificity of absorbed serum	CS biotype	IM biotype	ET biotype	CS biotype	IM biotype	ET biotype
Og CS	In CS	Og	80	160	110	< 10	< 10	< 10
Og IM	In IM	Og	40	80	40	< 10	< 10	< 10
Og ET	In ET	Og	40	80	40	< 10	< 10	< 10
In ET	Og ET	In	< 4	< 4	< 4	11	16	8
In-specific serum (Wellcome)			< 4	< 4	< 4	23	32	32

See footnotes to Table 1.

heterologous bacteria, however, the agglutination results formed a pattern very similar to that obtained with the vibriocidal test (above), as described below.

Table 4 shows the agglutinin titres of sera absorbed with organisms that were heterologous in both serotype and biotype. Serotype specificity is apparent, but the titres were not influenced by the biotype of the agglutinable suspension.

Table 5 records the effect of absorption with homologous serotype but heterologous biotype. With each serum, the titres against all three biotypes were reduced to a similar low level by a single absorption; after two or three absorptions, all detectable agglutinin had been removed, regardless of biotype. There was no evidence that the rate of absorption of agglutinin was related to biotype.

Finally, samples of serum were absorbed with suspensions of heterologous serotype but homologous biotype (Table 6). As in the vibriocidal test (Table 3), these sera showed obvious serotype specificity in agglutination; also, the commercial Inaba serum had similar activity to that of the locally produced Ogawa sera, but the activity of the locally produced Inaba serum was lower.

DISCUSSION

The absence of biotype specificity in the vibriocidal activity of antisera (Tables 1, 2) is hardly surprising: most of the properties which differentiate the three biotypes are unrelated to surface components of the bacteria. Nevertheless, recent evidence that the CS biotype may be more immunogenic than the ET biotype [13] supports the continued use of CS strains in the preparation of vaccine.

In contrast, Tables 1–3 re-affirm the important role of the serotype-specific antigens (Ogawa and Inaba) in the killing of cholera vibrios, regardless of biotype. Serotype specificity in immunity to cholera [8] is thus reflected in the vibriocidal test: but it is seen also in bacterial agglutination (Tables 4, 6). Moreover, these are lipopolysaccharide (LPS) antigens, which are probably involved not only in bacterial killing by antibody and complement: the cholera vibrio is one of several enteric pathogens in which adhesion to intestinal cells is LPS-associated [14]. Moreover, if any other adhesin were of greater importance than these LPS antigens, it seems unlikely that serotype-specific immunity would be demonstrable.

These findings emphasize the likely importance of a good response to both Ogawa and Inaba antigens, if vaccination is to be effective. However, Tables 3 and 6 show lower activity of the locally produced Inaba serum compared with Ogawa sera. This is consistent with previous reports [15–17] which showed that, compared with the Ogawa response to cholera vaccine, the Inaba response was weaker. The probable explanation has been provided by Kabir [18] who showed that, with Inaba LPS, a single band migrated faster in electrophoresis than the major band of Ogawa LPS, suggesting that it is a smaller component which would be less immunogenic.

Cholera vaccine has usually contained equal numbers of Ogawa and Inaba organisms. Maybe, efficacy would be increased by raising the proportion of Inaba cells, to produce a balanced response. The addition of adjuvant would also be beneficial: even without adjustment of the Ogawa-Inaba balance, inclusion of

aluminium hydroxide in whole-cell killed vaccine raised its efficacy to 75% for a period of 15 months in Indonesian children [19].

There is increasing evidence for the role of bacterial motility in the pathogenesis of cholera and other diseases [20,21]; so, further improvement might be achieved by preserving another surface antigen (flagella) in the vaccine. This would involve the killing of bacteria by chemicals rather than by heat, as in the preservation of H and Vi antigens in typhoid vaccine [22]. Moreover, as with pertussis and hepatitis B, a course of three doses of vaccine is probably necessary for a good immune response. However, there is no evidence that the addition of toxoid improved the efficacy of cholera vaccine [9–11, 17]. Nor is this surprising, in spite of the manifest role of enterotoxin in producing potentially lethal diarrhoea: unlike diphtheria toxin, the target cells for which are remote from the site of bacterial colonisation, cholera toxin offers little opportunity for neutralization by antitoxin before acting on the very enterocytes to which the vibrios are attached.

The recent explosion of cholera in Latin America, mostly with the Inaba serotype [23], calls for the development of an effective vaccine [2]. The need is even more urgent than previously, especially if treatment is delayed and 20% mortality occurs [24]. The present study confirms that vibriocidal tests can show whether the cross-absorbed sera of vaccinees reveal a balanced Ogawa–Inaba response. However, these tests are costly in time and materials; it is difficult to standardize the concentration of viable bacteria in suspensions, so that several concentrations of bacterial suspension must be tested against serial dilutions of serum, and the results are then read with the lowest concentration which gives satisfactory growth in the control cups. Comparison of Tables 4–6 with Tables 1–3 shows that the same evidence on serotype specificity, and lack of biotype influence, can be provided by the much easier and quicker test of bacterial agglutination in which a single suspension of killed bacteria can be standardized readily.

With recent developments in molecular immunology, it may be felt that purified Ogawa and Inaba extracts would give more precise data on the immune response. As with pertussis immunity [25] however, it would first be necessary to show that such extracts detected the same epitopes as those involved with whole cells in agglutination or vibriocidal activity.

The present study with rabbit sera has shown, both in agglutination and vibriocidal activity, the relative weakness of the Inaba response to vaccination. This parallel with the response of human vaccinees [15–17], mentioned above, suggests that the rabbit is a suitable model of the type-specific immune response in man; but, if desirable, the similarity could be assessed further by a comparison of the type-specific agglutinin and vibriocidal titres of human sera. If approved, adoption of the simpler agglutination test may encourage a wider investigation of the vital Ogawa and Inaba responses in vaccinees, and thus lead to the development and control of vaccines with higher efficacy.

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