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Type-specific action of vibriocidal antibody on *Vibrio cholerae*

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SUMMARY

The vibriocidal activity of Inaba and Ogawa antisera, from which cross-reacting agglutinin had been absorbed, was specific for *Vibrio cholerae* strains of the homologous serotype. No vibriocidal action against strains of the heterologous type was detected.

The sera appeared to be equally effective in killing organisms of different biotypes (classical, intermediate, and ElTor), provided that these were of the homologous serotype (Inaba or Ogawa). However, they had been raised against strains of the classical biotype only; and sera resulting from immunization with other biotypes had not been prepared.

The implications of these findings in immunity to cholera are discussed.

INTRODUCTION

Immunity to cholera is probably dependent, in part at least, on the development of vibriocidal antibodies which lyse the organisms in the presence of complement. These antibodies are directed against lipopolysaccharide of the cell wall which is the site both of the common (A) antigen and also of the type-specific B and C antigens of the serotypes Ogawa and Inaba respectively. Vibriocidal titres have been shown (Mosley, Benenson & Barui, 1968) to increase with increasing age, correlating almost linearly with the fall in morbidity in older people in endemic areas. Moreover, it was shown by Mosley, Ahmad, Benenson & Ahmed (1968) that family contacts of cholera patients were less likely to become infected if they had high titres of vibriocidal antibody.

Evidence is now accumulating to indicate that immunity to cholera is type-specific. According to Mosley *et al.* (1970), most patients were found by Goodner *et al.* (1962) to have agglutinating antibody to the common antigen but no type-specific antibody, at the onset of symptoms. Also, mutation from one serotype to the other has been reported during infection, leading to clinical relapse as the second serotype appeared (Gangarosa, Sanati, Saghari & Feeley, 1967).

Type-specific immunity may also explain the poor protection which some cholera vaccines have provided. Field trials carried out in East Pakistan where Inaba infection was prevalent (Mosley *et al.* 1970) showed that homologous monovalent (Inaba) vaccines provided protection, whereas heterologous (Ogawa)

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vaccine did not, although it stimulated antibody to the common (A) antigen. It is not known whether Inaba vaccine will protect against naturally occurring Ogawa infection.

We decided to investigate whether vibriocidal activity is type-specific and, if so, whether the specificity applies to both serotypes or only to one.

MATERIALS AND METHODS

Strains of Vibrio cholerae

Five strains were of Inaba serotype. One, A/GL obtained from Glaxo Laboratories, was of classical biotype; two, 82TN and 8351KA isolated during the current pandemic from human sources in Tehran and Kasauli respectively, were of intermediate biotype (Adibfar & Preston, 1974); two, B/GL from Glaxo Laboratories and 283MU from the Manchester University Collection of Bacteria (MUCOB), were of ElTor biotype.

Five strains were of Ogawa serotype. Two, 545CA isolated from a human source in Calcutta and 281MU from MUCOB, were of classical biotype; two, 37NA and 617CA isolated during the current pandemic from human sources in Nigeria and Calcutta respectively, were of intermediate biotype; one, 719CA isolated from fish in Calcutta, was of ElTor biotype.

Differential tests for V. cholerae

Voges–Proskauer test. The method of Barritt (1936) was used, with incubation at 30° C.

Haemolysin production. The original method of Greig (1914) was followed, except that a 5% suspension of sheep erythrocytes was used instead of goat erythrocytes (Adibfar & Preston, 1974).

Polymyxin B. Sensitivity was tested on nutrient agar with disks containing 50 international units. Strains with which the diameter of the zone of inhibition was at least 9 mm. were recorded as 'sensitive': those with which the diameter was less than 7 mm. were recorded as 'resistant'.

Cholera phage IV. Sensitivity to this phage (Mukerjee, 1963) was tested by spotting phage at routine test dilution on a nutrient agar plate seeded with a 2–3 hr. nutrient broth culture of vibrio.

Serological reagents

Sera. Wellcome monospecific absorbed rabbit antisera, prepared against classical strains of Inaba and Ogawa serotypes, were used. The Inaba antiserum was stated to have a titre of 80, the Ogawa 160, in tube agglutination tests, each having been absorbed to prevent cross-reaction at titres greater than 40.

Complement. Wellcome lyophilized guinea-pig serum was reconstituted with distilled water according to the manufacturer's instructions.

Diluent. Phosphate buffered saline (PBS) was used for preparing dilutions of serum and of bacterial suspension. This consisted of 0·85% aqueous sodium chloride, buffered to pH 7·3 with 0·07 M phosphate.

The vibriocidal test

The microtechnique of Benenson, Saad & Mosley (1968) was carried out in Cooke microtitre plastic plates with 'U' cups (Cooke Engineering Co., Alexandria, Va., U.S.A.). All reagents were dispensed with a dropping pipette delivering 0·02 ml.

Overnight growth of each strain on nutrient agar slopes at 36° C. was transferred to PBS with a cotton-wool swab, and suspended uniformly. Each was diluted to the opacity of Brown's standard no. 2, and twofold dilutions of these were held at 4° C. Each dilution of bacterial suspension was mixed with an equal volume of complement (1/5), and 0·02 ml. of the mixture was dispensed into each cup in a row across the microplate.

Twofold dilutions of Inaba and Ogawa antisera (from 1/20) were added in 0·02 ml. amounts, with the least diluted serum in the first cup of each row. The final dilution of complement in each cup was 1/20, and antiserum was in twofold dilutions from 1/40. The last two cups in the rows were serum controls (containing serum at a final dilution of 1/40, and PBS instead of complement) and complement controls (containing PBS instead of serum).

The plate was sealed tightly with plastic tape and floated in a water bath at 37° C. for 1 hr. to allow vibriocidal action by the antiserum. It was then removed and unsealed, and 7 drops of sterile nutrient broth were added to each cup. After re-sealing, it was returned to the water bath for 3 hr. to allow any surviving organisms to grow.

After leaving at 4° C. overnight, to allow bacteria to settle, the plate was examined by oblique light. Cups in which vibrios had been lysed contained clear fluid, whereas those in which they had survived and multiplied showed a button of organisms at the bottom of the cup.

Results were recorded as negative (no growth) if lysis had occurred, and positive if a button of organisms was present. The titre was recorded, for each dilution of vibrio suspension, as the reciprocal of the final dilution of antiserum in the last cup in which there was definite inhibition of growth, compared with the control cups for that dilution of suspension. To make a valid comparison of homologous and heterologous antiserum, the titres were compared in rows which had the same dilution of vibrio suspension for both antisera, and which showed satisfactory buttoning in the controls. In a few of the serum controls, unsatisfactory buttoning seemed to result from agglutination which caused the vibrios to settle in an irregular pattern over the bottom of the cup.

Neither plates nor diluent were sterile. Provided that a sufficient number of vibrios are present to outgrow any contaminants in the 3 hr. incubation period, sterile conditions are not necessary with this technique.

RESULTS

Identification of strains

The following observations were consistent with the probable identity of all ten organisms as strains of *V. cholerae*: they were all curved Gram-negative rods,

Table 1. *Serotypes and biotypes of ten strains of* Vibrio cholerae

Strain	Serotype	Voges–Proskauer test	Sensitivity to polymyxin B	Lysis by phage IV	Haemolysin production	Biotype
A/GL	Inaba	–	S	C	–	Classical
82TN	Inaba	+	R	Nil	–	Intermediate
8351KA	Inaba	+	R	Nil	–	Intermediate
B/GL	Inaba	+	R	Nil	+	ElTor
283MU	Inaba	+	R	Nil	+	ElTor
545CA	Ogawa	–	S	SC	–	Classical
281MU	Ogawa	–	S	C	–	Classical
37NA	Ogawa	+	R	Nil	–	Intermediate
617CA	Ogawa	+	R	Nil	–	Intermediate
719CA	Ogawa	+	R	Nil	+	ElTor

S = sensitive, R = resistant (see Materials and Methods). C = confluent lysis, SC = semi-confluent lysis.

Table 2. *Titration of strain* 617*CA of* V. cholerae *against homologous (Ogawa) antiserum*

Final dilution of bacterial suspension	Final dilution of antiserum (Ogawa) 1/40	1/80	1/160	1/320	1/640	Serum control	Complement control	Vibriocidal titre of antiserum
1/4	+	+	+	+	+	+	+	.
1/8	+	+	+	+	+	+	+	.
1/16	+	+	+	+	+	+	+	.
1/32	+	+	+	+	+	+	+	.
1/64	–	–	+	+	+	+	+	80
1/128	–	–	–	–	+	+	+	320
1/256	–	–	–	–	–	–	–	.
1/512	–	–	–	–	–	–	–	.

\+ = growth of vibrio, with formation of 'button' at bottom of cup.
– = no growth (clear fluid and no 'button').

arginine negative, and cholera-red positive; they produced acid without gas from sucrose and mannose but not from arabinose; and all were agglutinated rapidly on a slide by polyvalent cholera antiserum.

Strains A/GL, 82TN, 8351KA, B/GL and 283MU were agglutinated completely by Inaba antiserum within 3 min., but gave no reaction with Ogawa antiserum within 5 min.; strains 545CA, 281MU, 37NA, 617CA and 719CA were agglutinated completely by Ogawa antiserum within 3 min., but gave no reaction with Inaba antiserum within 5 min.

The biotypes of the ten strains are indicated by the results of tests recorded in Table 1.

Determination of optimal density of bacterial suspension

As shown in Table 2, serial twofold dilutions of a suspension of strain 617CA were titrated against serial twofold dilutions of homologous (Ogawa) antiserum in the presence of excess complement (final dilution 1/20). No inhibition was detected

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Table 3. *Titration of strain A/GL (Inaba) against Inaba and Ogawa antisera*

Final dilution of bacterial suspension	Final dilution of antiserum					Serum control	Complement control	Vibriocidal titre of antiserum
	1/40	1/80	1/160	1/320	1/640			
	Inaba antiserum							
1/64	−	+	+	+	+	+	+	.
1/128	−	+	+	+	+	+	+	.
1/256	−	−	+	+	+	+	+	.
1/512	−	−	±	+	+	+	+	160
	Ogawa antiserum							
1/64	+	+	+	+	+	+	+	.
1/128	+	+	+	+	+	+	+	.
1/256	+	+	+	+	+	+	+	.
1/512	+	+	+	+	+	+	+	<40

\+ = growth of vibrio, with formation of 'button' at bottom of cup.
± = slight growth (turbid fluid and small 'button').
− = no growth (clear fluid and no 'button').

unless the bacterial suspension had been diluted to less than 1/32. However, the vibriocidal titre increased with greater dilution of the suspension, though with the highest dilutions growth was not detectable in the control cups. Subsequent experiments were therefore performed with a range of dilutions of bacterial suspension from 1/64 to 1/512.

Determination of optimal dilution of complement

Strain 82TN was titrated in triplicate against homologous and heterologous antisera, using a different complement dilution (final dilutions 1/20, 1/40, 1/80) for each titration.

Dilution to 1/80 left insufficient complement to allow homologous antiserum to produce lysis. Dilutions of 1/20 and 1/40 gave titres with homologous antiserum that were not significantly different; and, with heterologous serum, no inhibition was detected. A moderate excess of complement (1/20) was chosen for use in subsequent tests, to mask any anti-complementary activity.

Comparison of vibriocidal effects of homologous and heterologous antisera on each strain

Examples of the results obtained with an Inaba strain and an Ogawa strain are shown in Tables 3 and 4 respectively. A summary of these and similar results with the other 8 strains is given in Table 5.

Heterologous antiserum for each of the 10 strains had a vibriocidal titre of less than 40, the lowest dilution of serum that was tested with the limited quantities available. In contrast, homologous serum had a titre of at least 160 for each strain. Thus, for every strain, the difference in vibriocidal activity between the two sera was at least 8-fold, and, in five cases, at least 32-fold.

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Table 4. *Titration of strain* 281*MU* (*Ogawa*) *against Inaba and Ogawa antisera*

Final dilution of bacterial suspension	Final dilution of antiserum 1/40	1/80	1/160	1/320	1/640	Serum control	Complement control	Vibriocidal titre of antiserum
	Inaba antiserum							
1/64	+	+	+	+	+	+	+	.
1/128	+	+	+	+	+	+	+	< 40
1/256	+	+	+	+	+	+	±	.
1/512	+	+	+	+	+	+	±	.
	Ogawa antiserum							
1/64	±	±	±	+	+	+	+	.
1/128	−	−	−	−	±	+	+	⩾ 640
1/256	−	−	−	−	−	+	±	.
1/512	−	−	−	−	−	+	±	.

\+ = growth of vibrio, with formation of 'button' at bottom of cup.
± = slight growth (turbid fluid and small 'button').
− = no growth (clear fluid and no 'button').

Table 5. *Vibriocidal titres of homologous and heterologous antisera for ten strains of* V. cholerae

Strain	Biotype	Serotype	Optimal* dilution of vibrio suspension	Vibriocidal titre of Inaba antiserum	Ogawa antiserum	Minimum difference between titres
A/GL	Classical	Inaba	1/512	160	< 40	8:1
82TN	Intermediate	Inaba	1/512	160	< 40	8:1
8351KA	Intermediate	Inaba	1/512	320	< 40	16:1
B/GL	ElTor	Inaba	1/512	⩾ 640	< 40	32:1
283MU	ElTor	Inaba	1/256	160	< 40	8:1
545CA	Classical	Ogawa	1/256	< 40	⩾ 640	1:32
281MU	Classical	Ogawa	1/128	< 40	⩾ 640	1:32
37NA	Intermediate	Ogawa	1/512	< 40	⩾ 640	1:32
617CA	Intermediate	Ogawa	1/512	< 40	⩾ 640	1:32
719CA	ElTor	Ogawa	1/64	< 40	160	1:8

* This was the highest dilution of vibrio suspension for which the controls were satisfactory. Titres of antisera were recorded for this dilution of suspension (see Tables 3 and 4).

The Inaba antiserum tended to give lower vibriocidal titres than the Ogawa, but it was of an older batch and had a lower agglutinating titre. A single experiment with a new batch of Inaba serum, kindly supplied for the purpose, gave a titre of at least 640 (compared with 160 for the old serum – Table 5) against the Inaba strain 82TN, whilst the titre of the Ogawa serum against this same suspension was, as usual, less than 40. It seems, therefore, that the lower vibriocidal titres of the Inaba serum recorded in Table 5 are a reflection on the potency of a particular batch of serum, rather than any indication that type-specificity of vibriocidal action operates more in one direction than in the other.

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Although the two sera (anti-Inaba and anti-Ogawa) had been prepared by immunization of rabbits with classical strains of the two serotypes, there was no obvious difference between their vibriocidal effects on strains of classical, intermediate and ElTor biotype, provided that the strains were of the same serotype. To this extent, the type-specificity of the vibriocidal action was related to serotype, not to biotype.

DISCUSSION

We have shown, with a microtechnique, that the vibriocidal action of each monospecific anti-cholera serum (Inaba and Ogawa) is significantly greater against bacteria of homologous than against those of heterologous serotype, the difference being at least 32-fold with most of the ten strains tested. The vibriocidal titre of homologous serum was not high against some strains, but it should be noted that we used only readily available commercial antisera which had been diluted by the manufacturer for routine use in the identification of serotypes by agglutination. In no case was any vibriocidal effect detected against organisms of heterologous serotype.

Some blurring of this type-specific action may be caused by antibody to the common (A) antigen. Unabsorbed anti-Inaba and anti-Ogawa sera, produced in rabbits, have been found to kill both types to almost the same titre (Finkelstein, 1962). But in man, natural infection (Benenson, Saad & Mosley, 1968) or vaccination (Mosley *et al.* 1970) with a single serotype may stimulate antibody which, although vibriocidal to both serotypes, has a higher titre for the homologous type. The heterologous reaction is presumably caused by antibody to the common (A) antigen, but appears to be of little protective value *in vivo*. Thus, Mosley *et al.* (1970) reported that Inaba vaccine gave only a low degree of cross-protection against Ogawa challenge in mice, whilst Ogawa vaccine was almost completely ineffective against Inaba challenge. Similarly in man, they showed that Ogawa vaccine failed to protect against Inaba infection, and our present findings give no reason to believe that the converse would not apply.

Hence, although vibriocidal antibody to the common (A) antigen has some killing effect for both serotypes *in vitro*, it is possible that this antigen may be masked *in vivo*, and therefore type-specific antibody to the infecting serotype may be needed to confer significant protection. It has been noted (Preston, 1975) that many batches of commercial cholera vaccine react well with Ogawa serum but poorly with Inaba. Our present study suggests that, if bivalent vaccine induces a poor type-specific response to either serotype, it may give poor protection.

The balance between Inaba and Ogawa in a vaccine may be disturbed by the addition of an ElTor component, and the need for this additional component is uncertain. In mouse experiments (e.g. Noble, 1965) cross-protection has been found between different biotypes, and our present results are consistent with this finding. However, although we have not detected any significant difference in the vibriocidal action of sera on bacteria of different biotypes, we have used only antisera raised against the classical biotype, and it would be useful to pursue this study with sera raised against other biotypes.

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