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Passive protection of serum from volunteers inoculated with attenuated strain 638 of *Vibrio cholerae* O1 in animal models

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Abstract

As part of the studies to obtain an oral vaccine against cholera disease, the protective effect of serum from volunteers inoculated in a controlled trial with a candidate live attenuated vaccine of *Vibrio cholerae* O1, El Tor Ogawa (638; CTXϕ mutant, *hap*::*celA*), was tested. It was confirmed that the serum, as well as the purified IgG and IgA from the volunteers had a protective effect in both of the animal models used, although the purified antibodies needed the presence of complement to be protective. These results emphasize the expectations about the protective potential of the candidate in challenge studies in humans to be conducted very soon. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Cholera; Vaccine; Animal models; Serum mediated protection; Genetically attenuated

1. Introduction

Vibrio cholerae O1 is the main causative agent of epidemic cholera, it exists as two biotypes, classical and El Tor, and as two main serotypes, Inaba and Ogawa. Cholera disease is caused mainly by the action of cholera toxin, which rises the electrolyte secretion and fluid loss from the small intestine through its stimulating action above of adenyl cyclase activity [1]. To cause infection, cholera vibrios must overcome the gastric acid barrier, attach to and penetrate the mucus coat, and reach the underlying epithelial cells [2]. Intestinal colonization in humans is diagnosticated by the detection of cholera vibrios in the stools, which reflect their multiplication in the small bowel and subsequent detachment. Vibrios are also taken up by M cells, in which they could interact with macrophages and lymphocytes to elicit immune response [3].

Since 1961, the seventh pandemic of cholera disease has been under development, due to *V. cholerae* O1, El Tor biotype. In 1992 in India, a new cholera outbreak appeared, produced by a different *V. cholerae* serogroup, the O139 [2]. This serogroup was characterized by possession of a large capsule, which confers resistance to the vibriocidal action of antibodies produced by O1 strains [4,5]. It has been reported that O139 strains evolved from El Tor strains, their similarity has even been demonstrated at genetic and molecular levels [6].

Studies have shown that the protective mechanism against cholera is directed against both bacteria and toxin [7–10]. The antibacterial response is mainly against the lipopolysaccharide (LPS), but also against other bacterial components, e.g. outer membrane proteins and pili [7,11–13]. Serum antibodies, directed against *V. cholerae* somatic antigens, are usually vibriocidal in nature [14,15]. However, the same may not be true for the secretory immunoglobulin A (IgA) class of antibodies, present in intestinal and other body secretions [16]. The secretory IgA class is believed to act through inhibition of intestinal attachment and subsequent colonization of vibrios [17].

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Identification of cell surface moieties of *V. cholerae*, responsible for attachment of the organism to intestinal epithelium, has remained elusive so far. Cholera patients who are refractory to subsequent infections mount immune responses against *V. cholerae* LPS in serum, milk and the intestine [18].

The recognition that protection against *V. cholerae* infection is highly dependent on stimulation of a mucosal immune response, has favored the concept of an oral vaccine [19]. CVD103HgR, a CTA^{-} CTB^{+} derivative of the classical biotype Inaba strain 569B, has been extensively tested in volunteers [20]. This vaccine is safe, well tolerated and highly protective. However, CVD103HgR is less protective against mild diarrhea due to challenge with El Tor vibrios [20]. Unfortunately, most El Tor biotype candidate vaccine strains, even those lacking the whole core region of the CTX element (ΔAce, ΔZot, ΔCT) have been considered to be too reactogenic for wide scale usage [19].

In animal challenge experiments, the presence of anti-LPS serum antibodies has been correlated with protection [21]. Apter [22] showed that intragastrically administered monoclonal antibody, (2D6) protected neonatal mice against a lethal oral dose of *V. cholerae* when given up to 2 h before challenge. Cynthia et al. [23] showed that oral administration of a monoclonal IgA, directed against a LPS component of the vibrio, protected neonatal mice against oral challenge.

In the National Center for Scientific Research, Havana, Cuba and in our laboratory, intensive work has been done to obtain an attenuated *V. cholerae* strain. From the wild type strain C7258 *V. cholerae* O1, El Tor biotype, Ogawa serotype, using recombinant techniques, the attenuated strain 81 (CTXΦ mutant) was obtained. Subsequently, in the genome of 81 strain it was inactivated the gene *hap* (codifying hemagglutinin/protease) by insertion of the gene *celA* (genetic marker that codify to *Clostridium thermocellum* endogluconase A) to obtain 638 strain (CTXΦ mutant, *hap*::*celA*) [24,25], our candidate to oral vaccine.

In the present study, total serum, as well as the purified IgG and IgA, from volunteers inoculated with the attenuated strain 638 of *V. cholerae* O1 El Tor Ogawa, were evaluated. Two tests were performed: the inhibition capacity of the assessed samples on the activity of different wild types strains O1 or O139, using the ileal loop model, and its protection ability in the neonatal mice model.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains C7258 and C6706 [26] are El Tor biotype strains of the Ogawa and Inaba serotypes, respectively. Strains 395 and 569B [27] are classic biotype strains of Ogawa and Inaba serotypes, respectively and strain 1837 [28] is serogroup O139. The strains were grown on brain heart infusion (BHI) agar, suspended in saline solution and adjusted spectrophotometrically at 0.6 units of OD at 540 nm using a Milton Roy Spectronic 401.

2.2. Preparation of positive and negative serum

Samples of serum were collected from volunteers inoculated with the attenuated 638 strain, that responded highly positive to four different immunochemical tests: ELISA for determination of anti-LPS Ogawa IgG antibodies, ELISA for determination of anti-LPS Ogawa IgA antibodies, ELISPOT method to quantify antibodies secreting cells and vibriocidal antibodies assay [24]. All samples were pooled and stored at −70°C and considered as the positive serum. This whole positive serum had the following features: 2.48 $\pm$1.12 and 2.52$\pm$1.15 as the logarithms of the reciprocal arithmetic mean titers for IgG and IgA antibodies respectively, titers defined as the dilution of serum, calculated by interpolation giving an absorbance value 0.4 unit above the background value, and 4135 (1280–10,240) as the geometric mean vibriocidal titer, titers defined as the reciprocal value of the highest dilution of serum causing complete inhibition of bacterial growth, as judged by visual color comparison of the culture medium with a control without serum [24].

The negative serum was obtained in the same way. In this case, from the volunteers that were inoculated with $NaHCO_3$ (1.5%) as placebo, and responded frankly negative to a wider battery of immunochemical tests, including those mentioned above, besides the analysis of the stools specimens by ELISA for detection of anti-LPS IgA and isolation/identification of *V. cholerae*.

2.3. Purification of IgG and IgA antibodies from positive serum

The antibodies were purified by a method described previously [29]. Briefly, the positive serum was diluted 1:3 with distillated water, precipitated with ammonium sulfate (50% saturation) and dialyzed against 0.01 M Na_2HPO_4 buffer, pH 8. The sample was applied into chromatographic column DEAE Sephasel (Pharmacia) and eluted with 0.01 M Na_2HPO_4 buffer, pH 8. IgG was obtained in the isocratic column volume. To obtained IgA, the column is subjected to lineal gradient elution (0–0.5 M NaCl in 0.01 M Na_2HPO_4 buffer, pH 8). The fractions that contained IgA were detected by the use the turbidimetric method [30], subsequently pooled and applied into affinity column jacalin–agar-

ose (Pearce) and eluted with 0.1 M Melibiose in phosphate-buffered saline (PBS) pH 7.4.

2.4. ELISA and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

With the objective of checking if the process of purification affected the antigen linked side, an indirect ELISA for the determination of anti-LPS Ogawa antibodies of *V. cholerae* O1 was carried out [24]. The antibody purity was determined by SDS–PAGE and Comassie blue R250 stain. For the IgA antibodies fraction, that were purified by jacalin extraction, an indirect ELISA for determination of IgM antibodies as contaminant was done using Ogawa LPS as solid-phase antigens and peroxidase-conjugated anti-human IgM (Sigma Chemical Co., St. Louis, MO, USA).

2.5. Rabbit ileal loop model

Ileal loops were performed as described [31]. Briefly, New Zealand white rabbits of 2–5 kg (age 9–11 weeks), locally supplied by the National Center for the Production of Laboratory Animals (CENPALAB), Havana, Cuba, were intravenously anesthetized with pentobarbital (25 mg/kg). The small intestine withdrawn and ligated approx. 10 cm from the appendix. The intestine was divided into 9 or 10 segments of 5–6 cm by ligatures. Each segment was injected with 0.5 ml of a mixture (v/v), which had been pre-incubated, in constant stirring during 1 h at 37°C. The mixtures consisted in 10^7 colony forming units (CFU) of the selected strain and one of the following alternatives: (i) positive serum in different dilutions (pure to 1:50); (ii) IgG purified from positive serum, 2–60 µg; (iii) IgA purified from positive serum, 5–30 µg; (iv) IgG or IgA, supplemented with negative serum, 20–60 and 5–30 µg respectively; (v) IgG or IgA, supplemented with de-complemented negative serum, 20–60 and 5–30 µg respectively.

The IgG and IgA ranges used to make the mixtures were selected considering the inclusion of the values of both of them, determined by the turbidimetry method [30], in the higher dilution of positive serum with best results in (i).

Two or three mixtures (at least two loops per mixture) were tested in three different animals and the fluid accumulation (FA) ratio mean value for each mixture was calculated. The last two alternatives [(iv) and (v)] were performed in order to evaluate the influence of complement in the reaction. The negative serum was de-complemented by incubation at 56°C for 30 min. Positive serum without cells, and 10^7 CFU of each strain in negative serum, was respectively used as negative and positive control.

After the incubation period (1 h), and immediately before being inoculated in the rabbit ligated intestine, samples of all the mixtures were cultured in thiosulfate–citrate–bile salt–sucrose agar (TCBS) and incubated at 37°C, overnight, to evaluate vibriocidal activity. After 16–18 h, the rabbits were sacrificed by a pentobarbital overdose and FA ratio was determined by measuring the amount of fluid per length unit of the loop. The results were expressed as the FA ratio mean value for each mixture induced by the tested sera.

2.6. Neonatal mouse protection and challenge

The experiments were carried out using 5-day-old Balb/c mice (CENPALAB, Havana, Cuba), distributed in groups of 10 animals and placed in independent boxes with mothers, until they reached 2.5 g average weight. Subsequently, the animals were removed from their mother 2 h before being inoculated intraperitoneally with 50 µl of one of the following variants: (i) positive serum in different dilutions (pure to 1:50); (ii) negative serum (negative control of the assay); (iii) purified IgG or IgA from positive serum, 1.5–40 and 0.625–10 µg respectively; (iv) IgG or IgA, 1.5–40 and 0.625–10 µg respectively, but supplemented with negative serum. In each variant a group of 10 animals was used. Each variant was tested three times.

The IgG and IgA ranges were selected following the same procedure that was described in rabbit ileal loop model experiments.

After 6 h of the inoculation, 10–15 LD_{50} of *V. cholerae* O1 or O139 strains (Table 1) was administered (in 50 µl) per mouse, by intragastric intubation via PE10 polyethylene tubing connected to a 1-ml syringe. Evans blue (Merck 0.01%) was added to allow visualization of the solution entering the stomach.

Six hours after the challenge, the mothers were returned to their breeding and 42 h later the survival of each group was evaluated. The results were expressed as survival % mean for each variant.

2.7. Statistic tests used

For the statistic processing of the data, the Variance

Table 1
LD_{50} of strains used in this study [39]

Strain	Biotype/serotype	LD_{50}
C7258	El Tor/Ogawa	1×10^4 cel.
C6706	El Tor/Inaba	3.6×10^4 cel.
569B	Classical/Inaba	2.3×10^5 cel.
395	Classical/Ogawa	6.06×10^3 cel.
1837	O139	3.7×10^3 cel.

analysis and the Student's *t*-test were used, with a 0.05% significance level.

3. Results

The serum of volunteers inoculated by oral route with the attenuated strain 638 *V. cholerae* O1 El Tor Ogawa, as well as the IgG and IgA antibodies purified, were assessed from the point of view of their potentials to inhibit the ability of virulent strains O1 and O139 to accumulate fluid in ligated intestine of rabbits, and to protect neonatal mice against a challenge by oral route with 10–15 LD_{50} of virulent strains O1 and O139.

The assays applied to evaluate the purity level of the purified IgG and IgA antibodies from positive serum (SDS–PAGE and IgM ELISA) demonstrated non-existence of contaminants of any nature. There was not any detectable structural damage that affect the recognition between antibodies and antigens (Ogawa LPS).

Table 2 shows the results obtained when the positive serum, pure and in dilutions up to 1:50, was combined with 10^7 CFU of the virulent strain C7258 *V. cholerae* O1 El Tor Ogawa and was inoculated in segmented rabbit ileum, as well as when neonatal mice were inoculated, by intraperitoneal route, with similar positive serum dilutions and were challenged 6 h later by oral route with 10–15 LD_{50} of the virulent strain C7258.

A significant inhibition of the FA ratio produced by this strain (1.42 ml/cm) was observed in the cases in which it was combined with the pure, 1:2, 1:10 and 1:20 positive serum, not so when it was combined with 1:40 and 1:50 dilutions.

A high survival percentage (90%) is observed when positive serum, pure and in 1:2, 1:5, 1:10 and 1:20 dilution, is inoculated, there being no significant differences between them ($p < 0.05$). However, when the positive serum in a higher dilution is inoculated, the survival rate obtained decreases significantly ($p < 0.05$) to levels similar to those obtained with the negative serum. Bearing in mind these results, it was decided to use the positive serum in a 1:20 dilution for the rest of the experiments. This was the higher serum dilution which significantly inhibited ($p < 0.05$) the ability of strain C7258 to accumulate fluid in ligated rabbit intestine at levels that are even similar to those obtained when pure serum was used, as well as, when the inoculation of the rabbit ileum was done without cells (negative control of the trial). In addition, the same dilution showed a high survival percentage in the neonatal mice model.

Fig. 1 shows the results obtained when the positive serum in a 1:20 dilution was combined with different virulent O1 and O139 strains. It can be observed that the greatest inhibition of the strain's fluid accumulating ability is obtained when the positive serum in 1:20 dilution is combined with the C7258 strain. However, when the same dilution of the positive serum was combined with C6706 and 569B strains, the inhibition obtained did not reach similar levels. On the other hand, it was observed that when the positive serum was combined with the 395 strain, a greater inhibition was obtained than that obtained for the strains of different serotype. The effect of the serum was even less for strain 1837 *V. cholerae* O139, since it was able to reduce the strain's activity to very small levels.

When the cultures in TCBS, of the samples ready to be inoculated into the intestinal segments, were assessed, growth was observed in all the samples that

Table 2
Results obtained when different positive serum dilutions, were combined with 10^7 CFU of the virulent strain C7258 and inoculated in segmented rabbit ileum, as well as when they were inoculated by intraperitoneal route to neonatal mice, which 6 h later were challenged by oral route with 10–15 times LD_{50} of the virulent strain C7258

Inoculum	FA (ml/cm) (S.D.), segmented rabbit ileum[a]	Survival % (S.D.), neonatal Mice[b]
Pure positive serum	0.29 (0.32)	90 (3.05)
Positive serum 1:2	0.36 (0.36)	100 (2.06)
Positive serum 1:5	Not evaluated	100 (1.42)
Positive serum 1:10	0.49 (0.34)	96.6 (2.5)
Positive serum 1:20	0.29 (0.38)	90 (3.01)
Positive serum 1:40	1.33 (0.16)	20 (1.72)
Positive serum 1:50	1.72 (0.25)	20 (1.33)
Positive serum 1:20 without cells	0.22 (0.2)	Not related with the assay
Cells with negative serum 1:20	1.42 (0.37)	Not related with the assay
Negative serum 1:20	0.22 (0.2)	16.6 (1.06)

[a] Two or three mixtures (at least two loops per mixture) were tested in three different animals and the FA mean value for each mixture was calculated.

[b] In each variant a group of 10 animals was used. Each variant was tested three times and the survival % mean for each variant was calculated.

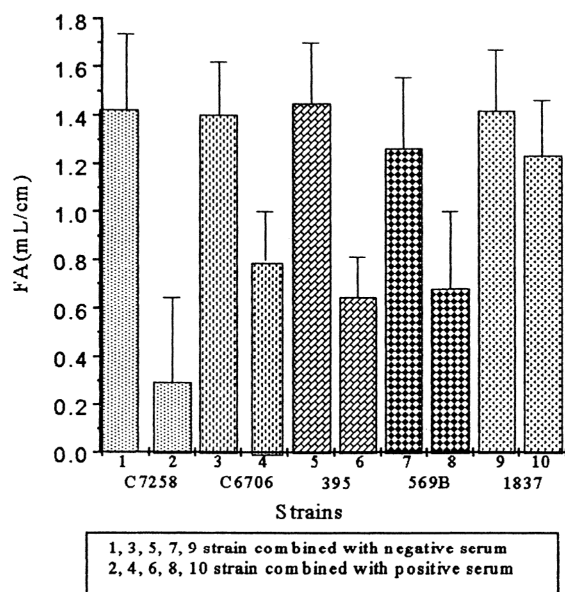

Fig. 1. Results obtained in rabbit ileal loop model, when negative serum (positive controls for each strain) and positive serum in a 1:20 dilution were combined with various virulent O1 and O139 strains.

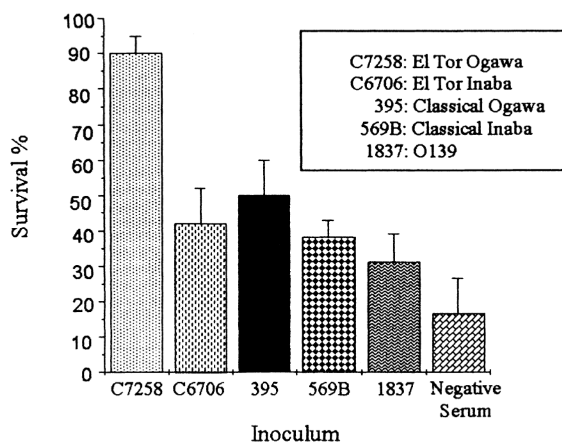

Fig. 2. Results obtained when neonatal mice were inoculated by intraperitoneal route with the positive serum in a 1:20 dilution and challenged by oral route with 10–15 LD_{50} of different virulent O1 and O139 strains.

did not contain complement, in the order of 10^7 CFU (a non-significant difference, $p < 0.05$ in relation to the count made of the samples free of antibodies and complement that were used for the control). However, when the number of cells was counted in samples that contained antibodies in the presence of complement, it was observed that the number of CFU decreased to 10^4 CFU (for the case of the positive serum pure and in dilutions up to 1:20, 20–60 μg of IgG, 20–30 μg of IgA). When 10^4 CFU of the strain C7258 were inoculated, in negative sera, into the intestinal segments, values of FA = 1.33 ml/cm (non-significant difference $p < 0.05$, with respect to the FA produced by an inoculum of 10^7 CFU of strain C7258) were detected.

Tables 3 and 4 show the results obtained when the IgG and IgA purified from the positive serum were combined in quantities of 2–60 and of 5–30 μg respectively, with 10^7 CFU of the virulent strain C7258.

It can be observed that for the quantities of IgG and IgA used, there was no inhibitory effect, since the values of FA obtained in these cases were similar to those obtained for this strain in the absence of positive serum.

To verify if the absence of complement, when the purified antibodies were used, was the reason why they did not have an inhibitory effect, negative serum as a complement source was added both to IgG and IgA. It was observed that, in the case of IgG, at least 20 μg were enough to significantly reduce the strain's activity, even at levels similar to those obtained with the positive serum in dilution up to 1:20 and with the negative control of the trial (inoculum without cells; Table 2). The IgA antibody attained a highly significant inhibitory activity only when 30 μg were used.

The IgA and IgG antibodies were used in the presence of de-complemented negative serum, being observed for this case that the inhibitory activity on the strain totally disappeared (Tables 3 and 4).

Fig. 2 shows the survival percentages obtained by

Table 3
Role of IgG purified from the positive serum in the inhibition of the FA ratio that virulent strain C7258 *V. cholerae* O1 El Tor Ogawa can produce in ligated rabbit intestine

IgG (μg)	FA[a] (S.D.)	Addition of complement FA[a] (S.D.)	Addition of de-complemented serum FA[a] (S.D.)
2	1.30 (0.21)	Not evaluated	Not evaluated
20	1.61 (0.29)	0.06 (0.05)	1.34 (0.20)
30	1.25 (0.25)	Not evaluated	Not evaluated
40	1.36 (0.26)	0.35 (0.19)	1.36 (0.22)
50	1.14 (0.22)	Not evaluated	Not evaluated
60	1.27 (0.29)	0.19 (0.14)	1.11 (0.29)

[a] FA of strain C7258 in the absence of positive serum = 1.42 m/cm; average of six determinations.

Table 4
Role of IgA purified from the positive serum in the inhibition of the FA ratio that virulent strain C7258 *V. cholerae* O1 El Tor Ogawa can produce in ligated rabbit intestine

IgA μg	FA[a] (S.D.)	Addition of complement FA[a] (S.D.)	Addition of de-complemented serum FA[a] (S.D.)
5	1.12 (0.25)	0.60 (0.15)	1.30 (0.28)
10	1.24 (0.28)	Not evaluated	Not evaluated
15	1.24 (0.30)	0.53 (0.20)	1.34 (0.20)
20	1.41 (0.28)	Not evaluated	Not evaluated
30	1.33 (0.20)	0	1.08 (0.31)

[a] FA of strain C7258 in the absence of positive serum = 1.42 ml/cm; average of six determinations.

inoculating the positive serum in a 1:20 dilution and challenging by oral route with 10–15 LD_{50} of virulent strains of *V. cholerae* O1 and O139.

A higher survival percentage (90%) is observed when the challenge is made with strain C7258, a lower survival percentage when the challenge is made with strain 395 and an even lower one when the challenge is made with strains C6706, 569B and 1837.

Table 5 shows the results obtained when IgG (1.25–40 μg) and IgA (0.6–10 μg), purified from the positive serum, were intraperitoneally inoculated and the animals were later challenged by oral route with 10–15 LD_{50} of the strain C7258.

It was observed that for concentrations lower or equal to 5 μg for IgG and 2.5 μg for IgA, no passive protection was obtained against the oral route challenge. At 10 μg for IgG and 5 μg for IgA, protection levels of 46.6 and 36.6% respectively are attained. However, for concentrations higher than the latter two, both for IgG and IgA, similar protection levels are not detected.

In order to verify if the absence of serum elements were the reason for the antibodies not showing the protective effect shown by the positive serum (90% survival rate), the IgG and IgA were supplemented with negative serum.

Table 6 shows the results obtained under these conditions. An increase of the survival percentage was observed in direct proportion with an increase of the concentration of IgG and IgA to levels of 5 and 2.5 μg respectively. Nevertheless, when the antibody concentration used was higher than these values, the survival percentage decreased.

4. Discussion

The results suggest that the potentials of the positive serum of volunteers inoculated with the attenuated strain 638, *V. cholerae* O1, El Tor Ogawa, to inhibit the ability of virulent strains O1 and O139 to accumulate fluid in ligated rabbit intestine and to protect in passive protection experiments against the oral route challenge in the neonatal mice model, is highly significant against strains of the same biotype and serotype as those of the strain used in the inoculation (El Tor Ogawa); is less significant against the same serotype but different biotype (Classic Ogawa); it is hardly observed against the same biotype but different serotype (El Tor Inaba), and even less against different biotype and serotype (Classic Inaba) and it is not at all observed against a different serogroup (O139).

It was considered that these results can be explained by the presence in the serum of a high titer of specific antibody against the LPS of Ogawa strains, that recognize Ogawa strains more effectively than Inaba strains and that do not recognize the LPS of O139 strains. Therefore, it is precisely

Table 5
Results of survival rate in passive protection trials with IgG and IgA purified from the positive serum[a]

Challenge strain[b]	IgG μg	Survival % (S.D.)	IgA μg	Survival % (S.D.)
C7258	1.25	0	0.625	0
	2.5	0	1.25	0
	5	0	2.5	0
	7.5	26.6 (5.77)	5	36.6 (5.77)
	10	46.6 (5.77)	7.5	26.6 (5.77)
	20	10 (0)	10	10 (0)
	40	10 (0)	Not evaluated	Not evaluated

[a] In these experiments, the survival % of the placebo group was 0. In each variant a group of 10 animals was used. Each variant was tested three times and the survival % mean for each variant was calculated.
[b] Doses: 10–15 LD_{50} in 50 μl by oral route.

against Ogawa strains that the greater activity of the serum can be observed.

It has been reported that the LPS of *V. cholerae* is the main antigen providing antibacterial immunity [32], and that when the anti-LPS antibodies are eliminated from the polyclonal antiserum produced against dead whole vibrios, that have been cultivated to a stationary phase, it results in a total loss of the serum's passive protection effects against experimental cholera [33]. On the other hand, it has been stated that the ability to induce crossed protective immunity of the LPS of the O1 Ogawa serotype with the Inaba serotype, is due to the generation of antibodies against antigen A, common to Inaba LPS and Ogawa LPS of *V. cholerae* O1 [34]. However, *V. cholerae* O1 does not offer any crossed protection to *V. cholerae* O139, since the most important protective antigen in both serogroups is the antigen O of the LPS, which differ in their length and composition [35]. In addition, the O139 strain used in this assay, characterized by possession a large capsule, which confers resistance to the vibriocidal action of antibodies produced by O1 strains [4,5] and it could explain the poor activity of positive serum on O139 strains in both assays.

Results obtained, when culturing the samples to be inoculated in the rabbit intestinal segments, showed the existence of 10^4 CFU that are not killed for the vibriocidal antibodies during the incubation period. This number of cells, in negative sera, was enough to produce an FA level similar to that obtained when 10^7 CFU were inoculated, that suggests that in the samples which showed inhibition of the strain's activity there are, besides vibriocidal antibodies, anti-adherence antibodies that neutralize the action of 10^4 CFU remaining.

The results obtained with the purified antibodies in the absence of complement, should not be explained by assuming that the IgG and IgA quantities used were not enough to achieve results similar to those when the positive serum in a 1:20 dilution was used, since the selected ranges included the IgG and IgA quantities in the 1:20 positive serum dilution (data not shown).

In the ligated rabbit intestine (ileal loop) method, the virulent action of the strain is favored, since one of the most important non-immune defense mechanism against this disease, such as intestinal peristalsis [33], is totally blocked, and undoubtedly this favors colonization of the strain, its cellular division and, along with these, an increase in the expression of virulent factors, which cannot be successfully neutralized by a limited amount of purified antibodies. This could be the reason why IgG and IgA, in the quantities used in these trials, do not show any inhibitory effect over the bacterial action in this model, requiring for this the presence of the complement proteins of the serum and their bactericidal activity (Tables 3 and 4).

In the trials regarding passive protection in neonatal mice, both the results with purified antibodies and the results with antibodies supplemented with negative serum, suggest that there is a dependence dose (Tables 5 and 6). In other words, there is a response to a given dose of antibodies, above or below which, the response drops and even disappears. The fact that high antibody concentrations, with or without negative serum, show very little protection or no protection at all against the oral route challenge, can be explained if the possibility of an inoculum with high protein concentration is taken into consideration. When this is administered by a very absorbing route, such as the intraperitoneal route, can produce reactions in the neonatal mouse, which combined with those produced as a result of the oral route challenge, bring about the death observed. These results also suggest that the presence of the serum elements makes the activity of both antibodies more effective, since a considerable protection of 70% for IgG and 50% for IgA is observed for concentrations in which the protection was not observed when the serum elements did not exist.

The determination of vibriocidal antibodies is a bactericide trial that requires the fixation of the complement by means of antibodies, which specifically binds

Table 6
Results of survival rate in passive protection trials with IgG and IgA purified from the positive serum, supplemented with negative serum[a]

Challenge strain[b]	IgG μg	Survival % (S.D.)	IgA μg	Survival % (S.D.)
C7258	1.25	10 (0)	0.625	0
	2.5	30 (10)	1.25	23.3 (5.77)
	5	70 (0)	2.5	50 (10)
	10	63.3 (11.54)	10	30 (10)
	20	43.3 (5.77)	Not evaluated	Not evaluated
	40	0	Not evaluated	Not evaluated

[a] In these experiments, the survival % of the placebo group was 0. In each variant a group of 10 animals was used. Each variant was tested three times and the survival % mean for each variant was calculated.

[b] Doses: 10–15 LD_{50} in 50 μl by oral route.

with *V. cholerae* [36]. It is broadly described in the literature that IgG antibodies fix complement and therefore unleash bactericidal activity [36]. However, there is a great controversy as to whether or not IgA fixes complement.

It has been stated that there are results from different laboratories that show that the alternative pathway in a number of species is activated by IgA aggregates [37]. It is also stated that in experiments where low serum concentrations are used, the alternative pathway of complement activation is not attained [37]. Complement activation by the IgA of other species has been studied at greater length and it has been demonstrated that it is a complex aspect that greatly depends on the system used, the aggregation state of the IgA and the complement source [37].

Our results suggest that low levels of IgA antibodies, in the presence of complement, do not show bactericidal activity, probably because the complement is not effectively fixed. Nonetheless, high levels of IgA antibodies, in the presence of complement, do show a significant bactericidal activity, which might be related to the formation of IgA aggregates, which, as it has been reported by other authors [37,38], have the ability of fixing complement and therefore show bactericidal activity.

The results we have presented in this paper show that serum of volunteers inoculated with the attenuated strain 638, *V cholerae* O1, El Tor Ogawa, as well as the antibodies IgG and IgA purified from it, as long as they are in the presence of the complement proteins, shield against the challenge with virulent strains of the O1 serogroup in two of the more widely used animal models in cholera research. These results are very encouraging if any correlation between our data and any protection in human challenge trials could be proven. Then the animal models and experimental designs used in this paper could be adopted for further evaluation of possible candidates to cholera vaccine.

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