Human immune response to the 18-kDa outer-membrane antigen of *Vibrio cholerae*

CARMEN V. SCIORTINO

Department of Pathology, Veterans Administration Medical Center and the University of Louisville, 800 Zorn Avenue, Louisville, KY 40206, USA

Summary. The serum IgG response of human volunteers challenged with *Vibrio cholerae* O1 was analysed for reactivity to *V. cholerae* O1 outer-membrane antigens by enzyme-linked immunosorbent assay (ELISA) and the immunoblot technique. Purified outer-membrane antigen preparations from vibrios grown in low-iron conditions were separated by SDS-PAGE. Specific immunoblot reactions of human sera showed that an 18-kDa antigen, cholera protective antigen, was the major antigen with which sera reacted. ELISA revealed an increase in antibody to the 18-kDa antigen in nine of 10 challenged volunteers. This response was independent of the biotype and serotype of the *V. cholerae* O1 challenge strain. Cholera protective antigen appears to be one of the major outer-membrane antigens involved in the human immune response to infection with *V. cholerae*.

Introduction

Patients who recover from cholera become immune to re-infection;¹ immunity is independent of the biotype and serotype of the infecting strain,² suggesting that a common antigen may be expressed in all pathogenic *Vibrio cholerae* strains.

The cholera enterotoxin is primarily responsible for the characteristic “rice-water” stools of cholera. This toxin has been widely studied as a potential source of a cholera vaccine, but immunity induced during recovery from cholera is greater than immunity to the toxin.^{3,4} Attenuated *V. cholerae* vaccines in which toxin production has been genetically deleted provide at least 50% protection from re-infection.^{3–7} This implies that another antigen may provide the stimulus against re-infection with *V. cholerae*.

One genetically engineered non-toxicogenic strain, *V. cholerae* CVD 103 HgR (*tox*A[–]B⁺), gave 62% protective efficacy against subsequent challenge with virulent *V. cholerae* in a North American volunteer trial in medical students.⁷ In a trial of this vaccine in Thailand, where epidemic cholera exists,⁸ 11 of 12 vaccinees developed serum vibriocidal titres (the best immunological correlate of protection) and nine showed a serum antitoxin response. The differences in antibacterial *versus* antitoxin immunity from those studies indicated that an unknown antigen was responsible for much of the antibacterial immunity.

We have developed monoclonal antibodies (MAbs) that recognise several outer-membrane antigens of *V. cholerae*,⁹ and protect suckling rabbits from lethal

infection with *V. cholerae*.¹⁰ Four different MAbs that recognised an 18-kDa antigen provided 98% protection from disease, whereas MAbs recognising lipopolysaccharide (LPS) or other outer-membrane antigens were only 20–40% protective. The antigen that reacted with the four protective MAbs was named cholera protective antigen (CPA).

In this study, ELISA and Western blotting were used to examine reactivity with CPA or *V. cholerae* outer-membrane antigens in human sera from the North American volunteer vaccine trial.⁷

Materials and methods

V. cholerae strains

Isolates of *V. cholerae* NIH 41, 569B, CVD 103 HgR and 3083TR² were used. Strain 569B (*tox*A⁺B⁺) is the Classical, Inaba parent of the genetically cloned CVD 103 vaccine strain.^{3,7} Strain 3083TR² (El Tor, Ogawa) is the virulent translucent colony type of *V. cholerae*.¹¹ *V. cholerae* strain NIH 41 (Classical, Ogawa) is a National Institutes of Health reference strain. In this study, *V. cholerae* was grown in 1 L of low-iron “T” medium¹² for 16 h at 37°C on a rotary shaker. Bacteria were harvested by centrifugation.

Antisera

MAbs SY15 (IgG) and NAK 258 (IgG₁) were prepared as previously described.^{9,10} MAb SY-15 was specific for *V. cholerae* LPS and MAb NAK-258 reacted specifically with CPA. MAbs were prepared by

ammonium sulphate (final saturation 50%) precipitation from hybridoma tissue culture supernates at 4°C. The precipitates were dialysed for 16 h against three changes of phosphate-buffered saline (PBS) and stored with thimerosal 0.1% at 4°C.

Human sera were provided by Dr J. B. Kaper (Center for Vaccine Development, University of Maryland, Baltimore, MD, USA). These sera were obtained in the course of a vaccine study in which volunteers were vaccinated by ingesting a single dose of *V. cholerae* CVD 103 (5×10^8 viable organisms) suspended in 120 ml of sodium bicarbonate buffer. Vaccinees were observed for 5 days and then given tetracycline for 5 days before discharge. A control group was not vaccinated. Six months later, volunteers were challenged by ingestion of *c.* 10^6 virulent *V. cholerae* organisms. Serum was collected before challenge and 28 days after challenge as previously described.⁷ For the present study, serum samples that had been stored at -70°C were transported on dry ice, thawed at 22°C and diluted as described below.

Peroxidase-conjugated antisera were purchased from Cappel Laboratories (Organon Technica, Durham, NC, USA), reconstituted in sterile de-ionised water as recommended by the manufacturer, distributed in 50- μ l amounts and stored at -70°C. For use, sera were thawed and diluted in PBS containing Tween 20 1%, non-fat dried milk (Carnation Co., Los Angeles, CA, USA) 5% and fetal calf serum (Rehatuin lot D82902, Armour Pharmaceutical Co., Kankakee, IL, USA) 3%.

Preparation of outer-membrane antigens

The procedure of Filip *et al.*,¹³ as modified by Sigel and Payne,¹² was used with the following additional variations: cell pellets were removed from storage at -70°C and thawed by addition of, and suspension in, 10 ml of sodium phosphate buffer containing Mg^{2+} . Samples were maintained at 4°C or submerged in an ice bath unless otherwise specified. Flagella were removed by vortex mixing the suspension vigorously with six glass beads (3 mm diameter), centrifuging the bacterial suspension at 4350 *g* and discarding the supernate containing flagella. The whole cell pellet was dispersed in 5 ml of TEAN buffer (5 mM Tris, 1 mM EDTA, 3 mM azide, 200 mM NaCl) containing DNAase I (Sigma) 200 μ g, and disrupted in a Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT, USA) with a micro-tip probe (output 6, duty cycle of 10) for 4 min. Unbroken whole cells were removed by centrifugation at 4350 *g* for 20 min. The supernate was brought to a volume of 10 ml with TEAN and centrifuged at 43700 *g* for 1 h at 4°C. The resulting pellet was extracted for 30 min at 37°C with 10 ml of TEAN containing N-sodium lauroyl sarcosine (SAR; Sigma) 1%, and recovered by centrifugation at 43700 *g* for 1 h at 4°C, after which the SAR extraction and centrifugation were repeated. Outer-membrane antigens were then washed with 10 ml of TEAN,

vortex mixed and centrifuged once more at 43700 *g* for 1 h at 4°C. The TEAN wash and centrifugation steps were repeated. The final pellet was collected by vigorous pipetting in 0.1 ml of TEAN buffer. Total protein was determined by a modification of the Lowry procedure¹⁴ with the addition of sodium dodecyl sulphate (SDS, BioRad) 0.2%. Outer-membrane antigen preparations were divided into portions and stored at -70°C.

Partial purification of CPA

Partially purified CPA was prepared by a modification of the above procedure. Frozen *V. cholerae* CVD 103 HgR cells (harvested from 1 L of medium) were thawed and lysed by the addition of 10 ml of TEAN buffer containing SAR 1%, and 200 μ g of DNAase I, vortex mixed and sonicated as described above. The lysate was incubated in a water bath at 37°C for 30 min to allow complete digestion of DNA and centrifuged at 43700 *g* for 20 min at 4°C. The pellet was resuspended in 10 ml of TEAN with SAR 1% and sonicated for 10 s. This suspension was vortex mixed, incubated at 37°C for 1 h and centrifuged at 43700 *g* for 1 h at 20°C. The supernate was discarded and 10 ml of TEAN with SDS 1% was added to the SAR-insoluble pellet. The suspension was again vigorously vortex mixed, incubated at 37°C for 1 h, and centrifuged. The pellet was washed twice in this way and then analysed by SDS-PAGE.

ELISA

The procedure was similar to that described previously.⁹ Partially purified CPA (10 μ g/well) was attached to polyvinyl-chloride (PVC) ELISA plates (Dynatech Laboratories Inc., Alexandria, VA, USA) by overnight incubation in bicarbonate coating buffer (pH 9.6) at 4°C. The following day, non-specific binding to PVC was blocked by incubation at 22°C for 1 h with 200 μ l of PBS containing Tween 20 1%, dried milk 5% and fetal calf serum 3%. Excess blocking reagent was removed by washing four times with PBS-Tween 20. To detect human serum antibody to CPA, sera were diluted 1 in 40 with blocking buffer and 100- μ l samples were reacted with CPA-coated plates at 37°C for 1 h. To establish the linearity and detection limits for the assay, serial 10-fold dilutions of CPA-specific MAb (NAK-258) (1 in 10 – 1 in 10000) were prepared in blocking buffer. Samples of 100 μ l were assayed in parallel with diluted human sera. After incubation, assay plates were washed four times with PBS-Tween 20 and incubated for 1 h at 37°C with homologous peroxidase-conjugated antisera diluted in blocking buffer. The conjugates were removed by washing four times with PBS-Tween 20 before the addition of the peroxidase substrate, 2,2'-azinobis-(3-ethylbenzthiazoline sulphonate) (Sigma). ELISA assays were performed twice in triplicate and

read on a Dynatech MR 700 ELISA spectrophotometer.

SDS-PAGE and Western blot analysis

Preparative electrophoresis was performed with a Hoefer SE 600 vertical slab gel electrophoresis unit. The procedure of Laemmli¹⁵ was followed. Samples of 40 µg of protein/lane were mixed with dissociation buffer and boiled for 2 min before loading lanes for SDS-PAGE. The stacking gel 3% and running gel 12% were electrophoresed at a stacking current of 15 mA and running current of 20 mA. After electrophoresis, gels were stained with silver stain (Pharmacia/LKB, Piscataway, NJ, USA).

For Western blot analysis, antigens were transferred to Immobilon P[®] membranes by use of a Hoefer TE52 transblot apparatus, with a voltage of 0.5 V for 3.5 h at 4°C. Lanes containing mol. wt standards and part of the antigen lane were excised from the blot, stained with Coomassie Blue R, and de-stained according to Laemmli.¹⁵ Non-specific binding sites of the unstained Immobilon blots were blocked with PBS containing Tween 20 1%, dried milk 5% and fetal calf serum 3%, for 1 h at 22°C. Blocking buffer was also used for dilutions of sera. Human sera or MAbs were diluted 1 in 20, and blots were incubated with sera for 1 h at 37°C with gentle shaking. Afterwards, blots were rinsed three times with PBS-Tween 20 and incubated with peroxidase-conjugated affinity-purified goat anti-serum to either mouse or appropriate human immunoglobulins. Blotted strips were rinsed three times with PBS-Tween 20 and exposed to the substrate, *o*-dianisidine (Sigma). Control sera and reagent controls were included for each blot. Immunoblots were performed twice for each antigen.

Results

Analysis of partially purified CPA

SDS-PAGE (fig. 1) showed that the final CPA preparation contained an 18-kDa protein and a second protein of > 200 kDa (fig. 1, lane C). Other fractions (fig. 1, lanes D and E), showed that the high-mol. wt protein band occurred in both SAR- and SDS-soluble preparations. Since the high-mol. wt protein could be differentiated from CPA by immunoblotting (see below), CPA was not further purified.

The detergent solubilisation procedure provided yields of 5–10 mg/L of cells. CPA was isolated with similar yields from *V. cholerae* strains 569B, 3083TR² and NIH 41. Growth of bacteria in low-iron 'T' medium increased the yields of CPA and reduced contamination with LPS and other outer-membrane antigens. CPA preparations sometimes contained two additional protein bands of *c.* 72 kDa and 32 kDa that were removed from the CPA preparation by the first SAR extraction and centrifugation (fig. 1, lane B); otherwise, this material co-purified with CPA.

Comparison of CPA, outer-membrane antigens and LPS within gels showed that the band corresponding to lipid A (*c.* 12 kDa) stained bright orange; the higher mol. wt repeating subunits of LPS stained brilliant yellow; and CPA stained a silver-grey colour. During the SAR solubilisation step, most LPS was removed from the CPA preparation (fig. 1, lane D). LPS was

Fig. 1. SDS-PAGE comparison of SAR-SDS purified CPA with purified *V. cholerae* LPS and outer-membrane antigens, stained with silver stain. Lane A, mol. wt markers; B, 72-kDa and 32-kDa outer-membrane antigens; C, SAR-insoluble, SDS-insoluble antigens (CPA is the 18-kDa protein); D, SAR-soluble proteins; E, SAR-insoluble, SDS-soluble outer-membrane antigens; F, *V. cholerae* LPS.

Fig. 2. CPA-ELISA of human sera. ELISA reactions of paired volunteers' sera were diluted 1 in 40 and reacted with purified CPA. Subject nos 1–10 were challenged with *V. cholerae*, and nos 11 and 12 were unchallenged controls. Error bars represent one standard deviation above the mean value ($n = 6$) observed in sera obtained before challenge.

Fig. 3. Western immunoblot of human sera reacted with outer-membrane antigens prepared from *V. cholerae* strain 569B grown under conditions of low iron availability. Lane A, Coomassie Blue R stained mol. wt standards; B, Coomassie Blue R stain of Imobilon-transferred antigens; C, immunoblot reacted with MAb NAK-258 (anti-CPA); D, immunoblot reacted with MAb SY-15 (anti-LPS); E, immunoblot reagent control. For all sera: lane a = serum before challenge with *V. cholerae*; lane b = serum 28 days after challenge. Subjects 1–5 were challenged with 10^6 *V. cholerae* N16961, El Tor:Inaba; and subjects 6–10 were challenged with 2×10^7 *V. cholerae* 569B, Classical:Inaba. Sera 11 and 12 were from unchallenged subjects.

Fig. 4. Western immunoblot of human sera reacted with CPA prepared from *V. cholerae* strain CVD 103-HgR grown under conditions of low iron availability. Lane A, Coomassie Blue R stained mol. wt standards; B, Coomassie Blue R stain of Imobilon-transferred antigens; C, immunoblot reacted with MAb NAK-258 (anti-CPA); D, immunoblot reacted with MAb SY-15 (anti-LPS); E, immunoblot reagent control. For all sera: lane a = serum before challenge with *V. cholerae*; lane b = serum 28 days after challenge. Subjects 1–5 were challenged with 10^6 *V. cholerae* N16961, El Tor:Inaba; and subjects 6–10 were challenged with 2×10^7 *V. cholerae* 569B, Classical:Inaba. Sera 11 and 12 were from unchallenged subjects.

not detected by silver stain in outer-membrane antigens (fig. 1, lane E), or in CPA preparations (fig. 1, lane C). CPA did not stain with Coomassie Blue R and stained weakly with silver stain. Therefore, the gel in fig. 1 was "over-developed" so that proteins in lanes B and C could be photographed. For this reason some lanes appeared to have more protein than others; however, each lane contained the same amount of protein.

ELISA

All human sera that were tested by ELISA showed some reactivity with CPA (fig. 2). The ELISA values of sera obtained after challenge with *V. cholerae* were more than two standard deviations above the mean value observed before challenge. An increase in serum ELISA reactivity was observed in the serum of all subjects after challenge, except subjects 7 and 11.

Western blotting

Western blots of outer-membrane antigens prepared from two *V. cholerae* strains of different biotype showed similar results with sera from volunteers. Sera from four of the 10 volunteers reacted weakly with outer-membrane antigens before challenge with *V. cholerae*. Six sera produced a stronger reaction with the 18-kDa antigen than with other outer-membrane antigens after challenge. The remaining sera reacted weakly with the 18-kDa antigen and with other outer-membrane antigens (fig. 3). No reactions were observed in immunoblots of pre-challenge sera obtained from subjects 11 and 12.

MAbs that were specific for CPA and LPS were included as controls in the immunoblots (fig. 3, lanes C and D). Immunoblots that were exposed to MAb SY-15 (fig. 3, lane D), showed bands coinciding with the high-mol. wt repeating subunits of LPS. Some of the sera that were treated with outer-membrane antigens showed weak reacting bands coinciding with LPS. Furthermore, the MAb controls provided evidence that the serum reactivity of CPA was separate and distinct from that of LPS. For instance, MAb SY-15 did not react with CPA in the immunoblots, and *vice versa*, MAb NAK-258 did not react with LPS in immunoblots of outer-membrane antigens (fig. 3).

Sera from volunteers were tested with the purified 18-kDa protein by immunoblotting to further demonstrate the independent reactivity of serum antibody with CPA (fig. 4). Eight of 10 sera obtained after challenge with *V. cholerae*, reacted with purified CPA. Serum from one of the unchallenged subjects also gave a positive reaction with CPA and outer-membrane antigens.

Discussion

The study of *V. cholerae* outer-membrane antigens is difficult since various physiological conditions regulate antigen expression. For instance, some antigens are expressed only *in vivo*,^{16,17} or in response to low-iron conditions in culture.^{12,17} Other proteins, such as the toxin co-regulated pilus, the 52-kDa *V. cholerae* outer-membrane protein and cholera toxin, are osmo-regulated.^{18,19} In this study, vibrios grown under conditions of low iron availability were used.

In the North American cholera vaccine trial⁷ from which sera were obtained for the present studies, one group of subjects was vaccinated with *V. cholerae* CVD 103 and another group was unvaccinated. Six months later, volunteers were challenged with virulent *V. cholerae*. Sera were collected 10 days before and 28 days after challenge. Vaccine efficacy in subjects who received CVD 103 HgR was 62%, and at six months

83% of the volunteers showed a rise (ELISA OD ≥ 0.2) in serum titre to cholera toxin, with a mean peak titre of 21.⁷

In the present study, sera from unvaccinated convalescent subjects reacted with purified CPA and other outer-membrane antigens. Increased Western blot reactions of sera with purified CPA strongly indicated that infection stimulated CPA antibody in volunteers. ELISA of sera diluted 1 in 40 showed that 80% of unvaccinated subjects exhibited a rise in serum antibody to CPA after challenge with *V. cholerae*. The high-mol. wt antigen was not reactive in Western blots, but it may have influenced ELISA results by increasing reactivity. Pre-existing serum reactivity with CPA and outer-membrane antigens occurred with the serum of some subjects before challenge and in some unchallenged subjects. However, in the original study from which these sera were obtained, pre-immune ELISA cross-reactivity with the vaccine preparations was not reported,⁷ but similar post-vaccination, pre-challenge reactivity was seen in Western blots (J. B. Kaper, personal communication).

To examine the possibility that binding of CPA with serum IgG was non-specific, the ELISA and Western blot results were compared. If the positive cut-off value of the ELISA was lowered to 0.15 OD, the results from Western blots and ELISA assays were in complete agreement. In Western blots, control sera nos. 11 and 12 and the pre-challenge sera all reacted similarly. We conclude from the two test results that the immunoblots gave specific results whereas the ELISA was quantitative but exhibited low-level non-specificity.

In a previous study, the serum IgM or IgA response of volunteers vaccinated with *V. cholerae* CVD 101 were measured by ELISA with whole vibrios, vibrio lysates, outer-membrane antigens, LPS, cholera toxin and the toxin co-regulated pilus.⁶ Among volunteers giving a positive test, a serological response occurred in the chronological order: cholera toxin before outer-membrane antigens before whole vibrios or LPS before vibrio lysate before toxin co-regulated pilus. The CPA component of the response to outer-membrane antigens was not investigated in that study, and Western blots were not done.

The present results provide evidence that the expression of CPA by challenge strains stimulates serum antibody which recognises CPA and that this reactivity is independent of biotype and serotype. The vibrio strains used in this study all contained CPA but more studies are needed to establish the role of CPA in cholera immunity. The biochemical identity of CPA and its prevalence in other bacteria is presently undergoing study.

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