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# Immunochemical Properties of the Major Outer Membrane Protein of *Vibrio cholerae*

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Antisera to the major outer membrane protein of *Vibrio cholerae* (molecular weight, 48,000) raised in rabbits (i) agglutinated several strains of *V. cholerae* and (ii) immunoprecipitated outer membrane proteins prepared from both the biotypes and serotypes of *V. cholerae*. Antibodies of all isotypes to the major outer membrane protein were detected in immune human sera by enzyme-linked immunosorbent assay. These results suggest that the major outer membrane protein was the common outer membrane antigen of *V. cholerae* which was immunogenic in humans.

*Vibrio cholerae* is a gram-negative bacterium which colonizes the small intestine of the gut, where it secretes an enterotoxin which binds to the mucosal epithelial cell and causes an outpouring of fluid into the intestinal lumen. Both antibacterial and antitoxic immunities are developed in the infected host (10). Although cholera is a toxin-mediated disease, recent evidence based on human volunteer studies suggests that antibacterial immunity plays a more important role in protecting humans than does antitoxic immunity (9).

*V. cholerae*, like other gram-negative bacteria, contains an outer membrane composed of both lipopolysaccharides (LPS) and proteins which provide the antibacterial immunity. In a recent paper, the composition of the outer membrane proteins of *V. cholerae* was described (4). It was observed that the outer membrane contained a major protein of molecular weight 48,000 (48K) which was present in all the biotypes (classical and El Tor) and serotypes (Ogawa and Inaba) of *V. cholerae*. This communication presents the immunochemical evidence which rigorously establishes that the 48K protein is the common outer membrane antigen of *V. cholerae*.

*V. cholerae* strains Inaba 10732 (classical), Inaba 3661 (El Tor), and Ogawa 10255 (El Tor) were obtained from the National Collection of Type Cultures, London, United Kingdom. *V. cholerae* Ogawa 395 (classical) was obtained as a gift from N. F. Pierce, The Johns Hopkins University, Baltimore, Md. *V. cholerae* classical strains Ogawa T-19765 and T-19767 and Inaba H-23448 and H-23055 and *V. cholerae* El Tor strains Ogawa U-12263, U-12512, U-12336, and U-12652 and Inaba U-12515, U-4286, U-12222, and U-12433 were obtained from the International Centre for Diarrhoeal Disease Research, Dacca, Bangladesh.

*V. cholerae* cultures were grown aerobically in semisynthetic or Syncase medium (3) at 37°C with continuous shaking up to the stationary phase of growth (8 h). The outer membrane proteins were prepared from the cell envelope fraction by Triton X-100 extraction as previously described (4). Membrane proteins were analyzed on 10% acrylamide slab gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the procedure of King and Laemmli (7).

*V. cholerae* 10732, a strain belonging to the Inaba serotype and the classical biotype, was used for the preparative isolation of the major outer membrane protein of molecular weight 48K. It was recovered from the polyacrylamide gel slab after electrophoresis as described previously (4). Briefly, the section of the gel containing the 48K protein was cut into slices and then eluted by shaking at 37°C for 12 h with 0.05 M $NH_4HCO_3$ containing 1% sodium dodecyl sulfate. After being extensively dialyzed against 0.05 M $NH_4HCO_3$, the extract was lyophilized.

Antisera to the 48K protein were raised in New Zealand white rabbits. The 48K protein suspension (100 μg in 0.25 ml of phosphate-buffered saline [pH 6.8]) was mixed with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). A sample of the emulsion (0.2 ml) was injected directly into the popliteal lymph nodes of rabbits. A booster dose was given on day 14. Rabbits were bled 1 week later. The immunoglobulins were isolated by precipitation of the immune serum with 33% ammonium sulfate (6).

Membrane proteins were solubilized at room temperature in Tris-hydrochloride (0.1 M; pH

6.8) containing 0.05% (wt/vol) sodium dodecyl sulfate and subjected to immunodiffusion according to the agar double diffusion technique of Ouchterlony (11). Peripheral wells were filled with 10 μl of the membrane protein solution. The central well was filled with 10 μl of the antiserum to the 48K protein. Diffusion was allowed to take place in a humidified chamber at room temperature.

To perform agglutination studies with the antiserum to the 48K protein, *V. cholerae* cultures were adjusted to $10^{10}$ cells per ml in 0.9% (wt/vol) sodium chloride. The antiserum was inactivated by heating at 56°C for 30 min. A twofold dilution of the antiserum was made in microtiter plates. Bacterial suspension (50 μl) was added to an equal volume of the appropriately diluted antiserum, and the agglutination pattern was recorded after incubation for 2 h at room temperature.

The enzyme-linked immunosorbent assay (ELISA) was applied to detect antibodies of all isotypes (immunoglobulin G [IgG], IgA, and IgM) to the 48K protein in human sera. The 48K protein was suspended in 0.05 M $NaHCO_3$ (pH 9.6) at the optimal concentration of 50 μg of protein per ml, determined by preliminary experiments. Individual wells of flat-bottom microtiter plates were coated with the suspension (100 μl) at room temperature for 16 h. The antigen was then discarded, and the wells were washed three times with phosphate-buffered saline (pH 7.4) containing 0.05% (vol/vol) Tween 20. ELISA was performed with human sera which had been preabsorbed with *V. cholerae* LPS belonging to both serotypes (Ogawa and Inaba) to remove anti-LPS antibodies. Immune sera (1 ml) were incubated with 6 mg of LPS, composed of equal amounts of the Ogawa and Inaba serotypes, at room temperature for 16 h, and the mixture was centrifuged at 105,000 × *g*. The absorbed sera were diluted in seven twofold steps from an initial dilution of 50, and the dilutions in duplicate were transferred to microtiter plates. The plates were incubated at room temperature for 1 h (IgG and IgA antibody determination) and also at 37°C for 2 h for the determination of IgM. Wells were then emptied and washed three times with phosphate-buffered saline (pH 7.4) containing 0.05% (vol/vol) Tween 20. Control wells containing no antigen were also filled with the antiserum. Swine anti-human IgG, IgA, or IgM alkaline phosphatase conjugate (Orion Diagnostica, Helsinki, Finland) was added and left at room temperature overnight. After three washes with phosphate-buffered saline (pH 7.4) containing 0.05% (vol/vol) Tween 20, 100 μl of the enzyme substrate (*p*-nitrophenylphosphate; Sigma Chemical Co., St. Louis, Mo.; 2 mg/ml of water) was added, and the plates were incubated at 22°C for 60 min. The enzyme reaction was read with a Titertek Multiscan plate reader at an absorbance of 405 nm.

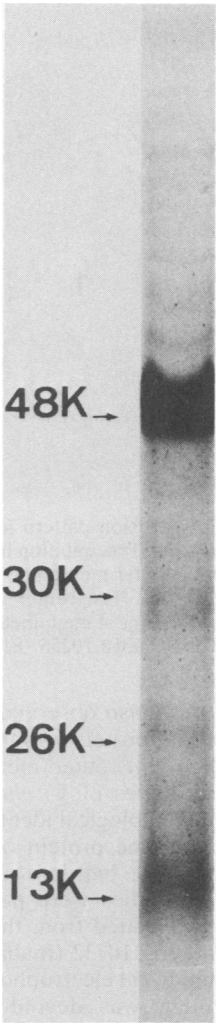


FIG. 1. Protein composition of the outer membrane of *V. cholerae* 10732 (classical). Electrophoresis was carried out on a slab polyacrylamide gel (10%) in the presence of sodium dodecyl sulfate according to the procedure of King and Laemmli (7).

Sera were collected from a healthy volunteer who received parenterally $8 \times 10^9$ killed cells of *V. cholerae*, composed of equal numbers of the Ogawa 50 (classical) and Inaba 48 (classical) serotypes. A negative control serum consisted of a pool of sera from newborn children from a cholera nonendemic region (Sweden).

The outer membrane proteins of *V. cholerae* 10732 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). The 48K protein was the major component. A few minor polypeptides of molecular weights 13K,

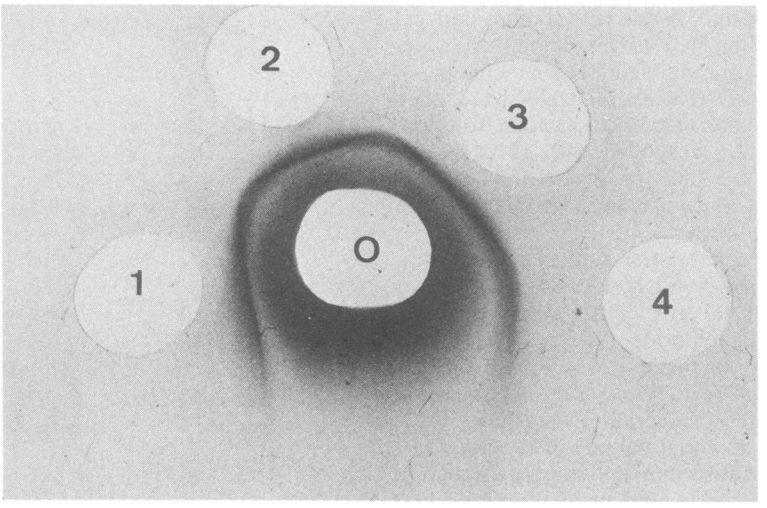


FIG. 2. Immunodiffusion pattern in agar gel showing the reactions of identity among the outer membrane proteins of *V. cholerae*. Precipitation lines were obtained by reacting the antiserum to the major outer membrane 48K protein with the outer membrane proteins prepared from a few strains of *V. cholerae* belonging to both the biotypes and serotypes. The center well (0) contained immunoglobulins to the major outer membrane 48K protein. Wells 1, 2, 3, and 4 contained outer membrane proteins prepared from strains Inaba 10732 (classical), Inaba 3661 (El Tor), Ogawa 10255 (El Tor), and Ogawa 395 (classical), respectively.

26K, and 30K were also observed. As reported previously, the 48K protein was the major component present in the outer membrane of all biotypes and serotypes of *V. cholerae* (4). To determine the immunological identity among the major outer membrane protein of *V. cholerae* belonging to both the biotypes and serotypes, immunodiffusion studies were performed. The 48K protein was isolated from the outer membrane of *V. cholerae* 10732 (Inaba) by preparative polyacrylamide gel electrophoresis. The purified 48K protein was devoid of any LPS moieties as detected chemically for carbohydrate moieties by the phenol-sulfuric acid method (1). Also, it did not produce any positive reaction when examined by the *Limulus* lysate gelation assay, a biological test used to detect endotoxic LPS moieties (8). Antisera to the 48K protein were raised in rabbits. To detect any anti-LPS activity, the antisera were checked by immunodiffusion against the LPS (Inaba) according to the method of Ouchterlony (11). No immunoprecipitation line was observed, suggesting that the antisera did not contain any anti-LPS activity.

Outer membrane proteins prepared from *V. cholerae* strains belonging to both the biotypes and serotypes were allowed to diffuse against the antisera to the 48K protein. As shown in Fig. 2, an immunoprecipitation line of identity was observed, which suggested that the 48K protein present in the outer membrane of various biotypes and serotypes of *V. cholerae* was immunochemically identical.

Quantitative agglutination studies were performed with the antisera to the 48K protein in microtiter plates. Sixteen strains of *V. cholerae* belonging to both the biotypes and serotypes were used. The antisera agglutinated all these bacteria; the titer remained at around 256. The preimmune serum did not cause agglutination. These results further demonstrate that the 48K protein was the common outer membrane antigen of *V. cholerae*.

Since the antisera to the 48K protein agglutinated *V. cholerae* of all biotypes and serotypes, it is evident that this protein was exposed on the bacterial cell surface and hence should be immunogenic in humans. To determine whether immune responses were developed to the 48K protein in humans, sera were collected from a healthy volunteer who had received parenterally a bivalent cholera vaccine consisting of equal amounts of Ogawa and Inaba strains. To detect immune responses to only anti-48K protein antibodies, anti-LPS antibodies were removed from the immune sera by absorption with the LPS from Ogawa and Inaba serotypes. The efficacy of absorption was checked by immunodiffusion, since the absorbed sera did not produce any immunoprecipitation reaction to the LPS, as determined by the Ouchterlony method (11). Applying the ELISA technique, antibodies to the 48K protein were detected in the immune human sera (Fig. 3). These antibodies belonged primarily to the IgG class, although a significant rise in IgA anti-48K protein titers was also observed. Since the volunteer had received the

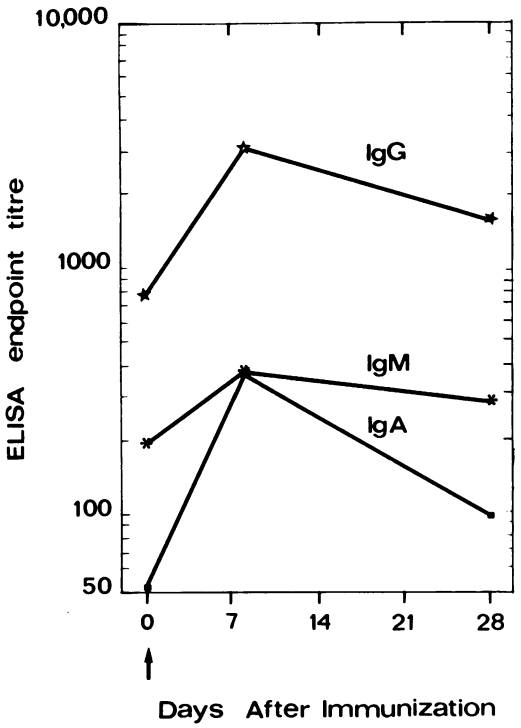


FIG. 3. Kinetics of antibody (IgG, IgM, and IgA) responses to the major outer membrane 48K protein of *V. cholerae* in human sera as determined by the microtiter ELISA technique. The volunteer was immunized with $8 \times 10^9$ cells consisting of equal numbers of Ogawa 50 (classical) and Inaba 48 (classical) strains. Immune sera were collected at different days after immunization.

cholera vaccine 6 months before the present immunization, the sera had significant levels of measurable anti-48K proteins before immunization. There was a rapid rise in the anti-48K titer on day 8 after immunization, which is characteristic of a secondary immune response. It is evident from these results that the 48K protein was immunogenic in a human. Since the volunteer was immunized with *V. cholerae* strains (Ogawa 50 and Inaba 48) different from the strain (Inaba 10732) from which the 48K protein was isolated, these results further demonstrate that the 48K protein was the common outer membrane protein antigen of *V. cholerae*.

This is the first paper in which the immune response to the major outer membrane protein of *V. cholerae* in a human is described. Currently, there is a great need for a cholera vaccine which would provide protection against all biotypes and serotypes of *V. cholerae*. Moreover, the antigen should be nontoxic. By raising antiserum to the 48K protein, it has been rigorously demonstrated in this investigation that the 48K protein is the common antigen of *V. cholerae*. The ability to induce the production of agglutinating antibody is considered a test for antigenic potency of a cholera vaccine (2). The antibodies to the 48K protein had agglutinating activities toward *V. cholerae* of all the biotypes and serotypes. In addition, previous investigations have demonstrated that the outer membrane proteins of *V. cholerae* lack toxic properties attributable to the LPS (5). All these criteria strongly suggest that the major outer membrane 48K protein should be considered as a suitable vaccine candidate against cholera.

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