CHAPTER IV
MATERIALS AND METHODS

1. Preparation of antigens

1.1 Crude and purified *V. cholerae* TcpA

1.1.1 Bacterial strains

The *tcp A* gene of *V. cholerae* O1 biotype El Tor strain O17SR has been cloned into pBlueScript KS vector (Ogierman *et al.*, 1993), subcloned into vectors pTrcHis2 A, B, C and subsequently transformed into *E. coli* DH5α. The recombinant clone that carried pTrcHis2 A expresses TcpA (Kalambaheti *et al.*, unpublished data). This clone was used for preparing crude and purified *V. cholerae* TcpA. The TcpA was prepared as follow:

1.1.2 Protein expression and purification

Ten ml of LB broth containing 50 μg ampicillin per ml was inoculated with a single recombinant *E. coli* colony and incubated overnight at 37°C with shaking (225-250 rpm). On the next day, 250 ml of LB containing 50 μg ampicillin per ml contained in a 1 liter flask was inoculated with 5 ml of the overnight culture. The culture was incubated at 37°C for 3 hr with aeration until the culture reached an OD_{600} at 0.4 (the cells were in mid-log phase). IPTG was added to a final concentration of 1 mM (0.5 ml of a 100 mM IPTG stock to each 50 ml of the LB culture) and the culture was incubated at 37°C for 5 hr with shaking. The preparation was centrifuged at 8,000 × *g* for 15 min at 4°C to precipitate the pellet. The pellet was resuspended in 8 ml of guanidinium lysis buffer, pH 7.8 and then slowly rocked the cells for 5-10 min at room temperature to assure thorough cell lysis. The cells were lysed either by using MSE ultrasonic disintegrater on ice for three of the 5-second pulses at high intensity (20 kHz) or French Pressure. The preparation was centrifuged at 3,000 × *g* for 15 min to

pellet the cell debris, and then the supernatant was transferred to a new tube. An aliquot of the supernatant (5 µl) was removed for SDS-PAGE analysis. The remaining supernatant (ready to be purified) was stored on ice or at -20°C.

The ProBond Purification System was used to purify 6 × His-tagged recombinant TcpA proteins by using the denaturing buffers, columns, and cell lysate. Eight ml of the recombinant *E. coli* lysate was added to a prepared purification column, allowed binding for 15-30 min at room temperature using gentle agitation (*e.g.*, on a rotating wheel) to keep the resin suspended in the lysate solution. The resin was settled by gravity or low speed centrifugation (800 × *g*), and the supernatant was carefully aspirated. The column was washed with 4 ml of Denaturing Binding Buffer, pH 7.8 by resuspending the resin in the buffer and rocking for two min, then the resin was settled by gravity or low speed centrifugation (800 × *g*). Then the supernatant was carefully aspirated. This step was repeated one more time. The column was washed with 4 ml of Denaturing Wash Buffer pH 6.0 by resuspending the resin and rocking for two min. The resin was settled by gravity or low speed centrifugation (800 × *g*) and then the supernatant was carefully aspirated. This step was repeated once more for a total of two washes with Denaturing Wash Buffer, pH 6.0. The column was washed with 4 ml of Denaturing Wash Buffer pH 5.3 by resuspending the resin and rocking for two min. The resin was settled by gravity or low speed centrifugation (800 × *g*) and then the supernatant was carefully aspirated. This step was repeated once more for a total of two washes with Denaturing Wash Buffer, pH 5.3. The column was clamped in a vertical position and snaped off the cap on the lower end. The protein was eluted by adding 5 ml of Denaturing Elution Buffer, pH 4.0. One milliliter fractions were collected and the elutions were monitored by taking OD_{280} readings of individual fractions. The fractions that contained the peak absorbance were pooled and dialyzed against 10 mM Tris, pH 8.0, 0.1% Triton X-100 overnight at 4°C to remove the urea. The dialyzed material was concentrated. The resulting protein preparation was ready for use as one of the oral cholera vaccine components.

Diagram 1 Steps of TcpA purification by ProBond purification system

Resuspend the ProBond™ resin by inverting and gently tapping the bottle repeatedly

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Pipette 2 ml of the resin into a 10-ml Purification Column

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Pellet the resin by low-speed centrifugation (800 × *g* for 1 min) and gently aspirate the supernatant

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Add 6 ml of sterile, distilled water and resuspend the resin by inverting and gently tapping the column

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Repellet the resin by low-speed centrifugation and gently aspirate the supernatant

↓

Add 6 ml of Denaturing Binding Buffer, pH 7.8 and resuspend the resin by inverting and gently tapping the column

↓

Repellet the resin by low-speed centrifugation and gently aspirate the supernatant and repeat this step one more time

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Add 8 ml of bacterial lysate to a prepared Purification column and bind for 15-30 min at room temperature with gentle agitation

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Repellet the resin by low-speed centrifugation and gently aspirate the supernatant

↓

Wash the column with 4 ml of Denaturing Binding Buffer, pH 7.8 by resuspending the resin and rocking for 2 min

↓

Repellet the resin by low-speed centrifugation and gently aspirate the supernatant and repeat this step one more time

Diagram 1 Steps of TcpA purification by ProBond purification system (Cont.)

Wash the column with 4 ml of Denaturing Wash Buffer, pH 6.0 by resuspending the resin and rocking for 2 min

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Repellet the resin by low-speed centrifugation and gently aspirate the supernatant and repeat this step one more time

↓

Wash the column with 4 ml of Denaturing Wash Buffer, pH 5.3 by resuspending the resin and rocking for 2 min

↓

Repellet the resin by low-speed centrifugation and gently aspirate the supernatant and repeat this step one more time

↓

Clamp the column in a vertical position and snap off the cap on the lower end and add 5 ml of Denaturing Elution Buffer, pH 4.0

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Collect 1 ml fractions and monitor the elution by taking OD_{280} reading of the fractions

↓

Pool the fractions that contain the peak absorbance and dialyze against 10 mM Tris, pH 8.0, 0.1 % Triton X-100 overnight at 4°C

1.2 Procholeragenoid (P)

P is produced by heat-mediated conversion of CT (cholera toxin/choleragen) to the natural toxoid (B subunits). This is done by heating CT at 65°C for 15 min. The toxicity after the heating should be less than 5% of the native CT. Prolonged heating up to 25 min reduced its residual CT-like toxicity to less than 1% (Pierce *et al.*, 1983).

1.2.1 Bacterial culture

A loopful from stock culture of *E. coli* MC1061 (pKTJ5-15X) harboring the cholera toxin gene, which was a kind gift from Professor Hisao Kurazono, Department of Medical Technology, University of Okayama, Japan, was streaked onto ampicillin agar plate (100 μg/ml) for isolation of colonies. The plate was incubated overnight at 37°C. A loopful of the organisms from the colonies was inoculated to each 10 ml volume of LB broth supplemented with ampicillin (100 μg/ml), and grown for 3 hr at 37°C in a shaking water bath. Seven ml of *E. coli* MC1061 culture was aseptically delivered to each 750 ml LB broth supplemented with ampicillin (100 μg/ml) contained in a 2 liter flask and incubated at 37°C for 18 hr with vigorous shaking.

1.2.2 Harvest of bacterial culture and preparation of crude CT

The bacterial growth in LB flask was harvested by centrifugation, then the bacterial pellet was resuspended in 0.9% NaCl in 10 mM Tris-HCl buffer, pH 8.6, and disrupted by sonication at 20 kHz per second for 2 min, five times. After removing the cell debris by centrifugation, the toxin was precipitated by adding solid ammonium sulfate to 65% saturation (430 g/L). The precipitate was then resuspended in TEAN buffer, pH 7.4, dialyzed against the same buffer and used as a crude cholera toxin. Protein content of the preparation was determined by Bradford's method (Bradford, 1976).

1.2.3 Purification of CT by using immobilized galactose column

Galactose affinity column was used for CT purification. The resin was commercially available from Pierce Co. (Rockford, USA). The resin was packed into a column (1.5 × 10 cm), equilibrated with TEAN buffer, pH 7.4. Crude CT was loaded and eluted by 1 M galactose in TEAN buffer, pH 7.4. The eluent was collected into fraction tubes; 1 ml per fraction. All fractions were measured at OD_{280} nm and monitored the purity of fractions by running on SDS-polyacrylamide gel electrophoresis. The purified fractions were collected, pooled together, concentrated and the amount of protein of the preparation was determined. This preparation should reveal homogeneous proteins, which migrated as two protein bands corresponding to the A and B subunits of CT on SDS-PAGE. P was produced by heating CT at 65°C for 15 min. The toxicity after the heating should be less than 5% of native CT (Pierce *et al.*, 1983).

Diagram 2 Steps of cholera toxin preparation

E. coli MC1061 was cultured in LB broth supplemented with ampicillin (100 μg/ml) for 18 hr at 37°C with vigorous shaking

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The bacterial cells were collected by centrifugation at 8000 × *g* for 15 min

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The cell pellet was resuspended in 0.9% NaCl in 10 mM Tris-HCl buffer, pH 8.6 and disrupted by sonication

↓

The cell debris was removed by centrification at 8000 × *g* for 15 min

↓

The toxin was precipitated by adding solid ammonium sulfate to 65% saturation

↓

The precipitate was resuspended in TEAN buffer, pH 7.4 and dialyzed against the same buffer

↓

The precipitation was used as crude cholera toxin

↓

The immobilized D-galactose was packed into a column (1.5×10 cm) and washed with excess volume of TEAN buffer, pH 7.4

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Crude CT was loaded and allowed binding to the resin for 30 min

↓

The excess crude cholera toxin was washed by TEAN buffer, pH 7.4 until OD_{280} reached 0

↓

The toxin was eluted by 1 M galactose in TEAN buffer, pH 7.4

↓

Collect 1 ml fractions and monitor the elution by taking OD_{280} reading of the fractions

Diagram 2 Steps of cholera toxin preparation (Cont.)

All fractions were determined the purity by running on SDS-PAGE

↓

The purified fractions were collected, pooled together, concentrated, and determined the amount of protein of the preparation

1.3 Commercialized cholera toxin (CT)

CT was purchased from Sigma (Sigma Chemical Co., USA) and used as one of the antigens for ELISPOT assay for ASC.

1.4 *V. cholerae* lipopolysaccharide (LPS)

1.4.1 Bacterial culture

A loopful from stock culture of *V. cholerae* El Tor biotype, streptomycin-resistant Ogawa 17 (O17SR) was streaked onto streptomycin agar plate for isolation of colonies and incubated overnight at 37°C. A loopful of the organisms from the smooth colonies was inoculated to each 10 ml-volume of trypticase soy broth (TSB), and incubated for 4 hr at 37°C with shaking. Three ml of the bacterial culture was aseptically delivered to each Roux bottle containing agar slant made of 250 ml of trypticase soy agar (TSA). The inoculum was spread over the surface of the agar by rocking the bottle. The Roux bottles were then incubated at 37°C for 48 hr.

1.4.2 Harvest of bacterial culture

The bacterial growth on the surface of the agar in Roux bottles was harvested using glass beads and cold normal saline solution (NSS). The harvested bacterial suspensions were washed with NSS by centrifuging three times at 10,000 × *g* for 20-25 min at 4°C. The final bacterial pellet was resuspended in ultra pure distilled water, collected into a 2-liter beaker stirred at room temperature. The dry weight of the bacterial suspension was determined and then adjusted to 10 mg/ml NSS.

1.4.3 Phenol water extraction

The bacterial suspension (10 mg/ml NSS) was preheated on a hot plate to about 50°C in a fume hood with stirring. An equal volume of prewarmed 90% phenol was added and the temperature was kept at 65-68°C for 30 min while the suspension is continuously stirred. This preparation was then cooled down to room temperature, and stored overnight at 4°C. The suspension was centrifuged at 5,000 × *g* for 20 min at 4°C. The upper aqueous layer was pipetted into a graduated cylinder and the volume

recorded. An equal volume of cold 95% ethanol was added to precipitate the nucleic acids which was then removed by filtering through layers of glass wool about 2-3 times. Four more volumes of ethanol was added to the preparation and stored overnight at 4°C. The preparation was centrifuged at 10,000 × *g* for 20 min at 4°C to precipitate the LPS. The ethanol was discarded. The pellet was dissolved in distilled water (crude LPS). This solution was dialysed overnight at 4°C against distilled water. After this, the solution was centrifuged at 10,000 × *g* for 20 min at 4°C to separate the solid impurities. The supernatant containing crude LPS was centrifuged at 100,000 × *g* for 3 hr at 4 °C (MSE Supraspeed 50 centrifuge). The wax-like deposit (crude LPS) was resuspended in ultra pure-distilled water to homogeneous solution. The dry weight of the preparation was determined. LPS preparation was subjected to SDS-PAGE and stained with Coomassie Brilliant Blue stain (should not see any stained material) and Silver stain (should reveal diffuse or ladder pattern brown bands).

Diagram 3 Steps of LPS preparation by using phenol-water method

V. cholerae O17SR was cultured in TSB for 4 hr at 37 °C with vigorous shaking

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Three ml of the bacterial cells were spread onto the Roux bottle containing agar slant of trypticase soy agar

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The roux bottles were incubated at 37°C for 48 hr

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The bacterial cells were harvested by using the sterile glass beads and NSS

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The harvested bacterial suspensions were washed three times with NSS by centrifugation at 10,000 × *g* for 20 min

↓

The bacterial pellet was resuspended in ultra pure distilled water and the dry weight was adjusted to 10 mg/ml NSS

↓

The bacterial suspension was preheated to about 50°C with stirring

↓

An equal volume of prewarmed 90% phenol was added and the preparation was heated at 65-68°C for 30 min

↓

The preparation was cooled down and stored overnight at 4°C

↓

The suspension was collected and centrifuged at 5,000 × *g* for 20 min at 4°C

↓

The upper aqueous layer was collected and the equal volume of cold 95% ethanol was added

↓

The preparation was filtrated through the layers of glass wool about 2-3 times

Diagram 3 Steps of LPS preparation by using phenol-water method (Cont.)

Four more volume of the cold 95% ethanol was added to the preparation and stored at 4°C for overnight

↓

The preparation was centrifuged at 10,000 × *g* for 20 min at 4°C

↓

The pellet was dissolved in DW and then repeat the extraction two more times

The preparation was dialyzed overnight against ultra pure distilled water

↓

The preparation was centrifuged at 10,000 × *g* for 20 min at 4°C to remove the cell debris

↓

The supernatant containing LPS was centrifuged at 100,000 × *g* for 3 hr at 4°C

↓

The wax-like deposit was resuspended in ultra pure distilled water to homogeneous solution

↓

The dry weight was determined

2. Protein determination

The protein content of a given preparation was determined by the Bradford method (Bradford, 1976). This method relies on the binding of the dye (Coomassie Blue G250) to protein. Bovine serum albumin (Bio-Rad Protein Assay Standard II, Bio-Rad, USA) was used as a standard range between 0-500 μg/ml. The standard was diluted two fold serially and 10 μl of each dilution as well as diluted or undiluted unknown samples were mixed individually with 200 μl of Bradford dye reagent (Protein Assay, Bio-Rad, USA). All mixtures were incubated at room temperature for 5 min and OD values were read *versus* blank (color reagent only) at 595 nm. Standard curve was plotted between the read-out OD and the concentrations of the standard. The protein concentrations of unknowns were read or calculated from the standard curve and multiplied by the dilution factor (if any).

3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is the most widely used method for qualitative analysis of protein mixture. It is particularly useful for monitoring protein purification. The SDS-PAGE was carried out in the 8.0 × 7.3 cm vertical slab gel prepared by using the casting apparatus (Mini-PROTEAN-III Cell, Bio-Rad, USA). A 4% acrylamide stacking gel and a 12% acrylamide separating gel were used. Approximately 5 ml of separating gel was poured per a slab gel, overlayered with distilled water and wait for polymerization at least 20 min. Unpolymerized gel monomers were removed and then replaced with the stacking gel mixture with the comb inserted from the upper side of the separating gel. The stacking gel in the casting apparatus was kept at room temperature for polymerization at least 45 min. The comb was removed, wells formed by comb were carefully washed with running buffer. Then the cassette was transferred to the electrophoretic chamber and ready for loading the samples.

Samples to be run on SDS-PAGE were boiled for 4 min in equal part of 4 × sample buffer (sample: 4 × sample buffer = 1:1) containing SDS with and without any reducing agent. SDS is an ionic detergent and binds strongly to, and denatures the protein. The treated samples were carefully applied into individual wells. Low molecular weight standard (Pharmacia Fine Chemicals, Sweden) or pre-stained SDS-PAGE broad range standard (Bio-Rad, USA) was included in one slot of each slab gel.

Care was taken not to contaminate the adjacent wells with the samples. Electrophoresis was done in an electrophoretic chamber and with an electric power supply (model 3,000/300, Bio-Rad, USA) at a constant voltage (200 volts). After electrophoresis, the gel was removed from the glasses. It was then stained by either Coomassie Brilliant Blue or Silver stain.

4. Staining

Protein bands were visualized by soaking the SDS-PAGE gel in Coomassie Brilliant Blue staining solution at room temperature for 1-2 hr(s) on a platform rocker. After staining, the excess stain was removed and the gel was destained in a destaining solution, high methanol and low methanol, respectively, until the background is clear by changing the destaining solution frequently. If the color of the protein bands faded, it could be restored by restaining. When destaining is completed, the gel was either immediatedly photographed, scanned and stored the photo in computer or dried on the paper (Cellophane membrane backing, Bio-Rad, USA).

Silver staining is very sensitive staining method, which can detect nanogram quantities of proteins, glycoproteins or glycolipids. First, the gel was fixed by fixative reagent for 20 min with gentle rocking. The gel was then washed with ultra-pure distilled water 2 times, 10 min each, with gentle rocking and then transferred to silver reagent. The gel was developed until the solution turned yellow or until brown-smoky precipitate appeared. The silver reagent was poured off and the reaction was stopped with 5% acetic acid (v/v) for 5 min. The gel was stored in 1% glycerol in ultra-pure distilled water prior to gel drying or scanning.

5. Preparation of antisera

5.1 Preparation of rabbit anti-cholera toxin serum

Cholera toxin (Sigma Chemical Co., USA) was injected intravenously to a 2-2.5 kg rabbit in 1 ml doses using normal saline solution as diluent. The toxin was administered as the following immunization schedule:

Day	Concentration of CT (μg per ml)
0	4
8	10
15	20
33	40
43	40
50	40

The rabbit was bled on the 64th day through the heart. After blood has coagulated, the serum was separated; the titer of the serum was determined by indirect ELISA using CT as antigen and then stored at -70°C in 2-ml aliquots.

5.2 Preparation of rabbit anti-TcpA serum

5.2.1 Preparation of TcpA for rabbit immunization

Ten ml of LB broth containing 50 μg ampicillin per ml was inoculated with a single recombinant *E. coli* colony and incubated overnight at 37°C with shaking (225-250 rpm). On the next day, 50 ml of LB containing 50 μg ampicillin per ml was inoculated with 1 ml of the overnight culture. The culture was incubated at 37°C for 3 hr with aeration until the culture reached an OD_{600} at 0.4 (the cells were in mid-log phase). IPTG was added to a final concentration of 1 mM (0.5 ml of a 100 mM IPTG stock to each 50 ml of the LB culture) and the culture was incubated at 37°C for 2 hr with shaking. The preparation was centrifuged at 8,000 × *g* for 15 min at 4°C to precipitate the pellet. The pellet was resuspended in 8 ml of guanidinium lysis buffer,

pH 7.8 and then slowly rocked the cells for 5-10 min at room temperature to assure thorough cell lysis. The cells were lysed either by using MSE ultrasonic disintegrater on ice for three of the 5-second pulses at high intensity (20 kHz) or by using French Pressure unit. The preparation was centrifuged at 3,000 × *g* for 15 min to pellet the cell debris, and then the supernatant was transferred to a new tube.

The recombinant *E. coli* lysate was added to a prepared purification column, allowed binding for 15-30 min at room temperature using gentle agitation (*e.g.*, on a rotating wheel) to keep the resin suspended in the lysate solution. The resin was settled by gravity or low speed centrifugation (800 × *g*), and the supernatant was carefully aspirated. The column was washed two times with 4 ml of Denaturing Binding Buffer, pH 7.8 by resuspending the resin in the buffer and rocking for two min, then the resin was settled by gravity or low speed centrifugation (800 × *g*). Then the supernatant was carefully aspirated. The column was then washed two times with 4 ml of Denaturing Wash Buffer pH 6.0 by resuspending the resin and rocking for two min. The resin was settled by gravity or low speed centrifugation (800 × *g*) and then the supernatant was carefully aspirated. The column was washed two times with 4 ml of Denaturing Wash Buffer pH 5.3 by resuspending the resin and rocking for two min. The resin was settled by gravity or low speed centrifugation (800 × *g*) and then the supernatant was carefully aspirated. The column was clamped in a vertical position and snaped off the cap on the lower end. The protein was eluted by adding 5 ml of Denaturing Elution Buffer, pH 4.0. One milliliter fractions were collected and the elutions were monitored by taking OD_{280} readings of individual fractions. The fractions that contained the peak absorbance were pooled and dialyzed against 10 mM Tris, pH 8.0, 0.1% Triton X-100 overnight at 4°C to remove the urea. The dialyzed material was concentrated and the amount of protein was determined. The preparation was subjected to run on SDS-PAGE by using ten-well comb. The TcpA bands located at about 25 kDa from the TcpA separated-polyacrylamide gel were collected by cutting the gel using a clean and sharp scalpel in minimal size of the gel slice. Then the gel slices (total number =4) were minced with sterile pestle and mortar and resuspended in a small volume of sterile normal saline solution.

5.2.2 Rabbit immunization

The rabbit was injected subcutaneously on days 1, 14, 28, 42, and 56 with the TcpA in minced gel. The rabbit was bled on the 70th day through the heart. After blood has coagulated, the serum was separated and the titer of the antiserum was determined by indirect ELISA using pure TcpA as antigen. The antiserum was then stored at -20°C in 2-ml aliquots.

5.3 Anti-*V. cholerae* O1 LPS antibody

The monoclonal antibody produced by the hybridoma clone 27E10 (MAb 27E10) which reacts specifically to the whole cell homogenate and LPS of all *V. cholerae* O1 strains (Chaicumpa *et al.*, 1995) was used to detect and enumerate anti-LPS producing cells.

6. Preparation of liposome-associated vaccine plus bacterial CpG DNA

Antigens of *V. cholerae*, namely LPS, crude TcpA, and P were entrapped in liposomes by the bath-sonication method. Each dose of this vaccine for one rat consisted of *V. cholerae* antigens, *i. e.* 5 mg of LPS, 5 mg of crude TcpA, 200 μg of P, and 100 μg of CpG DNA (ODN#1826). Liposomes were prepared from two batches of lipid solution (batches X and Y). Each batch consisted of 10 mg of bovine brain sphingomyelin and 4 mg of cholesterol dissolved in 1.5 ml of chloroform and 1 ml of ether. The antigens of one dose of vaccine were dissolved in 0.5 ml of distilled water and mixed with batch X of the lipids, while 0.5 ml water alone was put into batch Y of the lipids. The preparations of both tubes were subjected separately to sonication at 20 kHz per second for 3 min each three times or until each formed a homogeneous single phase emulsion. The contents of the two tubes were mixed together and dried at 40°C under low vacuum in a rotary evaporator. When the preparation is dried to a gel-like consistency, 3 ml of 5% $NaHCO_3$ (vaccine diluent) was added to make a homogeneous preparation and mixed with 100 μg CpG DNA.

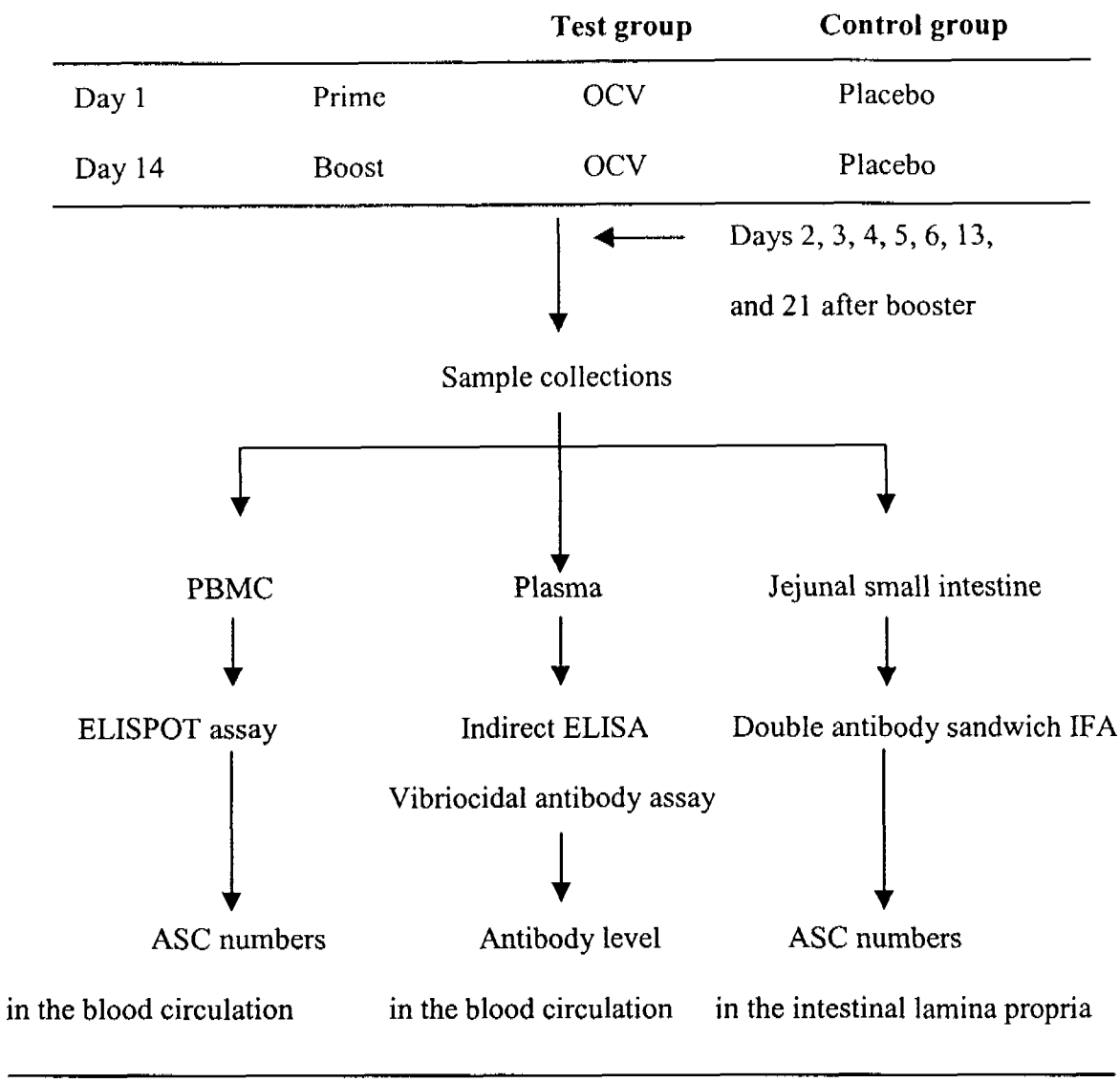
7. Experimental animals and oral immunization

Eight-week-old male Wistar rats were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon-Pathom Province, Thailand. The animals were treated well and allowed to familiarize themselves with the new domesticated conditions for one week before commencing the experiments.

One day before immunization, the rats were starved for at least 15 hr and 1 ml of 5% $NaHCO_3$ was given orally to each of them to reduce gastric acidity prior to vaccination. They were then divided into two groups, test and control groups, of 21 rats and 7 rats, respectively. At day 1, the rats of either test or control group were primed orally with the vaccine contained in 3 ml of 5% $NaHCO_3$ (vaccine diluent) or placebo (3 ml of the vaccine diluent alone), respectively. All rats were returned to the domesticated cages with adequate supply of food and water in an air-conditioned room (25°C). Fourteen days later, the primed rats were boosted appropriately with the same vaccine or placebo, and at days 2, 3, 4, 5, 6, 13, and 21 after the booster immunization, PBMC, plasma, and small intestine were collected from each rat (three rats of test group and one rat of control group per each time point). The steps for the immunization and specimen collection are summarized in Diagram 4.

For oral immunization, the cannula with a Luer fitting and a rounded metal end was used for oral dosage to rat. The rat was held with its head backward so that the throat and esophagus were lined up as straight as possible. The cannula was gently inserted down the throat as far as the lower esophagus. If resistance is encountered, the cannula may have entered the trachea. In that case, the cannula was then withdrawn, and a second attempt was made.

Diagram 4 Steps for animal immunization, sample collection, and antibody assessment



OCV: Oral cholera vaccine

Placebo: 5% $NaHCO_3$ (3 ml)

8. Sample collection and antibody assessment

At days 2, 3, 4, 5, 6, 13, and 21 after the booster immunization, anticoagulated whole blood (please see section 8.1 below) and small intestine were collected from each rat as mentioned in section 7. Food was withheld from all rats whereas drinking water was supplied. After starving for 15 hr, each rat was ether anesthesized. The ether jar was arranged with grid over the cotton wool soaked in ether, so that the animal only breathes the fumes, but was not come in contact with the diethyl ether which will irritate the skin. The rat was sufficiently anesthethized when the breathing was regular and slow, and when it did not move. Individual blood was collected by aseptically heart puncturing into heparinized tube before it was killed by cervical dislocation. The abdominal cavity of each rat was opened, the whole length of small intestine was collected and the serosal surface was washed with cold NSS to get rid of blood and then the mucosal surface was washed three times in excess volume of cold 0.01 M PBS, pH 7.2 in order to remove the intestinal content. The middle part of small intestine (28-32 cm from pyrolus) was cut into pieces of 1 cm in length and then were fixed overnight in the fixative solution (paraformaldehide). Each of these jejunal segments was then placed in disposable mold containing embedding medium (O.C.T. Compound, Miles Scientific, USA) and kept at -70°C.

PBMC were used for ELISPOT assay to enumerate antibody secreting cells against all immunogens in the blood circulation. Plasma samples were used for indirect ELISA and vibriocidal antibody assay to determine serum antibody levels. Intestinal tissues were used in a double antibody sandwich immunofluorescence (IF) for enumerating antibody secreting cells (ASC) against all immunogens in the intestinal lamina propria.

8.1 Isolation of mononuclear cells

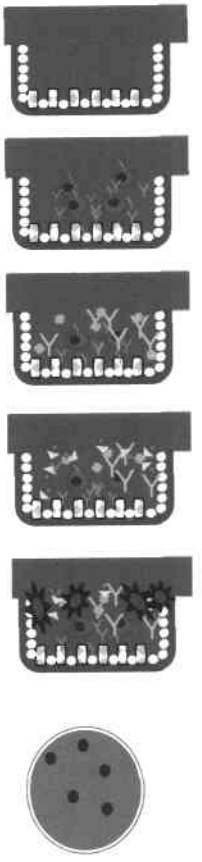
Peripheral blood mononuclear cells (PBMC) were isolated from the anticoagulated blood samples of individual rats collected in the heparinized tubes by Ficoll-Paque (Ficoll-Paque Research Grade, Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation (density = 1.077). Two volumes of RPMI-1640 medium was added to the blood sample, mixed and the mixture was overlayed onto the Ficoll solution. The volume of the Ficoll used was approximately 1/3 volume of the blood/RPMI-1640 mix. The preparation was centrifuged for 30 min at 300 × *g*. The plasma fraction (upper most layer) was collected to the sterile microtube and stored at -70°C for indirect ELISA and vibriocidal antibody assays. PBMC were collected from the interface and were then washed twice with RPMI-1640 and resuspended in 1 ml complete medium (RPMI-1640 with 5% fetal bovine serum). Viable cells were identified by trypan blue exclusion and counted with a hemocytometer. Cell suspension was adjusted to the final concentration of 5×10^5 viable cells per ml of complete RPMI-1640 medium (Appendix B).

8.2 Antigen-specific antibody-secreting cell (ASC) assay

All steps were performed under sterile condition. MultiScreen-IP plate (Millipore, Bradford, USA) was coated with 50 µl of 1 µg /well of individual antigens (CT, LPS or purified TcpA [pTcpA]) in 0.01 M PBS, pH 7.4 at 4°C overnight. The plate was then washed five times with 0.01 M PBS, pH 7.4 and blocked with 3% BSA in 0.01 M PBS, pH 7.4 for 2 hr at 37°C. The plate was washed five times with 0.01 M PBS, pH 7.4 and 200 µl of the cell suspension was added to each well at a concentration of 5×10^5 cells per ml. The plate was incubated at 37°C with 5% CO_2 with humidity for 16 hr and was then washed three times with 0.01 M PBS, pH 7.4 followed by three additional washes with 0.05% Tweeen-20 in 0.01 M PBS, pH 7.4 (PBST). Fifty microliters of a biotinylated anti-rat immunoglobulin diluted with a diluent (Appendix B) (1:1,000) were then added to each well and incubated for 1.5 hr at 37°C. The plate was washed five times with PBST. Fifty microliters of streptavidin-alkaline phosphate diluted with a diluent (1:1,000) were then added to each well and incubated for 1.5 hr at 37°C. The plate was washed three times with

PBST followed by three additional washes with 0.01 M PBS, pH 7.4. Fifty microliters of BCIP-NBT substrate (Bio-Rad) were added to each well, and spots were developed for 8 min at room temperature. Substrate was emptied from the wells and then the wells were rinsed with distilled water to stop the reaction. The plate was allowed to air dry and then spots were enumerated by ELISPOT reader. Each experiment was set in duplicate. The results were transformed to numbers of spot-forming cells per 10^6 mononuclear cells.

Diagram 5 Steps of ELISPOT assay for enumerating ASC in the blood circulation



LPS or CT or purified TcpA coated well

↓ Wash and Block at 37 °C for 2 hr
Wash

Add PBMC (5×10^5 cells/ml)

↓ Incubate at 37 °C for 16 hr
Wash

Add anti-rat Ig-biotin conjugate

↓ Incubate at 37 °C for 1.30 hr
Wash

Add Streptavidin-Alkaline Phosphatase

↓ Incubate at 37 °C for 1.30 hr
Wash

Add BCIP-NBT substrate

↓

ELISPOT reader

↓

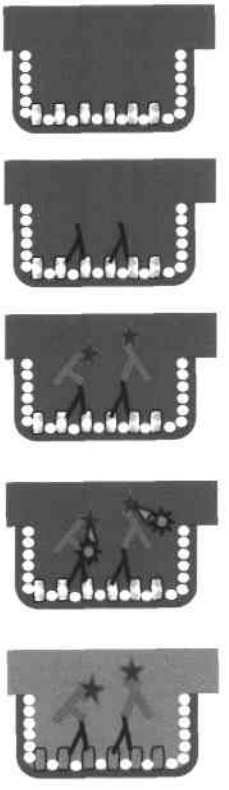
The number of spot-forming cells per 10^6 PBMC

8.3 An indirect ELISA

This method was used for detection of antibodies and determination of antibody titers in the immune rabbit sera or mouse monoclonal antibody. The indirect ELISA was carried out by the following procedure:

Individual wells of the microtiter ELISA plates (NUNC, Denmark) were coated with 100 µl of appropriate antigens diluted in a coating buffer (carbonate-bicarbonate buffer, pH 9.6) at appropriate concentrations. The plates were incubated in an incubator at 37°C for 2 hr in a humid chamber. The antigen-coated plates were washed thoroughly with 0.01 M PBS, pH 7.4, containing 0.05 % Tween-20 (PBS-T) solution to remove unbound materials, and 200 µl PBS-T containing 1.0 % bovine serum albumin were added to each well to block the sites that were not occupied by the antigens. The plates were incubated at 37°C for 1 hr. After another wash as above, 100 µl of samples (two-fold serial dilution) were added to appropriate wells while PBS, pH 7.4 was added to the blank wells to serve as negative controls. The antigen-antibody reaction was allowed to occur at room temperature for 2 hr and then the non-reacted materials were washed off by the PBS-T. After washing, 100 µl of goat anti-rabbit/anti-mouse immunoglobulin IgG-horseradish peroxidase conjugate (SEROTEC) diluted 1:1,000 in diluent were added into each well and incubated at 37°C for 1 hr in order to determine antibody titers in the immune rabbit sera or mouse monoclonal antibody, respectively. In case of antibody titers in the experimental rats, 100 µl of anti-rat Ig- biotin conjugate diluted 1:1,000 in diluent were added into each well and incubated at 37°C for 1 hr and then wash and 100 µl of streptavidin-horseradish peroxidase conjugate diluted 1:2,000 in diluent were added into each well and incubated at 37°C for 1 hr. The excess conjugate was washed away, 100 µl of *p*-phenylene-diamine dihydrochloride (PPD) (Sigma Chemical Co., USA) substrate solution which was freshly prepared was added to each well and the plates were kept at room temperature in the dark for 30 min. The enzyme-substrate reaction was stopped by adding 50 µl of 1 N NaOH solution. The optical density (OD) of the content in each well was determined at 492 nm using an ELISA reader. The OD at 492 nm of 0.05 or more was taken as positive reaction.

Diagram 6 Steps of an indirect ELISA for determining antibody titers of the experimental rats



LPS or CT or purified TcpA coated well

↓ Block at 37 °C for 2 hr
Wash

Add plasma (2-fold serial dilution)

↓ Incubate at 37 °C for 1 hr
Wash

Add anti-rat Ig-biotin conjugate

↓ Incubate at 37 °C for 1 hr
Wash

Add streptavidin- horseradish peroxidase conjugate

↓ Incubate at 37 °C for 1 hr
Wash

Add substrate solution

↓

Measure the OD at 492 nm

8.4 Vibriocidal antibody assay

Plasma samples from individual vaccinated or control rats were diluted (serially 2 fold) with 0.1% peptone saline solution in the glass tubes set in a rack on ice. Log phase-broth culture of *V. cholerae* biotype El Tor strain O17SR was diluted to give 2×10^4 viable organisms/ml of 1:10 guinea pig serum previously adsorbed with glutaraldehyded fixed *V. cholerae* (cold peptone saline solution was used as serum and bacterial diluent). Equal volume of the bacteria in the 1:10 diluted guinea pig serum was added to each dilution of the plasma and control tube which contains peptone saline solution alone. After mixing by vortexing, the tubes were incubated at 37°C for 1 hr. The test tubes in the rack were kept in 13°C ice bath and 20 µl of all samples were plated onto streptomycin agar plates. The vibriocidal antibody titer of the plasma was taken as the dilution that results in at least 50% killing of the bacteria as compared to the number of bacterial colonies from sample from the control tube (mixture without rat plasma).

Diagram 7 Steps of Vibriocidal antibody assay (complement-mediated vibrio lysis)

Individual plasma was heat-inactivated at 56 °C for 30 min

↓

Heat-inactivated plasma was diluted with 0.1% peptone saline solution (2-fold dilution)

↓

Equal volume of $2x10^4$ live bacteria/ml in 1:10 guinea pig serum was added to each dilution of the plasma and control tube and then mixed

↓

The preparation was incubated at 37 °C for 1 hr

↓

Twenty µl of all samples were plated onto streptomycin agar plates

↓

Vibriocidal antibody titer was taken as the highest dilution of Ab giving 50% killing of the added vibrios