

# Traffic of Antibody-Secreting Cells after Immunization with a Liposome-Associated, CpG-ODN-Adjuvanted Oral Cholera Vaccine

Srinuan Somroop<sup>1</sup>, Pongsri Tongtawe<sup>1</sup>, Urai Chaisri<sup>2</sup>, Pramuan Tapchaisri<sup>1</sup>, Manas Chongsa-nguan<sup>2</sup>, Potjane Srimanote<sup>1</sup> and Wanpen Chaicumpa<sup>1</sup>

**SUMMARY** An oral cholera vaccine made up of heat-treated recombinant cholera toxin (rCT), *V. cholerae* lipopolysaccharide (LPS), and recombinant toxin-co-regulated pili subunit A (rTcpA), entrapped in liposomes in the presence of unmethylated bacterial CpG-DNA (ODN#1826) was used to orally immunize a group of eight week old rats. A booster dose was given 14 days later. Control rats received placebo (vaccine diluent). The kinetics of the immune response were investigated by enumerating the antigen specific-antibody secreting cells (ASC) in the blood circulation and intestinal lamina propria using the ELISPOT assay and a histo-immunofluorescence assay (IFA), respectively. ASC of all antigenic specificities were detected in the blood of the vaccinated rats as early as two days after the booster dose. The numbers of LPS-ASC and TcpA-ASC in the blood were at their peak at day 3 post booster while the number of CT-ASC was highest at day 4 after the booster immunization. At day 13 post immunization, no ASC were detected in the blood. A several fold increase in the number of ASC of all antigenic specificities in the lamina propria above the background numbers of the control animals were found in all vaccinated rats at days 6 and 13 post booster (earlier and later time points were not studied). Vibriocidal antibody and specific antibodies to CT, LPS and TcpA were detected in 57.1% and 52.4%, 14.3%, and 19.0% of the orally vaccinated rats, respectively. The data indicated that rats orally primed with the vaccine could produce a rapid anamnestic response after re-exposure to the *V. cholerae* antigens. Thus, a single dose of the vaccine is expected to elicit a similar anamnestic immune response in people from cholera endemic areas who have been naturally primed to *V. cholerae* antigens, while two doses at a 14 day interval should be adequate for a traveler to a disease endemic area.

Cholera, a severe watery diarrheal disease of humans caused by the bacterium *Vibrio cholerae*, continues to be a major public health problem in developing countries where the disease has established its endemicity. In many endemic areas, cholera outbreaks are seasonal such as after the monsoon season in Bangladesh and during the hot-dry months in Thailand. A cholera upsurge, however, can also be due to social strife, famine and crowding such as in refugee camps where hygiene and sanitation are sub-

standard. Although the disease can be treated relatively simply by oral and/or intravenous fluid and electrolyte replacement,<sup>1</sup> prompt and sufficient access to such measures is inadequate in many areas during large epidemics which usually results in some

From the <sup>1</sup>Faculty of Allied Health Sciences, Thammasat University, Rangsit Center, Pathumthani 12121, Thailand, <sup>2</sup> Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand. Correspondence: Wanpen Chaicumpa  
E-mail: tmwcc@mahidol.ac.th

case fatality. Antibiotics, especially tetracycline have been used with success in shortening the duration of diarrhea and in the reduction of *V. cholerae* in the patient's stool.<sup>2</sup> Nevertheless, resistance to antimicrobial agents has been increasingly found.<sup>3</sup> Cholera caused by serogroups O1 and O139 can spread rapidly and even assume epidemic or pandemic proportions. The usual preventive strategies for cholera outbreaks include disease surveillance, health education, and improvement of water sanitation and personal and food hygiene. However, these measures are difficult to implement in many areas of the world where the disease is endemic. Alternatively, vaccination against cholera is required for inhabitants of and travelers to endemic areas. Cholera vaccine efforts have included the development of both parenteral and oral vaccines.<sup>4</sup> However, because of the nature of the *V. cholerae* infection, oral vaccination which induces intestinal immunity is believed to be more effective than the immunity elicited by a parenteral vaccine.<sup>4,5</sup>

In this study, an oral vaccine made up of three antigens, *i.e.* heat-treated recombinant cholera toxin (CT), *V. cholerae* O1 lipopolysaccharide (LPS), and recombinant toxin-co-regulated pilus subunit A (rTcpA) has been formulated using liposomes as an antigen delivery vehicle and unmethylated CpG-ODN as an adjuvant. The kinetics of the specific antibody secreting cells (ASC) in the peripheral blood of vaccine primed-rats were studied in order to determine the mucosal memory response to the vaccine.

## MATERIALS AND METHODS

### Experimental animals

Eight week-old male Wistar rats were purchased from The National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom Province, Thailand. The animals were allowed to adapt to the new environment in the animal house of The Faculty of Allied Health Sciences, Thammasat University, for one week before commencing the experiment.

Animal experiments were performed following the guideline of the National Research Council of Thailand and were approved by the Ethical Committee of the Faculty of Allied Health Sciences, Thammasat University, Thailand.

### Preparation of vaccine components

The recombinant toxin co-regulated pilus subunit A (rTcpA) was prepared as previously described.<sup>6</sup> A gene encoding the protein of *V. cholerae* O1 biotype El Tor strain O17SR was cloned into a pBluescript KS vector, subcloned into a pTrcHis plasmid and subsequently transfected into an *E. coli* DH5 $\alpha$ . The recombinant TcpA was prepared from a selected transformed *E. coli* clone. Briefly, a single colony of *E. coli* grown overnight on a TSA plate was inoculated into 10 ml of LB broth containing 50 mg ampicillin/ml (LB-A). Five ml of the overnight culture were inoculated into 250 ml of LB-A contained in a one liter-flask and incubated at 37 °C with aeration until the OD at 600 nm was 0.4. IPTG was added to the culture to 0.1 M and the culture was incubated for further five hours. The bacteria were collected after centrifugation at 8,000 x g, at 4°C for 15 minutes. The cell pellet was added to 8 ml of guanidinium lysis buffer pH 7.8, subjected to sonication by using an MSE ultrasonic disintegrator (20 kHz, 4°C, for 3 minutes, three times) and centrifuged as above. The clear supernatant was mixed with the matrix of the ProBond Purification System (Invitrogen, CA, USA) which had been washed with and equilibrated in a denaturing binding buffer pH 7.8. The mixture was kept rotating at 25°C for 30 minutes and was then centrifuged at 800 x g for 10 minutes. The resin was collected and washed thoroughly with denaturing washing buffers pH 6.0 and 5.3, respectively, until the OD at 280 nm was nil. The resin was packed into a column and the bound protein was eluted into one-ml fractions using denaturing elution buffer pH 4.0. The fractions with a detectable OD at 280 nm were pooled, dialyzed against distilled water and concentrated by lyophilization. The purity of the rTcpA preparation was checked by SDS-PAGE and Coomassie Brilliant Blue-G250 (CBB) staining.

### Heat-treated recombinant cholera toxin (rCT)

Recombinant cholera toxin (rCT) was prepared from *E. coli* MC1061 (pKTJ5-15X) harboring cholera toxin genes, which was a kind gift from Professor Hisao Kurazono, Osaka Prefecture University, Japan. The *E. coli* were streaked onto an agar plate containing 100  $\mu$ g ampicillin/ml and the plate was incubated at 37 °C for 8 hours. A loopful of the bacteria from the plate was inoculated into 10 ml of LB-

A, and incubated at 37 °C for 3 hours with shaking. Seven ml of the culture were inoculated into 750 ml LB-A in a two liter-flask and incubated as above for 18 hours. The bacteria were collected by centrifugation at 10,000 x g, 4°C, 30 minutes. The cell pellet was resuspended in 0.9% NaCl in 10 mM Tris-HCl buffer pH 8.6 and subjected to sonication at 20 kHz per second, 4°C, 2 minutes, twice. The debris was removed by centrifugation, the supernatant was collected and ammonium sulfate was added to the preparation until the saturation reached 65%. The precipitate was dissolved and dialyzed in TEAN buffer. The protein content of this crude CT preparation was determined.<sup>7</sup> Recombinant CT was purified using a galactose column (Pierce Co., Rockford, USA). Briefly, the resin was packed into a column (10 x 10 cm) and equilibrated with TEAN buffer pH 7.4. Crude rCT was loaded into the column and after washing to get rid of unbound material, the rCT was eluted out using 1 M galactose in TEAN buffer. The eluent was collected in one ml-fractions. All fractions with a significant OD read-out at 280 nm were collected. Individual fractions containing pure rCT were detected by running a SDS-PAGE and CBB-staining. Fractions containing pure rCT were pooled, dialyzed against distilled water and lyophilized. The rCT was heated at 56°C for 15 minutes to prepare procholeraenoid (P). The toxicity of the CT after heating (P) should be less than 5% of the native toxin.<sup>8</sup>

### Lipopolysaccharide (LPS)

LPS of *V. cholerae* O17SR was prepared by the phenol-water extraction method of Westphal and Jann.<sup>9</sup> The LPS extraction procedure was repeated until no protein could be detected in the LPS preparation by SDS-PAGE, CBB-staining and Bradford method.<sup>7</sup>

### Unmethylated CpG-ODN

CpG-DNA (CpG-ODN#1826) was sourced from the Coley Pharmaceutical Group, Wellesley, USA. The nucleotide sequence was 5'-TCCATGACGTTCTGACGTT-3'.

### Formulation of liposome associated-oral cholera vaccine

Each dose of the liposome associated-cholera vaccine was prepared as previously described.<sup>6,10</sup>

Antigens, *i.e.* heated rCT (P), rTcpA, and LPS were entrapped in a liposome by the bath-sonication method.<sup>9</sup> Each dose of the rat vaccine contained 200 µg of P and 5 mg each of LPS and rTcpA in 3 ml of 5% NaHCO<sub>3</sub> (vaccine diluent). Liposomes were prepared as previously described<sup>6,10</sup> from two batches of lipid solution (Batches X and Y). Each batch consisted of 10 mg bovine brain sphingomyelin and 4 mg cholesterol dissolved in 1.5 ml of chloroform and 1.0 ml of ether. The antigens for 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 of distilled water alone was mixed with batch Y of the lipids. The mixtures in both tubes were individually sonicated at 20 kHz per second, 4°C, 3 minutes, three times. The homogeneous single phase emulsions in both tubes were mixed well and dried in low vacuum in a rotary evaporator. Three milliliters of the vaccine diluent were added to the gel-like material to make a homogeneous preparation. CpG-ODN (100 µg) was added before immunizing a rat.

### Animal immunization

Experimental rats were starved for 15 hours and one ml of 5% NaHCO<sub>3</sub> was instilled *via* a stomach tube to each animal in order to reduce gastric acidity. The rats were divided into two groups. Each rat of the first group (21 rats) received oral liposome associated-cholera vaccine plus CpG-ODN, while rats of group 2 (7 rats) were individually fed with three ml of the vaccine diluent. All rats were returned to the domesticated cages with adequate supply of water and food pellets. Fourteen days later, the vaccine primed-rats were given a vaccine booster while the controls received a placebo.

### Sample collection

Whole blood and plasma samples were collected from three rats of group 1 and one rat of group 2 on days 2, 3, 4, 5, 6, 13, and 21 after the booster. The numbers of ASC in the peripheral blood samples at these time points were determined by ELISPOT assay. Levels of antibodies to each vaccine component in the plasma samples were measured by indirect ELISA. Vibriocidal (antibody-complement mediated vibrio-lysis) antibody levels in the plasma, which are an indirect correlate of mucosal immunity, were also determined. Small intestines of rats of both groups were collected on days 6 and 13 post-booster

for enumeration of ASC in the lamina propria (LP) by IFA.

#### **Preparation of rabbit polyclonal antibodies to rCT and rTcpA**

Cholera toxin (Sigma Chemical Co., USA) in one ml of normal saline solution was injected intravenously into a New Zealand White rabbit (weighed ~ 2.0 kg). The immunization was repeated five additional times on days 8, 15, 33, 43, and 50. The following amounts of toxin were used respectively: 4, 10, 20, 40, 40 and 40 µg. The rabbit was bled on day 64 and the serum anti-CT titer was determined by indirect ELISA.<sup>10</sup> A satisfactory titer was obtained; then the rabbit was bled *via* cardiac puncture. Serum immunoglobulin was extracted from the rabbit immune serum by ammonium sulfate precipitation. After dialysis in 0.01 M PBS pH 7.4 the protein content and antibody titer of the immunoglobulin preparation were determined.

Recombinant TcpA (100 µg) was subjected to SDS-PAGE in a mini-PROTEAN electrophoresis apparatus (Bio-Rad, USA) using a 7 x 7 cm separating gel. A rabbit was immunized subcutaneously (two sites) with a homogenate of two polyacrylamide gel strips containing bands of rTcpA, which were cut from the SDS-PAGE separated-rTcpA and minced into two ml of PBS. The immunization was repeated four more times on days 14, 28, 42 and 56 *via* the same route using the same amount of immunogen. The rabbit was bled on day 70. The immunoglobulin was extracted, the protein content and the indirect ELISA titer against the purified TcpA were determined.

#### **Monoclonal antibody to *V. cholerae* lipopolysaccharide**

The murine hybridoma clone 27E10 secreting monoclonal antibody specific to antigen A in the LPS of *V. cholerae* serogroup O1<sup>11</sup> was cultured in a serum-free medium. The spent culture medium of the hybridoma gave an indirect ELISA titer of 1:256 against the purified LPS.

#### **ELISPOT assay for enumeration of the antibody secreting cells (ASC) in peripheral blood**

At days 2, 3, 4, 5, 6, 13 and 21 after the booster dose, about three ml of peripheral blood were aseptically collected from each rat into a

heparinized-tube. Peripheral blood mononuclear cells were isolated from the blood by using Ficoll-Hypaque medium (Pharmacia Biotec, Uppsala, Sweden). The cells were washed twice with RPMI-1640 medium supplemented with 5% de-complemented fetal bovine serum (FBS). Viable cells were enumerated by the trypan blue exclusion method in a hemacytometer and adjusted to  $5 \times 10^5$  cells/ per ml in the supplemented-RPMI medium. The wells of a MultiScreen-IP plate (Millipore, Bradford, USA) were individually coated with 1 µg of antigen per well (rCT, LPS, or rTcpA) in 50 µl of 0.01 M PBS pH 7.4 at 4°C overnight. All wells were washed thoroughly with the PBS and incubated with 200 µl of 3% BSA at 37°C for 2 hours. After the excess BSA was removed and the wells were washed as above, 200 µl of the PBMC (10,000 cells) were added to each well. The plate was incubated at 37°C in a 5% CO<sub>2</sub> incubator with humidity for 16 hours. All wells were washed with PBS pH 7.4 followed by thoroughly washing with PBS containing 0.05% Tween-20 (PBS-T). Fifty microliters of biotinylated-anti-rat immunoglobulin (Amersham Pharmacia Biotec, UK) diluted 1:1,000 in a diluent (0.01 M PBS pH 7.4 containing 1% FBS) were added to each well and the plate was incubated at 37°C for 90 minutes. After washing to remove the second antibody, 100 µl of streptavidin-alkaline phosphatase conjugate (DAKO A/S, Denmark) diluted 1:1,000 with the diluent were added to each well. The plate was incubated further at 37°C for 90 minutes, washed with PBS-T, after which a substrate, *i.e.* 50 µl of BCIP-NBT (Bio-Rad, USA) was added to each well and incubated for 8 minutes. The substrate was discarded and all wells were washed with distilled water and then air-dried. The number of spots (number of ASC to each antigen) was counted by using an ELISPOT reader and the number of ASC per  $10^6$  PBMC was calculated. Each experiment was set in duplicate.

#### **Enumeration of ASC in the intestinal lamina propria**

The numbers of ASC specific to CT, TcpA and LPS in the lamina propria were determined using an immunofluorescence assay as previously described.<sup>6,10</sup> Briefly, a cryostat section (5 µm thick) of jejunum was fixed to a microscopic slide using a gradient of 90% and absolute methanol. The tissue section was then covered with a solution of antigen

(Sigma CT 2  $\mu\text{g/ml}$ , rTcpA 10  $\mu\text{g/ml}$ , or LPS 10  $\mu\text{g/ml}$ ). The slide was incubated at 25°C in a humid box for 30 minutes. Excess fluid was rinsed-off with PBS pH 7.2. The tissue section was then incubated with either rabbit polyclonal anti-CT or anti-TcpA, or mouse monoclonal antibody to LPS antigen A at 25°C for 30 minutes. After the excess antibodies were discarded, the slide was thoroughly rinsed with PBS then the tissue section was incubated with fluorescein labeled-anti-rabbit/anti-mouse immunoglobulins and incubated as above for further 30 minutes. The fluorescein was removed by washing, the slide was blot-dried, mounted with buffered glycerol pH 7.6, and covered with a cover slip. The ASC were visualized under an Olympus fluorescence microscope using 10x ocular and 20x objectives. Two intestinal sections from each rat were used for counting the number of the antigen specific ASC (anti-CT, anti-TcpA and anti-LPS) from 50 microscopic fields (25 microscopic fields from each tissue section).

#### Indirect ELISA and vibriocidal assay

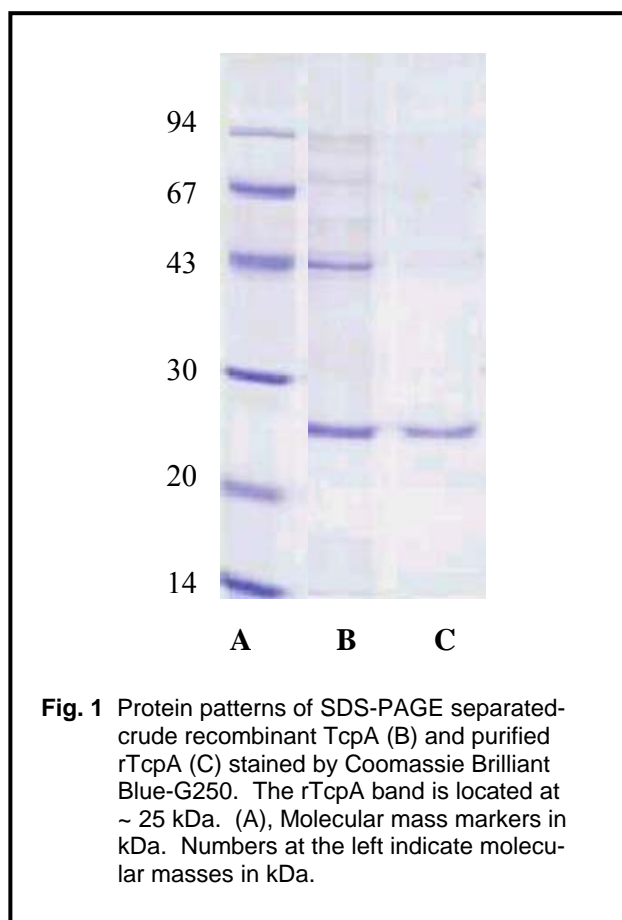
The antibody titers to CT, TcpA and LPS in the plasma samples of all rats were determined using an indirect ELISA. The vibriocidal antibody titer in each plasma sample was measured using a previously described method.<sup>12</sup>

#### Statistical analysis

The differences in the means of the ASC numbers and antibody levels between the immunized and placebo groups were statistically analyzed by using the Student's t test.

### RESULTS

Figs. 1 and 2 show protein bands of rTcpA and rCT preparations after SDS-PAGE and CBB staining. The rTcpA preparation revealed a single protein band at  $\sim 25$  kDa (Fig. 1, lane C) while the rCT preparation showed bands of A subunits at  $\sim 30$  kDa and B subunits at  $\sim 11$  kDa (Fig. 2, lanes C and D). The purified LPS contained no protein, as no band was detectable after SDS-PAGE and CBB staining (Fig. 3A, lanes B, C, and D) but the LPS smear pattern was revealed after Silver staining (Fig. 3B, lanes B, C, and D). The results indicate a high degree of purity for all three antigens.



**Fig. 1** Protein patterns of SDS-PAGE separated crude recombinant TcpA (B) and purified rTcpA (C) stained by Coomassie Brilliant Blue-G250. The rTcpA band is located at  $\sim 25$  kDa. (A), Molecular mass markers in kDa. Numbers at the left indicate molecular masses in kDa.

Fig. 4 illustrates the appearance of the multi-lamella liposomes by using negative staining with 2% uranyl acetate under transmission electron microscopy. Their sizes, as determined by using a sub-micron particle analyzer (Coulter<sup>®</sup> Model N4D, Germany), were  $55.5 \pm 8$  nm (5%),  $298 \pm 57$  nm (57%) and  $3,010 \pm 38$  nm (38%).

The ELISPOT assay could detect ASC of all antigenic specificities, *i.e.* anti-CT, Anti TcpA and anti-LPS in the blood circulation of the vaccine primed-rats as early as day 2 post-booster (earlier time was not studied). The numbers of ASC to TcpA and LPS were highest on day 3 post-booster, while the anti-CT ASC number peaked at day 4 post-booster. Thereafter, the numbers of ASC in the blood declined markedly. At days 13 and 21 post-booster, no ASC was detected in the peripheral blood of the immunized rats (Fig. 5a, 5b, and 5c).

The antibody titers to CT, TcpA and LPS in the plasma samples of the vaccinated and control rats were determined by indirect ELISA. Among the 21

vaccinated rats 11 (52.4%), 4 (19.0%) and 3 (14.3%) rats showed significant anti-CT, anti-TcpA, and anti-LPS levels in the plasma compared to the controls. The highest titers of anti-CT, anti-TcpA and anti-LPS were 1:5,120, 1:320 and 1:320, respectively (Fig. 6a, 6b and 6c).

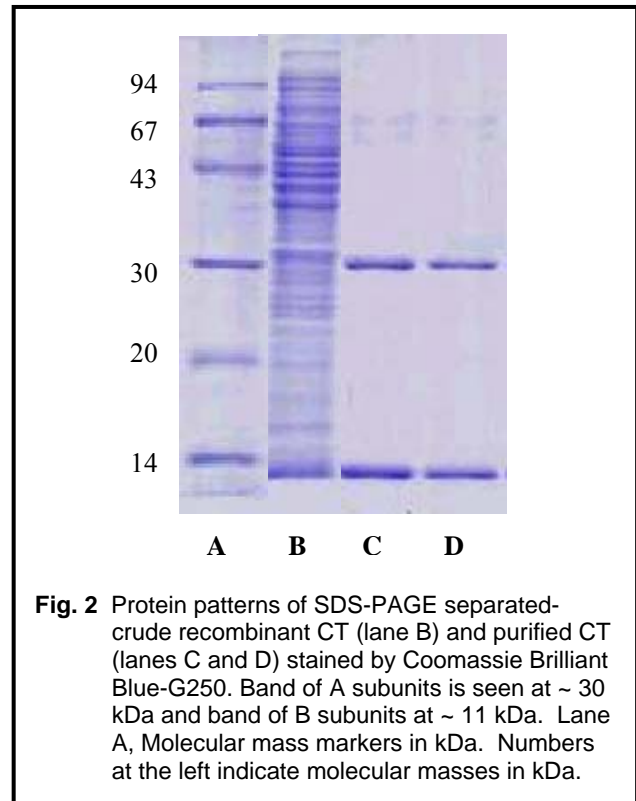
Fig. 7 shows the vibriocidal titers in the plasma samples of the vaccinated and control rats. It was found that 12 of 21 (57.1%) vaccinated rats had significant vibriocidal antibodies in their plasma compared to the controls.

Only few ASC to LPS (2-5 cells per one microscopic field), and no anti-TcpA or anti-CT were found in the LP of control rats. The numbers of ASC of all antigenic specificities in the LP of the vaccinated rats out-numbered by several folds those in the controls at days 6 and 13 after the booster (data not shown).

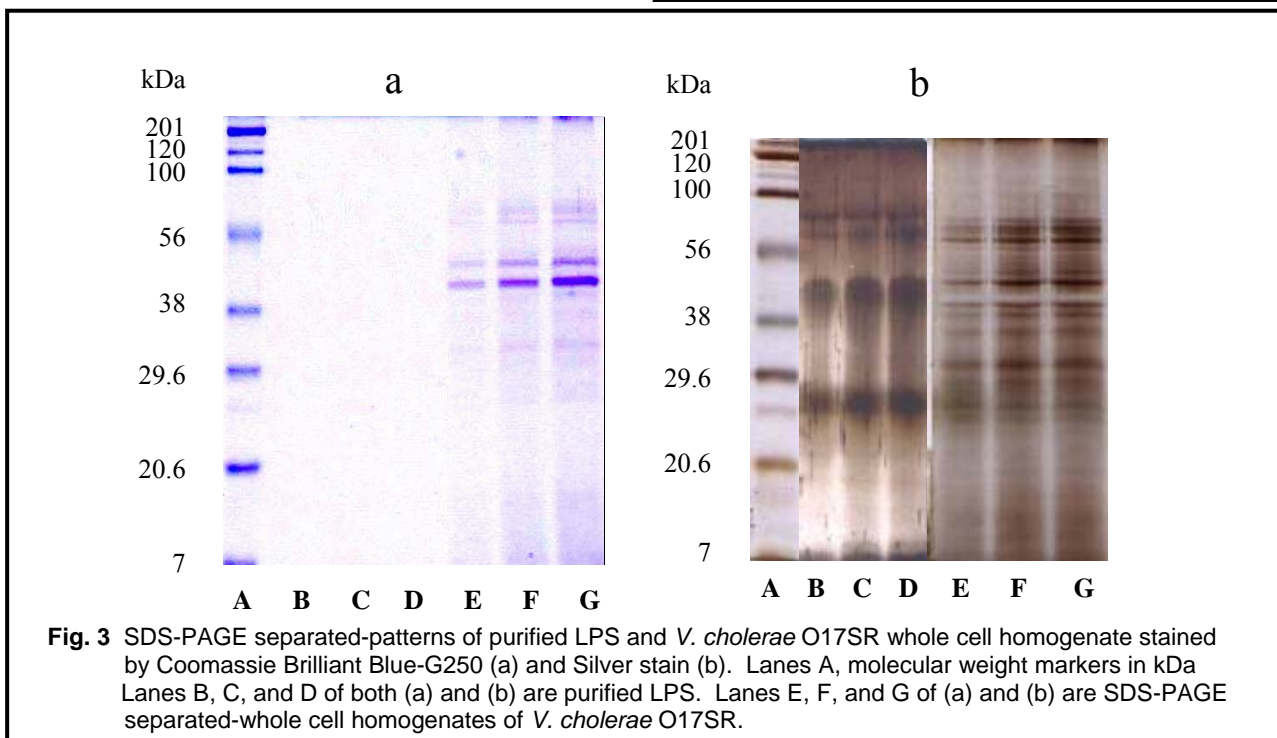
## DISCUSSION

In this study, three refined antigens, *i.e.* heat-treated-recombinant CT, *V. cholerae* O1 LPS and recombinant TcpA were combined and formulated with an antigen delivery vehicle, *i.e.* liposomes made

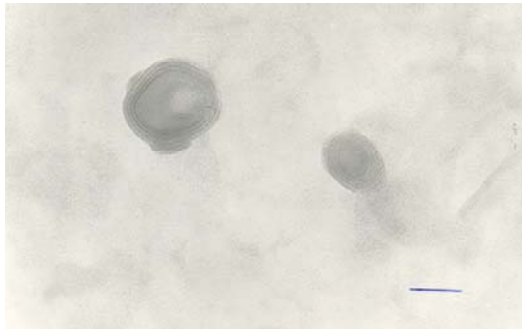
of sphingomyelin and cholesterol, into an oral vaccine against cholera using unmethylated CpG-DNA, *i.e.* CpG-ODN#1826, as an adjuvant. The recombinant TcpA and LPS were expected to elicit mucosal



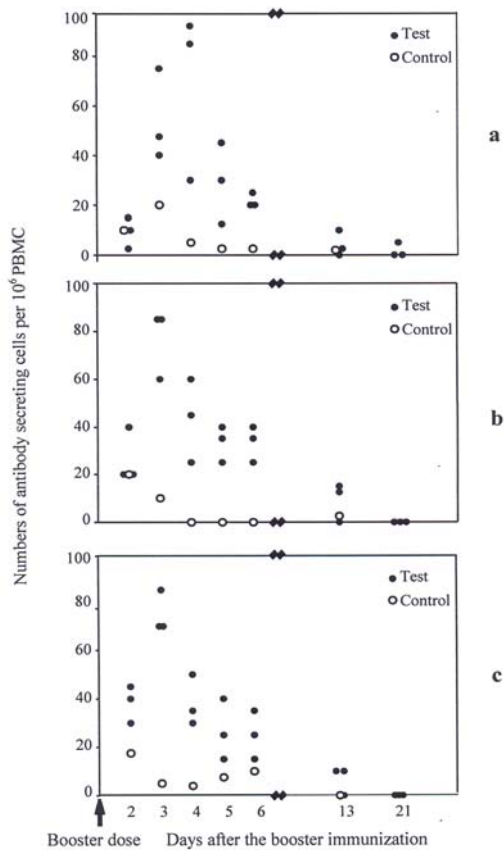
**Fig. 2** Protein patterns of SDS-PAGE separated-crude recombinant CT (lane B) and purified CT (lanes C and D) stained by Coomassie Brilliant Blue-G250. Band of A subunits is seen at ~ 30 kDa and band of B subunits at ~ 11 kDa. Lane A, Molecular mass markers in kDa. Numbers at the left indicate molecular masses in kDa.



**Fig. 3** SDS-PAGE separated-patterns of purified LPS and *V. cholerae* O17SR whole cell homogenate stained by Coomassie Brilliant Blue-G250 (a) and Silver stain (b). Lanes A, molecular weight markers in kDa Lanes B, C, and D of both (a) and (b) are purified LPS. Lanes E, F, and G of (a) and (b) are SDS-PAGE separated-whole cell homogenates of *V. cholerae* O17SR.

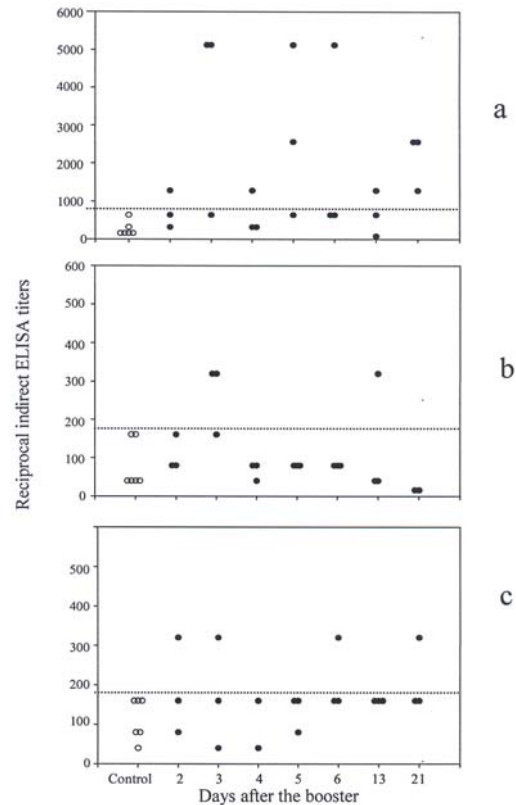


**Fig. 4** Appearance of the multilamellar liposomes revealed by transmission electron microscopy after negative staining by 2% uranyl acetate. Bar length is 10 nm.



**Fig. 5** Numbers of ASC per  $10^6$  PBMC of vaccinated and control rats at different days after the booster. (a) anti-CT; (b) anti-LPS; (c) anti-TcpA.

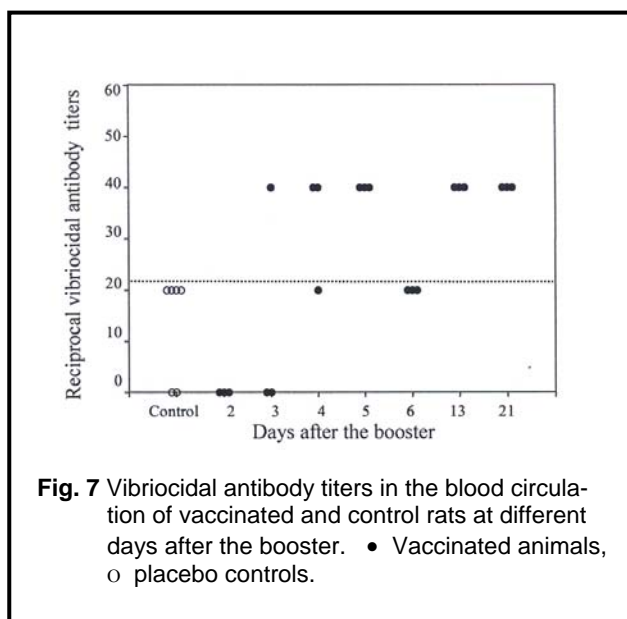
antibodies that prevent attachment of the *V. cholerae* to the intestinal epithelium by means of interfering



**Fig. 6** Indirect ELISA titers of antibodies to CT (a), LPS (b) and TcpA (c) in the plasma of vaccinated and control rats at different days after the booster.

with the *V. cholerae* adhesion to the epithelial brush border and/or causing bacterial agglutination and thus retaining the pathogens within the intestinal lumen or in the mucus and glycocalyxes. The *V. cholerae* bacteria would then be moved on to the large intestine by intestinal peristalsis where they may be killed by bacterial antagonism or excreted with the feces. The anti-CT antibody especially the anti-B subunit should prevent binding of the toxin to the GM1 receptor on the enterocyte membrane. The immunogens in the oral vaccine, *i.e.* *V. cholerae* LPS, rTcpA and heat-treated CT (procholeragenoid) with or without liposomes have been previously shown to synergize each other's immunogenicity in both experimental animals and human volunteers.<sup>10,12,13</sup>

Data in the literature have indicated that liposomes are suitable delivery vehicles for drugs and vaccines.<sup>14-20</sup> The liposomes used in this study were multilamellar with a relatively large aqueous phase



**Fig. 7** Vibriocidal antibody titers in the blood circulation of vaccinated and control rats at different days after the booster. • Vaccinated animals, ○ placebo controls.

as shown in Fig. 4; thus they are suitable as vaccine carrier. Hydrophilic molecules (*e.g.* rTcpA and heat-treated rCT) may be entrapped within the liposome aqueous phase, while hydrophobic molecules (*e.g.* LPS) may attach to or be integrated in the liposome lipid bilayers. Regardless of their lipid composition and sizes and the way by which they carry their cargo, the liposome lipid bilayers can adhere to the plasma membrane of the cognate mammalian cell and deliver their passenger directly into the cytoplasm. The liposomes used in this study were heterogeneous in size, *i.e.*  $55.5 \pm 8$  nm (5%),  $298 \pm 57$  nm (57%) and  $3,010 \pm 520$  nm (38%). The large liposomes (several microns) were likely to be trapped by the microfold (M) cells of the epithelium overlying the Peyer's patch (PP), while smaller liposomes or free antigenic molecules (if there were any) should be absorbed and transported *via* the absorptive intestinal epithelial cells.<sup>21</sup> Particles larger than  $10 \mu\text{m}$  would not be taken up.<sup>22</sup> It is known that the PP is the inductive site of the immune response to the mucosal antigens, especially particulate antigens.<sup>23</sup> M cells transport undegraded antigenic particles *via* cytoplasmic vesicles to the antigen presenting cells (mostly dendritic cells; DC) in the dome area of the PP where T-cell priming can occur either locally within the patch or in the DC draining mesenteric lymph nodes (MLN).<sup>24-27</sup> Antigen stimulated lymphocytes originated in the PP (predominantly IgA-committed B-lymphocytes and antigen primed T-cells) migrate out *via* the efferent lymphatics to the MLN. They and their counterparts originate in the

MLN and subsequently drain to the thoracic duct, circulate in the arterial blood, and eventually settle (by means of integrins and chemokine receptors on the lymphocytes and mucosal addressin as well as by chemokine produced in the intestinal lymphoid tissue) mainly at the intestinal lamina propria (LP) and partly at the effector sites of other mucosal lymphoid tissues.<sup>28-30</sup> Thus, in order to prime the antigen specific lymphocytes with the vaccine components in the PP and MLN, the immunogens should be entrapped in rather large liposomes. Unfortunately, only 38% of our liposomes met the requirement for the M cell entrapment. Nevertheless, all of the vaccine primed-rats showed good and rapid memory response upon being boosted with the vaccine 14 days after the primary immunization. As soon as two days post vaccine booster, ASC of all antigenic specificities (anti-CT, anti-LPS, and anti-TcpA) were detected in the blood circulation indicating their traffic to the destination at the mucosal effector sites, principally the LP. Significant numbers of the ASC of all antigenic specificities were present in the LP of the vaccinated rats at days 6 and 13 post-booster (earlier and later times were not studied). These memory cells should be ready to exert their immune function upon encountering the homologous antigens from natural infection.

In the intestines, the predominant immunoglobulins are secretory IgA. This bias is believed to be due to the influence of the locally produced cytokines, *i.e.* TGF $\beta$ , IL-10 and IL-4, which promote class-switching of the naïve IgM/IgD B-cells in the PP to IgA-committed B-cells.<sup>31,32</sup> The unmethylated CpG-ODN #1826 and liposomes used in this study were found previously to be effective and synergistic oral adjuvants for refined *V. cholerae* immunogens.<sup>6</sup> The unmethylated CpG-ODN and LPS contained in the formulated vaccine are pathogen associated molecular patterns (PAMPs) for TLR9 and TLR4, respectively. The proinflammatory cytokine response to these PAMPs produced by DC and other APC should give rise to an enhanced adaptive immune response and production of other classes of specific antibodies (*e.g.* IgG2a).<sup>32</sup> Moreover, heat-inactivated CT should enhance the mucosal as well as the systemic immune responses to the co-administered antigens.<sup>32</sup> After two doses of immunization with the vaccine, vibriocidal antibodies were detected in the plasma samples of 57.1% of the immunized animals implying that the vaccine induced also the comple-



ment fixing antibody, *e.g.* IgG2a and IgG3 to the *V. cholerae* somatic antigen, *i.e.* LPS.

In this study, the vaccine primed rats recalled their previous exposure to *V. cholerae* antigens by having specific B lymphocytes in the peripheral blood as early as two days after antigen re-exposure (earlier time was not studied). Thus, a single dose of the vaccine is expected to elicit a similar anamnestic immune response in inhabitants of cholera endemic areas who have been naturally primed to *V. cholerae* antigens, while two doses of the vaccine at a 14 day interval should be adequate for *V. cholerae* naive travelers to disease endemic areas. However, studies in human volunteers should be carried out to pinpoint the kinetics of the immune response and the effectiveness of the vaccine.

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