

# CpG DNA, Liposome and Refined Antigen Oral Cholera Vaccine

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*Vibrio cholerae* superficially infect the intestinal epithelium of humans, produce toxins and ultimately generate a massive outpouring of water and electrolytes into the intestinal lumen and diarrhea (cholera) ensues. The pathogenicity of *V. cholerae* involves three important steps. Firstly, the vibrios which arrive at the small intestinal lumen via food and/or liquids, use their single polar flagella to propel through the mucus and glycocalyxes towards the intestinal epithelium.<sup>1,2</sup> The vibrios may produce certain enzymes, e.g. mucinase, fibronectinase, to facilitate their direct movement.<sup>3,4</sup> Secondly, the vibrios attach to the microvilli of the enterocytes using their surface adhesins.<sup>5,6</sup> This attachment prevents removal of the vibrios by the normal intestinal peristalsis to the large intestine where they might die from local bacterial antagonism or are excreted with the stool. Attachment to the small intestinal epithelium also allows them to multiply (colonize) near to the site of action of their toxins. Thirdly, once colonized, the vibrios produce toxins, e.g. cholera toxin (CT), Zonula Occludens toxin (Zot), and accessory cholera enterotoxin (Ace). The CT B subunits bind

**SUMMARY** An oral cholera vaccine made up of three *Vibrio cholerae* antigens, i.e. lipopolysaccharide (LPS), recombinant toxin co-regulated pili (rTcpA) and heat-treated cholera toxin (H-CT) has been developed in six different formulations. Eight-week-old Wistar rats were divided into nine groups and immunized as follows: the first group received the oral vaccine 1 consisting of the three antigens (LPS, rTcpA and H-CT) associated with a liposome (L) and bacterial CpG-DNA (ODN#1826). The rats of groups 2 and 3 received oral vaccines 2 and 3 consisting of the liposome-associated three antigens with and without non-bacterial CpG-DNA (ODN#1982), respectively. Rats of groups 4 received oral vaccine 4 consisting of the three antigens mixed with the ODN#1826, similar to vaccine 1, but without liposome. Rats of groups 5 and 6 received oral vaccines 5 and 6 consisting of the three antigens with and without ODN#1982, respectively, similar to vaccines 2 and 3, but without liposome. Rats of groups 7, 8 and 9 received oral placebos, namely liposomes (L), ODN#1826 (CpG), and vaccine diluent, i.e. 5% NaHCO<sub>3</sub> solution, respectively. All vaccines were given in three doses at 14-day intervals. It was found that the combination of liposome and ODN#1826 in vaccine 1 evoked the highest immune response to *V. cholerae* antigen compared to other vaccine formulations and placebos, as measured by the appearance of antigen-specific antibody-producing cells in the intestinal lamina propria. The immunogenicity according to the magnitude of the immune response was: V1>V2=V3>V4>V5=V6>V7=V8=V9. The results of this study indicate that CpG-DNA and liposome are effective mucosal adjuvants for an oral cholera vaccine prepared from refined *V. cholerae* antigens and their combination seems to be synergistic. The potential role of liposome as a vaccine delivery vehicle has been confirmed.

to specific receptor sites facilitating translocation of the active A subunit into the enterocyte cytosol where it activates an adenylate cyclase complex by ADP-ribosylation which causes an increase in the cAMP level, and eventually massive secretion of fluid and electrolytes into the intestinal lumen.<sup>7,8</sup>

It is known that immune responses to vaccines are influenced

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by the route of the immunization, the form of the antigen and the presence of immunobiologically active elements in the vaccine that mediate specific cell/tissue tropisms. Vaccines may also contain adjuvants, vectors and/or delivery vehicles that affect the quality and quantity of the immune responses.<sup>9-11</sup> For cholera, oral immunization is the best way to elicit an intestinal mucosal immune response in the form of specific antibodies which have been found to be a protective immunological correlate.<sup>12-15</sup> An oral vaccine consisting of *V. cholerae* lipopolysaccharide, cell-bound hemagglutinin and procholeraegenoid has been developed and tested in both animals and humans.<sup>16,17</sup> The three antigenic components exhibited marked immunological synergism when given together, as shown by an increase in the number of antibody producing cells (AbPC) of all three antigenic specificities, as compared to the vaccines consisting of only one of the three antigens.<sup>16</sup> The vaccine was found to be safe without untoward reactions but with only moderate immunogenicity after two or three oral doses. Thus, an adjuvant and/or a safe delivery vehicle were needed. Subsequently, a liposome associated oral vaccine, which elicited higher immunogenicity in both animals and human volunteers than a vaccine consisting of the *V. cholerae* antigens alone or a heat-killed whole cell vaccine, has been tested in both animals and humans.<sup>17-20</sup> The liposome appears to be a safe and promising oral antigen delivery vehicle,<sup>17,21</sup> although the uptake efficiency by the M cells of the Peyer's patches and the normal absorptive epithelial cells above the lamina propria as well as the subsequent amount of a secretory IgA response

remain inconclusive.<sup>22</sup> Recently, bacterial CpG DNA has been found to be a highly effective adjuvant of a mucosal vaccine administered intranasally and orally to mice.<sup>23,24</sup> The bacterial CpG DNA induces B cells to proliferate and secrete immunoglobulin.<sup>25</sup> In addition, CpG DNA is a T-cell independent B-cell mitogen that can drive more than 95% of resting B-cells into the cell cycle.<sup>26</sup> The CpG DNA has been reported to protect B-cells against Fas-mediated apoptosis.<sup>27</sup> Moreover, CpG DNA drives B-cells to express increased levels of cell surface co-stimulatory molecules, i.e. B7 and also class II MHC molecules that lead to improved antigen presentation.<sup>28,29</sup> Bacterial CpG DNA motifs promote antigen-specific T-cell responses through a co-stimulatory effect by ligation of T cell receptors (TCR) resulting in high levels of T-cell proliferation and cytokine production.<sup>29</sup>

In this study, the effectiveness of bacterial CpG DNA in enhancing the immune response to a combined-antigen oral cholera vaccine with and without liposome association was assessed in an animal model.

## MATERIALS AND METHODS

### Preparation of the *V. cholerae* antigens

#### Lipopolysaccharide (LPS)

LPS was extracted by the phenol-water method<sup>30</sup> from a smooth, streptomycin resistant variant (strain O17SR) of El Tor, Ogawa *V. cholerae*. LPS obtained from the first extraction was re-extracted twice more in order to eliminate all protein contaminants. The absence

of contaminating proteins in the triple-extracted LPS was confirmed by the Bradford method<sup>31</sup> and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining. The dry weight of the pure LPS was determined.

#### Cholera toxin (CT)

CT was purchased from Sigma Chemical Company, St. Louis, MO, USA.

#### Heat treated-cholera toxin (H-CT)

H-CT was produced by a heat-mediated conversion of CT (cholera toxin/choleraegen) to the natural toxoid (B subunits). This was done by heating recombinant CT (rCT) at 65°C for 15 minutes. Briefly, the CT was prepared from *E. coli* MC1061 (pKTJ5-15X) harboring the cholera toxin gene.<sup>32</sup> *E. coli* MC1061 was cultured and harvested by centrifugation, 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 minutes for five times. After the cell debris was removed by centrifugation, the toxin was fractionated by adding solid ammonium sulfate to a saturation of 65% (516 g/l). The precipitate was then dissolved in TEAN buffer, pH 7.4, dialyzed against the same buffer (crude CT). The protein content of the crude CT was determined by the Bradford method.<sup>31</sup> A galactose affinity column was used for rCT purification.<sup>32</sup> The resin was commercially obtained from Pierce Co., Rockford, USA.

#### Toxin co-regulated pili (TcpA)

The *TcpA* gene of *V. cholerae*

O1, biotype El Tor, strain O17SR, was cloned into a pBlueScript KS vector,<sup>33</sup> subcloned into vectors pTrcHis2A and subsequently transformed into *E. coli* DH5 $\alpha$ . The *E. coli* clone that harbored the pTrcHis2A was used for preparing crude and purified *V. cholerae* TcpA. Briefly the recombinant *E. coli* DH5 $\alpha$  was cultured at 37°C for 3 hours to an OD<sub>600</sub> of 0.6 (the cells were in the mid-log phase). IPTG was added to a final concentration of 1 mM (0.5 ml of a 100 mM IPTG stock to 50 ml of LB broth) and the culture was incubated at 37°C for 2 hours. The preparation was then centrifuged at 8,000  $\times$  g for 15 minutes at 4°C to precipitate the pellet. The pellet was re-suspended in 8 ml of guanidinium lysis buffer, pH 7.8, after which the preparation was slowly rocked for 5-10 minutes at room temperature to assure thorough cell lysis. The cell lysate was sonicated on ice with three 5-second pulses at high intensity. Then it was centrifuged at 3,000  $\times$  g for 15 minutes to pellet the cellular debris, and the supernatant (crude recombinant TcpA) was transferred to a fresh tube. Five microliters of the supernatant were removed for SDS-PAGE analysis. The TcpA was purified from the remaining supernatant by the Pro-Bond Purification System (Invitrogen, CA, USA). 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 by lyophilization. The resulting protein (recombinant TcpA; rTcpA) was then used as one of the three oral cholera vaccine components.

### Preparation of the vaccines

#### *Liposome-associated refined antigen formulation with CpG DNA (vaccine 1)*

The 3 antigens of *V. cholerae*, namely LPS, crude rTcpA, and H-CT were entrapped in liposomes or inserted in lipid bilayers by the bath-sonication method<sup>19,34</sup> to produce a water-in-oil emulsion. Each per rat dose of this vaccine consisted of 5 mg of LPS, 5 mg of crude TcpA, 200  $\mu$ g of H-CT and 100  $\mu$ g of CpG DNA (ODN#1826 with the sequence 5'-TCCATGAC-GTTCCTGACGTT-3') (Coley Pharmaceutical Group, Wellesley, MA, USA). Briefly, liposomes were prepared from two batches of lipid solutions (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. *V. cholerae* 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 water alone was mixed with the batch Y. Both preparations were sonicated at 20 kHz per second for 3 minutes, until each formed a homogeneous single phase emulsion. The two preparations were mixed together and dried at 40°C under a low vacuum in a rotary evaporator. When the preparation was dried to a gel-like consistency, 3 ml of 5% NaHCO<sub>3</sub> (vaccine diluent) was added to create a homogeneous preparation, which was then mixed with 100  $\mu$ g CpG DNA. Thus one dose of vaccine 1 was prepared. Vaccine 1 was used for immunizing rats of group 1.

#### *Liposome-associated refined antigen formulation with non-CpG DNA (vaccine 2)*

Vaccine 2 was prepared similarly to vaccine 1 except the diluted vaccine was mixed with non-CpG DNA (ODN#1982), instead of the CpG DNA (ODN#1826). Vaccine 2 was used for immunizing rats of group 2.

#### *Liposome-associated refined antigen formulation (vaccine 3)*

The three antigens of *V. cholerae* (LPS, rTcpA and H-CT) were entrapped in liposomes or inserted in lipid bilayers as described above for vaccine 1. One dose of vaccine 3 was prepared in 3 ml of vaccine diluent without adding any CpG DNA. This vaccine was used for immunizing rats of group 3.

#### *Non-liposome vaccine with CpG DNA (vaccine 4)*

Vaccine 4 consisted of the three antigens in a vaccine diluent mixed with ODN#1826 and was used for immunizing rats of group 4.

#### *Non-liposome vaccine with non-CpG DNA (vaccine 5)*

Vaccine 5 consisted of the three antigens mixed with ODN #1982 in a vaccine diluent and was used for immunizing rats of group 5.

#### *Non-liposome vaccine (vaccine 6)*

An oral cholera vaccine composed of only the three *V. cholerae* antigens in 3 ml vaccine diluent was prepared for immunizing rats of group 6.

**Vaccine 7 (control/placebo 1)**

Vaccine 7 composed of only liposomes in vaccine diluent was prepared for immunizing rats of group 7.

**Vaccine 8 (control/placebo 2)**

Vaccine 8 composed of only CpG DNA in vaccine diluent was prepared for immunizing rats of group 8.

**Vaccine 9 (the negative control)**

Vaccine 9 composed of 5% NaHCO<sub>3</sub> solution (3 ml per dose) was prepared for immunizing rats of group 9.

**Immunization of rats**

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 more than one week before commencing the experiments. The rats were starved for at least 15 hours, and 1 ml of 5% NaHCO<sub>3</sub> was given orally to each of them to reduce gastric acidity prior to vaccination. They were then divided into nine groups of five rats each. Each of the group 1 rats received vaccine 1 three times at 14-day intervals. The rats of the other groups were given the respective vaccines or placebo controls (vaccines 2-9) using the same immunization schedule. The animals had adequate supply of food and water at all times.

**Sample collection**

Six days after the last immunization, food was withheld from

all rats, only drinking water was supplied. After fasting for 24 hours, all rats were sacrificed by cervical dislocation. The abdominal cavity of each rat was opened, the whole length of the small intestines was collected and the intestinal lumen was quickly flushed three times with an excess volume of cold PBS, pH 7.2, in order to remove the intestinal content. The middle part of the small intestines (28-32 cm from the pylorus) was cut into pieces of 1 cm in length. These jejunal segments were kept individually in disposable molds at -70°C.

**Preparation of rabbit polyclonal antibodies to CT and TcpA**

Cholera toxin (Sigma Chemical Co., USA) was injected intravenously into a rabbit (weighed 2-2.5 kg) in 1 ml of normal saline solution. The concentrations of CT injected at days 1, 8, 15, 33, 43 and 50 were 4, 10, 20, 40, 40 and 40 µg/ml, respectively. The rabbit was bled on day 64 and the serum anti-CT titer was determined by an indirect ELISA. When a satisfactory level of the antibody was found, the animal was bled via heart puncture. Serum immunoglobulins were obtained after ammonium sulfate precipitation. After dialysis, the protein content and the antibody titer of the preparation were determined. The anti-CT indirect ELISA titer of the preparation was 1:8,000.

Rabbit polyclonal antibodies to *V. cholerae* TcpA were prepared by injecting another rabbit subcutaneously with a homogenate of polyacrylamide gel strips containing bands of purified rTcpA protein cut from an SDS-PAGE separated-recombinant *E. coli* DH5α lysate. The rabbit was re-immunized simi-

larly on days 14, 28, 42 and 56 and was bled on day 70; the immunoglobulins were precipitated as for the rabbit anti-CT immunoglobulins. The protein content and the anti-TcpA titer were determined. The anti-rTcpA indirect ELISA titer of the preparation was 1:2,000.

**Mouse monoclonal antibodies to *V. cholerae* O1 LPS**

The hybridoma clone 27E10, which produced monoclonal antibodies specific to *V. cholerae* O1 LPS<sup>35</sup> was cultured in a serum free medium. Each ml of the culture supernatant contained 2,560 indirect ELISA units against the homologous LPS.<sup>35</sup>

**Enumeration of antibody producing cells (AbPC)**

A double antibody sandwich immunofluorescence (IF) method was used for enumerating cells which produced antibodies to LPS, rTcpA and CT in the intestinal lamina propria of the experimental rats. A cryostat section (5 µm) of the jejunum was applied to a microscopic slide and fixed first with 90% methanol, followed by absolute methanol. The section was then covered with an appropriate antigen at a specific concentration (10 µg/ml of pure LPS for enumerating anti-LPS producing cells, 10 µg/ml of pure rTcpA for enumerating anti-rTcpA producing cells and 2 µg/ml of CT for anti-CT producing cells). The reaction was allowed to take place for 30 minutes at room temperature. Then left-over materials were washed off with PBS, pH 7.2. The section was overlaid with either 80 indirect ELISA units of mouse monoclonal antibodies to *V. cholerae* O1 LPS, 1:100 rabbit anti-

TcpA or 1:100 rabbit anti-CT for detecting anti-LPS producing cells, anti-TcpA producing cells or anti-CT producing cells, respectively, for 30 minutes in a humid chamber at room temperature. The slides were washed in PBS, pH 7.2 for 30 minutes and incubated for 30 minutes at room temperature with 1:40 rabbit anti-mouse immunoglobulin-FITC for the anti-LPS producing cells or 1:40 swine-anti rabbit immunoglobulin-FITC for the anti-TcpA and anti-CT producing cells. The slides were washed as above and blotted. They were then mounted with buffered glycerol, pH 7.6, and covered with cover slips. The mucosal immune response of rats was determined by enumerating the positive antibody-producing cells in the jejunal lamina propria with an Olympus fluorescence microscope (Olympus, Japan) using 10× ocular and 20× objectives. Two intestinal sections from each rat (5 rats per group) were used for counting the positive cells of each type, *i.e.* two sections for anti-LPS producing cells, two sections for anti-TcpA producing cells and two sections for anti-CT producing cells. The mean and standard deviation of the number of antibody producing cells were calculated for each antigenic specificity in 250 microscopic fields per group of rats.

#### Statistical analysis

The differences in the means of the antibody producing cell counts among the groups of rats were statistically analyzed by using one way analysis of variance (ANOVA) and least significant difference (LSD).

### RESULTS

The appearance of antibody producing cells (AbPC) in the in-

testinal lamina propria is shown in Fig. 1. Rats of group 1 which received liposome-associated antigens plus 100 µg of CpG DNA#1826 gave higher anti-LPS, anti-TcpA, and anti-CT cell counts in the lamina propria than rats given liposome-associated antigens plus non-CpG DNA#1982 (group 2) or rats given only liposome-associated antigens (group 3). The average number of antibody producing cells per one microscopic field, *i.e.* 10× ocular, 20× objective (mean ± standard deviation from 250 microscopic fields) (Table 1) for anti-LPS, anti-TcpA, and anti-CT producing cells for group 1 were 118.72 ± 2.47, 47.70 ± 0.54, and 111.73 ± 2.55, respectively. While for group 2, the respective average cell counts were 109.23 ± 2.59, 43.10 ± 0.67, and 94.38 ± 2.13, respectively, and for group 3 the respective average cell counts were 108.06 ± 2.27, 43.17 ± 0.64, and 93.51 ± 2.27, respectively. The respective cell counts of group 1 were significantly higher than those of groups 2 and 3 ( $p < 0.05$ ). Rats of group 4 which received free antigens plus 100 µg of CpG DNA #1826 gave higher anti-LPS, anti-TcpA, and anti-CT cell counts than rats given free antigens plus non-CpG DNA#1982 (group 5) or rats given only free antigens (group 6). The average cell counts for anti-LPS, anti-TcpA, and anti-CT producing cells for group 4 were 86.85 ± 2.11, 37.30 ± 0.58, and 83.44 ± 2.21, respectively. While for group 5, the average cell counts were 80.11 ± 2.20, 34.24 ± 0.65, and 74.74 ± 2.16, respectively, and for group 6 the average cell counts were 80.08 ± 2.58, 34.68 ± 0.43, 74.80 ± 2.02, respectively.

Rats which received lipo-

some-associated antigens in all groups (group 1, group 2, and group 3) gave significantly higher anti-LPS, anti-TcpA, and anti-CT cell counts than rats given free antigens in similar doses without liposomes, *i.e.* group 4, group 5, and group 6.

Groups 1, 2, 3, 4, 5, and 6 which received cholera antigens gave significant immune responses as opposed to rats that did not receive the antigens (group 7, group 8, and group 9). The rats of group 7, group 8, and group 9 gave statistically similar AbPC averages even though rats of group 7 and group 8 received empty liposomes and CpG DNA#1826, respectively, while rats of group 9 only received vaccine diluent.

### DISCUSSION

In this study, the mucosal immune responses to different formulations of oral cholera vaccine prepared from refined *V. cholerae* antigens were measured by enumerating the specific antibody producing cells (anti-LPS, anti-TcpA and anti-CT) in the intestinal lamina propria of the vaccinated rats. These cells which contained antibodies in their cytoplasm were revealed by a sandwich immunofluorescence assay. As early as seven days after the third oral dose of vaccination, there was a good seeding of the lamina propria with specific antibody producing cells in rats which received antigens with and without adjuvants. Whether fewer doses of the individual vaccine formulations are similarly effective still needs to be studied.

Liposomes made of sphingomyelin and cholesterol have been

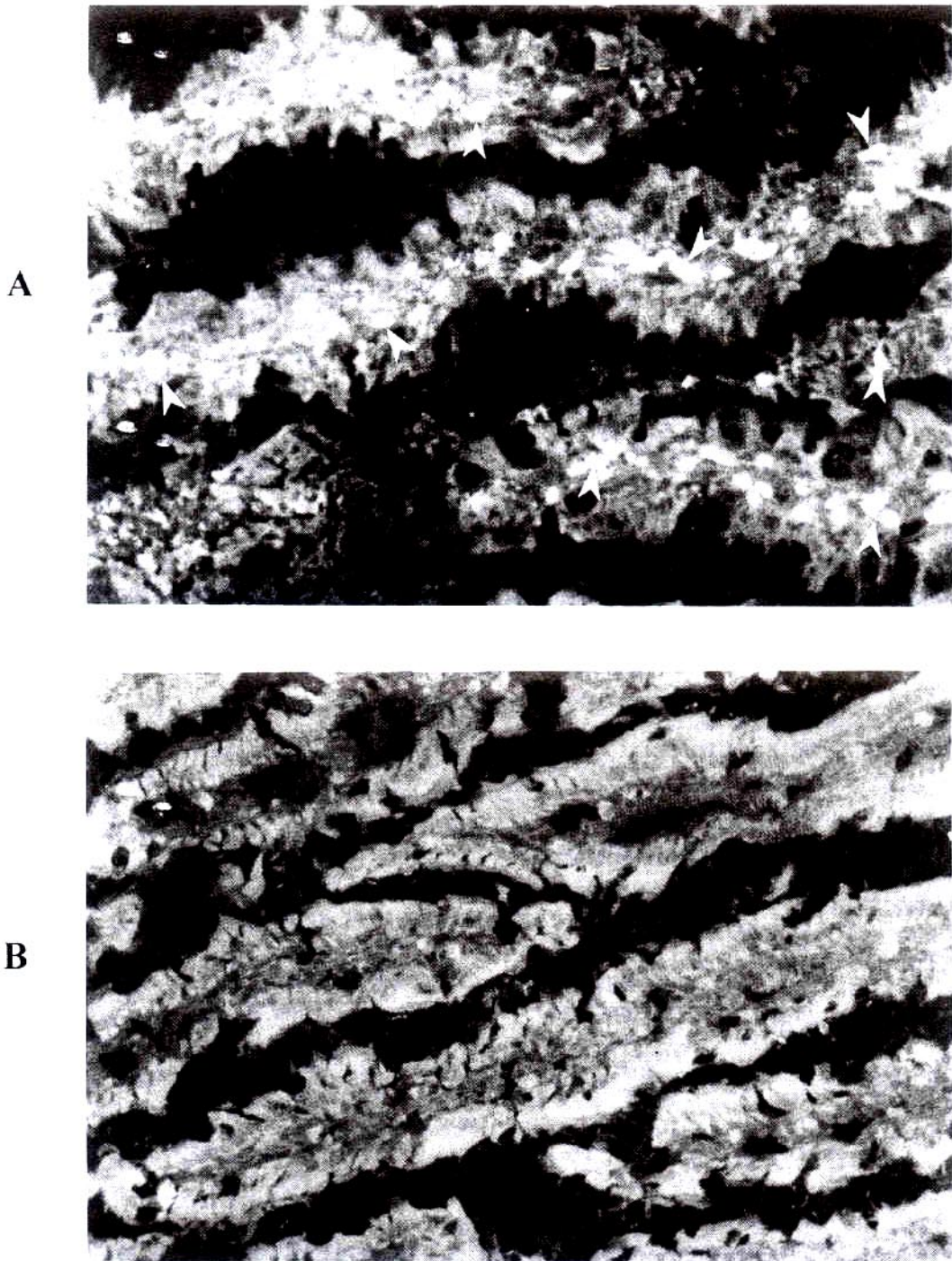


Fig. 1 (A) Jejunal lamina propria of a rat of group 1 showing anti-LPS producing cells (arrows). (B) Jejunal lamina propria of a rat of group 9 which served as negative control.

found previously to be good mucosal adjuvants and antigen delivery vehicles for an oral cholera vaccine consisting of *V. cholerae* LPS, cell bound hemagglutinin and procholera-eragenoid.<sup>19</sup> In this study, the potential adjuvanticity of CpG DNA alone or in combination with liposomes for an oral cholera vaccine prepared from combined antigens of *V. cholerae* (LPS) and *E. coli* clones harboring the *V. cholerae* *ctx* or *tcpA* gene (rTcpA and H-CT), was studied. The immune responses of rats which were orally given the liposome-associated antigens alone, or together with CpG DNA or non-CpG DNA were compared. The average counts of antibody producing cells of all three antigenic specificities in rats vaccinated with the liposome vaccine in the presence of CpG DNA (group 1) were higher than those given the liposome vaccine alone (group 3) or liposome vaccine with non-CpG DNA (group 2). The differences were statistically significant ( $p < 0.05$ ). There was no difference in the average cell counts for anti-LPS, anti-TcpA, and anti-CT producing cells between rats of groups 2 and 3. The CpG DNA alone without liposome conferred adjuvanticity to the vaccine consisting of free antigens (LPS, TcpA and H-CT); i.e. the number of antibody producing cells in rats given antigens mixed with CpG DNA (group 4) were significantly higher than in rats given the antigens alone (group 6) or with non-CpG DNA (group 5). There was no difference in the average cell counts between rats of groups 5 and 6.

The results of our experiments established that liposome and CpG DNA (ODN#1826) were effective and synergistic adjuvants for the refined antigen oral cholera

**Table 1** Number of antigen-specific antibody-producing cells (AbPC) in the jejunal lamina propria of all groups of rats as determined by an antibody sandwich immunofluorescence assay

Group no.	Average number of AbPC /1 microscopic field		
	anti-LPS	anti-TcpA	anti-CT
1 <sup>a</sup>	118.72 ± 2.47**	47.70 ± 0.54	111.73 ± 2.55
2 <sup>b</sup>	109.23 ± 2.59	43.10 ± 0.67	94.38 ± 2.13
3 <sup>b</sup>	108.06 ± 2.27 <sup>b</sup>	43.17 ± 0.64	93.51 ± 2.27
4 <sup>c</sup>	86.85 ± 2.11	37.30 ± 0.58	83.44 ± 2.21
5 <sup>d</sup>	80.11 ± 2.20	34.24 ± 0.65	74.74 ± 2.16
6 <sup>d</sup>	80.08 ± 2.58	34.68 ± 0.43	74.80 ± 2.02
7 <sup>e</sup>	12.15 ± 0.39	7.10 ± 0.23	9.04 ± 0.41
8 <sup>e</sup>	8.48 ± 0.31	5.70 ± 0.19	5.59 ± 0.44
9 <sup>e</sup>	8.02 ± 0.41	5.74 ± 0.17	5.01 ± 0.18

\*Numbers of AbPC in groups with similar superscript are not statistically different at  $p < 0.05$ .

\*\*Average numbers (the mean ± standard error) from 250 microscopic fields (10× ocular, 20× objective).

vaccine. Liposomes, regardless of their composition and size, can adsorb to most cells and release their incorporated/associated substances which can then interact with the cells.<sup>37-39</sup> Liposomes were shown to be taken up by macrophages and other phagocytic cells in the blood, lymph, and various tissues (e.g. lymph nodes, spleen and liver) with the subsequent release of the associated antigen(s). The liver and spleen took up nearly all liposomes introduced by the intravenous route, whereas most liposomes injected via the subcutaneous or intramuscular route were retained at the site of injection and were taken up by filtering macrophages. Besides the adsorption capability, another explanation for the immunoadjuvanticity of liposomes is that antigen presenting cells (APC) are more capable to efficiently take up and process antigens when they are associated with liposomes and thus produce an enhanced immune re-

sponse. It has also been suggested that liposomes may directly present antigen to lymphoid cells for the induction of an immune response. Liposomes can also serve as an immunoadjuvant for mucosal immunization.<sup>17,19,40-43</sup> Liposomes provide for an effective uptake by the M cells of the Peyer's patches and for subsequent interaction with antigen presenting cells in the subepithelium dome region. Although the uptake efficacy by M cells and the subsequent induction of the mucosal antibody (secretory IgA) response remain unclear, it has been shown in most cases however that co-administration of antigens (and adjuvant) in liposomes can significantly improve the immune response.<sup>17,19,43</sup> The proposed mechanism for the delivery properties of liposomes is their ability to protect the antigen from hostile environments, e.g. acids and proteolytic enzymes of the host. Although liposomes are not completely resistant to the lipases and

bile salts found in the small intestine, polymerized liposomes or liposomes made up of cholesterol, like the one used in this study, are at least partially resistant.<sup>44,45</sup>

Mucosal adjuvanticity of unmethylated CpG DNA has been demonstrated previously in mice where hepatitis B virus surface antigen was co-delivered intranasally.<sup>46</sup> The adjuvanticity of the CpG ODN was found to be superior to that of CT for the induction of both humoral and cell-mediated systemic immunity as well as for the induction of mucosal secretory IgA responses in the lungs and in feces. Moreover, synergy was observed between CpG and CT. When administered together, CT and CpG induced ten times stronger responses than either CT or CpG administered alone.<sup>46-48</sup>

The mechanisms by which oral CpG DNA works are not yet understood. It has been shown that foreign DNA ingested by mice can be detected in columnar epithelial cells, Peyer's patches, liver, spleen and peripheral blood cells, i.e. B cells, T cells and macrophages. Using CpG DNA in oral vaccines may enhance the immunogenicity of poor oral antigens (i.e. subunit vaccines) or allow to use lower doses of strong antigens. The development of safe and effective oral vaccines that induce both mucosal and systemic immunity would provide better protection against mucosal pathogens in an easy and cost-effective manner, thus reducing morbidity and mortality worldwide. Therefore our findings that CpG DNA and liposome are able to enhance the intestinal immune responses in rats vaccinated with refined *V. cholerae* antigens is of considerable interest

for the future development of oral vaccines, not only against cholera, but also against numerous other infectious diseases.

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