CHAPTER VI
DISCUSSION

Strategies for prevention and control of cholera are health education to promote good personal hygiene emphasizing proper hand washing and food preparation, construction and maintenance of proper sewage disposal facilities, and provision of safe and plentiful water including simple and inexpensive methods of domestic water disinfection and storage (Tauxe *et al.*, 1995; Sanchez and Taylor, 1997). These ideal strategies are not feasible in many of the developing countries where the disease is endemic, due to lack of resources and proper attention and concerns. Thus, the use of an effective vaccine has been considered as a promising alternative in the prevention and control of cholera (Levine *et al.*, 1981).

An ideal vaccine would provide effective immunogenicity which includes high, sustainable immune response and good memory. Besides, it should be safe with none or acceptable untoward reaction, available at reasonable cost, and easily delivered to populations at risk especially in developing parts of the world, and it should require no booster. Moreover, other factors contributing to the success of vaccination should be taken into account. These include the route of immunization (parenteral *versus* mucosal), the form of the antigen (lived, killed, peptide subunit, *etc.*), and the usage of biologically active elements (delivery vehicle and/or adjuvant) that mediate specific tissue tropism (Walker *et al.*, 1994; Spriggs *et al.*, 1996).

Since the pathogenic mechanisms of *V. cholerae* infection are confined to the intestinal mucosa, protection against cholera by immunological factors should be incited locally, *i.e.* induction of specific local immune responses at the mucosa associated-lymphoid tissues (MALT). This is most effectively achieved by direct application of a vaccine to the mucosal surfaces, *i.e.* oral immunization, which when the antigens are delivered appropriately resulting in the optimal induction of humoral and/or cell-mediated immune response (Dietrich *et al.*, 2003). Oral vaccination represents the most promising vaccination strategy to induce mucosal immunity and to prevent gastrointestinal infections or infections that start from the gastrointestinal mucosa (Moldoveanu *et al.*, 1995).

Several oral cholera vaccines have been developed. Live, attenuated oral cholera vaccine candidates have been produced by either chemical treatment of wild type *V. cholerae* such as Texas Star SR (Honda and Finkelstein, 1979; Levine *et al.*, 1984) or by genetic engineering such as CVD103-HgR (Mekalanos *et* al., 1983; Kaper *et al.*, 1984a, 1984b). The mutants provoke immune response that closely mimics that produced by the natural infection. This is because the live attenuated strains can transiently propagate and colonize the gut of the vaccines; hence providing adequate supply of antigen to the MALT. The live-attenuated whole organism vaccine provides prolonged exposure of the immune cells to several epitopes of the attenuated organisms, thus, resulting in high immunogenicity and good memory (Levine and Kaper, 1993). As a consequence, a single dose of these vaccines was found to give adequate immunity. This eliminates the need for boosters (Kotloff *et al.*, 1992; Gottuzzo *et al.*, 1993; Tacket *et al.*, 1999), which is a major advantage to the third world countries, where epidemiologic studies have shown that roughly 20% of individual fails to return for each subsequent booster (Goldsby *et al.*, 2000). However, the drawbacks of the live-attenuated vaccines are their potential to cause adverse reaction in vaccinees and the ability to reverse to pathogenic state of the attenuated vaccine strains (Levine *et al.*, 1988; Herrington *et al.*, 1988; Kaper *et al.*, 1995).

In an effort to ensure non-reactogenicity while conferring protection, a killed whole-cell cholera vaccines containing CTB subunits, *e.g.* WC-BS (Holmgren and Svennerholm, 1990) and WC-rBS (Sanchez *et al.*, 1994) have been developed. Although, these vaccines have been proved to be safe with no adverse reaction (Clemens *et al.*, 1990; Jertborn *et al.*, 1992; Sanchez *et al.*, 1993), they confer low immunogenicity and thus multiple (at least two and possibly three) spaced, large antigenic doses (Sanchez *et al.*, 1994; Trach *et al.*, 1997) are required to prime and boost the gut immune system. This would make a vaccine relatively costly. Besides, community vaccination programs in the endemic areas (most developing countries) are much more complicated for vaccines that need boostering than a single dose of the live-vaccine.

To overcome obstacle from live-attenuated and killed whole-cell vaccines, a refined vaccine consisting of specific and purified macromolecules derived from

pathogens was developed. The idea is to use highly immunogenic, well-defined epitopes, either individually or their cocktail thereof as vaccine component(s).

From the *V. cholerae* pathogenesis, it is speculated that three functional types of antibodies would be required for complete protection against cholera. The first antibody, namely the agglutinating antibody, would agglutinate the newly arrived vibrios in the intestinal lumen. Vibrio antigens that elicit the production of antibody with this kind of activity include antigens on the surface of the vibrios, *e.g.* flagella, outer membrane protein, LPS; among which LPS is the most predominant and easiest one to prepare (Chitnis *et al.*, 1982; Jacob *et al.*, 1993; Sinha *et al.*,1993; Leelawongtawon *et al.*, 2003). The second antibody, *i.e.* antibody to vibrio adhesive factor, would be required to prevent the adhesion of the vibrios to the mucosal epithelial cells and thus prevent the bacterial colonization. The antigens which can elicit the rise of anti-adherence antibody include CHA and TcpA (Foo and Chaicumpa, 1981; Tayer *et al.*, 1987; Herrington *et al.*, 1988; Chaicumpa *et al.*, 1990; 1998). Once the vibrios have attached to the intestinal epithelium, they multiply and produce toxins, *e.g.* cholera toxin. The antitoxin would, then, afford protection against cholera by blocking the binding of the B subunits of CT to the Gm1 receptors on the host cell. Immunogens responsible for stimulation of antitoxin production include CT holotoxin, B subunits or procholeragenoid (P) (Pierce *et al.*, 1983; Chaicumpa *et al.*,1987; 1998). Besides, cholera antigens, *i.e.* LPS, CHA, and P, have been found to confer antigenic synergism when given orally together to animal or human volunteers (Chaicumpa *et al.*, 1987, 1998). The vaccine composed of the three antigens elicited better immune response over the killed whole *V. cholerae* vaccine (Chaicumpa *et al.*, 1998; Kalambaheti *et al.*, 1998). This may be due to the fact that the former contained only immunogenic components while the whole-cell organism vaccine may, as well, contains certain immune suppressive epitopes, and thus stimulates regulatory/suppressive T cell response.

A major challenge to successful oral immunization also requires the fulfillment of the following two requirements. First, vaccine antigens need to be delivered effectively to mucosal inductive lymphoid tissues. Second, immune responses induced within the lymphoid tissues need to be enhanced through co-administration of appropriate mucosal adjuvants. These manipulations lead to reduction of the size and

frequency of the vaccine dose, protect the vaccinees from untoward reactions which may be induced by vaccine components, and enhance the immunogenicity of the vaccine. Liposome and CpG DNA represent possible delivery vehicles (Allison and Gregoriadis, 1974; New *et al.*, 1985; Chaicumpa *et al.*, 1990, 1998) and effective adjuvants for oral vaccines (Davis *et al.*, 1998; McCluskie *et al.*, 1998; Deml *et al.*, 1999), respectively.

Oral cholera vaccines are aimed to be implemented both in endemic and non-edemic areas. The vaccines are used to prevent and control the outbreaks of cholera among naturally exposed inhabitants in the endemic parts, whereas they will be used to protect the cholera naïve travellers, who will travel to the cholera endemic areas.

Unmethylated bacterial CpG DNA (ODN#1826) and liposome were found to be effective and synergistic mucosal adjuvants for the refined antigen oral cholera vaccine, when three doses at 14 day intervals were administered orally to experimental rats, as shown by an increase in the number of ASC of all three antigenic specificities as compared to the vaccine alone, liposome associated vaccine or vaccine plus CpG DNA (Leelawongtawon *et al.*, 2003). In this study, the liposome-associated oral cholera vaccine prepared from *V. cholerae* antigens, LPS, P, and TcpA, in the presence of unmethylated bacterial CpG DNA (ODN# 1826) was analysed, by means of the kinetics of the antigen-specific ASC in the blood circulation of the cholera antigen primed-rats. The study was carried out with eight-week old Wistar rats which had been orally primed with the vaccine in order to emulate the natural condition of the people in the endemic areas which usually have been naturally exposed to cholera antigens. The numbers of specific ASC (anti-CT, anti-LPS, and anti-TcpA) in blood of boosted rats were enumerated at different times by ELISPOT assay after a vaccine booster dose was given to each rats 14 days after priming.

Antigens of *V. cholerae*, namely LPS, crude TcpA, and P were entrapped in liposomes by the bath-sonication method. Sizes of liposome that were measured by using sub-micron particle analyzer (Coulter® Model N4D, Germany) range from 55.5 nm to 3,010 nm that are taken up by specialized M cells of Peyer's patch. The small (submicron) colloidal particles are believed to be absorbed and transported via the intracellular pathway through the enterocytes, while larger particles (several microns) are absorbed exclusively by Peyer's patches M cells (Chen and Langer, 1998).

Eldridge *et al.*, demonstrated in their study that the particles greater than 10 μm were not taken up (Eldridge *et al.*, 1990). Therefore, a multilamellar liposome in this study could be taken up by Peyer's patches M cells after oral administration. These antigens might be attached to the liposome surface (*i.e.*, LPS) or be entrapped in the aqueous compartment (*i.e.*, P and TcpA). Therefore, this vehicle that encapsulate the antigens within the particles could protect the antigens from degradation in the gastrointestinal tract.

All (100%) of antigen-primed rats revealed immune responses to all vaccine components. The presence of ASC of all antigenic specificities could be detected in blood circulation of all primed-rats indicating high immunogenicity. The kinetics of ASC numbers in blood to all three immunogens revealed similar trend. Anti-LPS and anti-TcpA ASC started to appear on day 2 and peaked on day 3 after the booster. The CT-specific ASC appeared also on day 2 and maximum number was found on day 4 after the booster. Thereafter, all immunogen-specific ASC declined markedly. None of the tested rats revealed detecable ASC in blood at day 13 after the booster immunization (the times between days 6 and 13 were not studied). Appearance of ASC in the blood circulation very soon after a booster dose of the vaccine implies that the antibody committed B lymphocytes left the Peyer's patches (inductive site) and recirculated before homing to the intestinal lamina propria and other mucosa, which is a regular traffic of the lymphocytes of the MALT; they are activated at mucosal inductive site migrate through the blood circulation to the another and/or remote effector site(s) of the mucosal lymphoid tissues after completing their maturation (as mentioned previously in Chapter II). In this study, the antibody committed lymphocytes in the blood circulation of the vaccinated rats would be expected to home at the mucosal effector sites, *i. e.* lamina propria, and ready to secrete protective antibodies upon re-exposure to the same antigens that reach the lymphoid tissue *via* to the intestinal lumen. The specific antibody committed cells at the lamina propria are believed to be committed for the recall memory upon re-exposure to antigen. They would be able to exert their activity rapidly in order to protect the host in time after exposure to *V. cholerae* and before the onset of cholera as it is known that the incubation period of cholera is about 3-5 days after ingesting contaminated food or water.

Orally administered vaccine has been known to stimulate mucosal immune response effectively. However, serum response can also be expected if the antigen reached other distant lymphoid organs, *e.g.* lymph nodes or spleen, or if the locally produced antibodies escaped the secretory process of the intestine and entered the blood circulation. However the systemic antibodies are ineffective in prevention of cholera.

Results of serum ELISA in this study revealed that the antigens were able to generate serum antibody response in some of the immunized rats. Anti-CT response was found in 11/21 (52.4%) of the vaccinated rats, with the highest reciprocal titer at 5120. While, only 14.3% and 19% of the vaccinated rats showed a significant increase of anti-LPS and anti-TcpA, respectively. It seems as if the liposome-associated vaccine confined the immune response, mainly to the mucosal (intestinal) lymphoid tissue.

Complement-mediated antibody-specific bactericidal activity has been described as the activation of complement by antibodies bound to cell surface of the target bacteria resulting in bacteriolysis and cell death (Alain *et al.*, 2003). The vibrio antigens against which the vibriocidal antibodies were directed, have been shown to lie in the stable polysaccharide determinants of LPS and in a proteins commonly found in varying amounts closely associated with them (Neoh and Rowley, 1970). Proteins that have been shown to be protective in animal studies include outer membrane proteins, flagellar protein, flagellar sheath proteins, cell associated haemagglutinin, a 30-kDa surface protein, TCP, and fimbriae (Steele *et al.*, 1974; Chaicumpa and Atthasistha, 1977; Ehara *et al.*, 1991). The serum vibriocidal antibody is believed to be a reliably predictor of resistance to cholera and vibriocidal assay has been used as the only valid serologic assay that correlates with protection (Mosley *et al.*, 1969). The presence of this antibody is thought to be a marker for protective intestinal antibodies which may be directed against the same antigens (Kaper *et al.*, 1995).

In this study, the vaccine could elicit response in all immunized rats as shown by ELISPOT and IFA of ASC in the lamina propria. However, it could induce vibriocidal antibody only in 12 of 20 vaccinated rats (57.1%) (only twofold rising). The results tended to indicate that the liposome-associated oral cholera vaccine with unmethylated bacterial CpG DNA limited the systemic immunity, both serum ELISA

and vibriocidal antibodies. As vibrios are the pathogen of gastrointestinal tracts, triggering a commitment of mucosal immunity characterized by the production of secretory IgA antibodies from ASC is the main protective immune mechanism against the disease (Pierce and Koster, 1990). Logically, the vaccine as the one of this study that could enhance the specific ASC activity, although showed limiting systemic immunity (conferring the low serum ELISA and vibriocidal antibody responses) should be good enough as one of the public health tools.

It has been known that the most potent mucosal adjuvants are the bacterial toxins, *i.e.* CT produced by *V. cholerae* or LT produced by enterotoxic *E. coli* (Holmgren *et al.*, 2003). Oral administration of holo CT, its individual B subunits (CTB), or heat-treated CT, *i.e.* procholeragenoid (P) elicits strong serum and mucosal antibody responses (Pierce *et al.*, 1983). Procholeragenoid (P) obtained by heating the CT at 65°C for at least 15 min is as immunogenic as the parental toxin while remaining only 5% or less of its original toxicity (Finkelstein *et al.*, 1971). Liposome-associated oral cholera vaccine prepared from LPS, CHA and P generated strong serum anti-CT antibodies in Thai volunteers (Chaicumpa *et al.*, 1990, 1998). This finding suggested that P, through orally administered, might be able to reach the circulation and activated the systemic immune response, whereas LPS and TcpA elicited the immune response mainly locally (Chaicumpa *et al.*, 1998). In this study, antibody responses were not evaluated and followed up until the end of the experiment (day 21 after the booster immunization), which may give the information about the maximum antibody response, due to the limited animal number. A large number of rats make it very difficult to handle during doing experiment. However, the pattern of antibody responses had trend that correlated with the findings in human study (Chaicumpa *et al.*, 1998).

The background antibodies to cholera antigens were found in the sera of control rats which received placebo. Also, few ASC were found in their lamina propria. These suggest that the rats have been exposed to cholera antigens previously perhaps through food or drinking water. The condition emulates the people of the cholera endemic areas.