CHAPTER I
INTRODUCTION

Cholera continues to be a major public health problem in nearly all developing countries. The disease is endemic in some areas, while in other areas, epidemic can result from social strife and crowding conditions such as in refugee camps. Although the disease can be treated relatively simply by oral and intravenous rehydration, many developing countries do not have sufficient access to the volumes of rehydration fluids during large outbreaks. Antibiotics such as tetracycline have been used with success to shorten duration of diarrhea, but resistance to antimicrobial agents has been increasing throughout the world (Kaper *et al.*, 1995). Since cholera outbreaks involve large numbers of people within a short period, it is one of the diseases which prevention is better than cure. The usual preventive approaches such as health education, sanitation, and improved water supplies are highly ideal, but not feasible in many of the developing countries where the disease is endemic, due to lack of resources or proper attention and concerns. For these reasons, the use of an effective vaccine has been considered a promising alternative in the control of cholera.

Cholera vaccine efforts have included the development of both parenteral and oral vaccine candidates. Parenteral, killed whole-cell vaccines have been available since the end of the nineteenth century. These vaccines produce a short-term protection and significant adverse side effects (Ryan and Calderwood, 2000). It is known that the routes of antigens acquired by individual lymphoid tissues affect the outcome of an immune response and also the effectiveness of the response. For example, antigens in blood are filtered, trapped, processed and presented in strategic blood/tissue interfaces in the spleen, which result in peripheral response (immunity) characterized by the appearance of specific IgM and/or IgG in the blood, most often effectively protect against the systemic pathogens. Numerous studies have indicated that induction of systemic immunity through parenteral immunization can effectively clear systemic infections; however, it usually fails to protect the mucosal infections (Mestecky, 1987). On the other hand, mucosal antigens, *e.g.* antigen in the lumens of

gastrointestinal tract (*e. g. V. cholerae* antigens) are trapped by M cells presented on the surface of intestine and transcytosed onto lymphoid cells in Peyer's patches which trigger mucosal immune response characterized by the production of secretory IgA (sIgA) antibodies, the main protective factor against mucosal infections. For this reason and also the mucosal nature of *V. cholerae* infection, development of oral vaccines that might result in mucosal immune responses and immunity is more appropriate for protection against cholera. Presently, both live attenuated and killed whole-cell vaccines have been evaluated and some have been commercialized. However, most of these vaccines still provide limited protection. Moreover, a variety of undesirable symptoms such as mild diarrhea, malaise, cramps, fever, and headache also have been noted (Nataro and Levine, 1999).

Refined *V. cholerae* antigens (subunits or macromolecules) have been isolated and tested as vaccine components. Animal and human trials of oral vaccines prepared from combinations of such refined antigens have been conducted. In 1987, Chaicumpa and coworkers developed an oral bacterial fraction vaccine consisting of LPS, cell-bound haemagglutinin (CHA), and procholeragenoid (P) (a heat-treated, high molecular weight CT having <5% of the toxicity of the holotoxin). The LPS was expected to elicit agglutinating antibodies that would agglutinate the newly arrived vibrios in the intestinal lumen. The CHA is for eliciting the anti-adhesin antibodies that will inhibit the attachment of the vibrios to the intestinal epithelium and the P is for eliciting the anti-cholera toxin antibodies. The vaccine components showed antigenic synergism by increasing the total number of antibody-producing cells of all antigenic specificities compared with the vaccine consisted of one antigen alone, when two doses at 14 days intervals were administerated orally to animals (Chaicumpa *et al.*, 1987). A similar vaccine (combined LPS, CHA and P) was tested in Thai volunteers and found to confer high rates of intestinal antibody production of all antigenic specificities (Chongsa-nguan *et al.*, 1991).

The oral vaccines prepared from the killed vibrios or refined antigens are safe with no untoward reaction as compared to live vaccines, but may be less immunogenic. Therefore, there is an urgent need for the development of potent and safe adjuvant and/or delivery vehicle for oral vaccines. Liposomes represent one possible delivery system, which protects antigens from acids and proteolytic enzymes

of the gastrointestinal tract. It may increase antigen absorption, processing and presentation. These concentric spheres of phospholipid multilayers can deliver drug or antigens entrapped in the aqueous phase, incorporated into lipid multilayers, or adsorbed to liposomal surfaces and subsequently enter the cell cytosol (Michalek *et al.*, 1999). In 1990, an oral liposome-associated cholera vaccine consisting *V. cholerae* LPS, CHA and P was tested in rats by Chaicumpa and coworkers. The vaccine showed enhanced immune response, as measured by the appearance of specific antibody-producing cells in the lamina propria of the rats (Chaicumpa *et al.*, 1990). An oral liposome-associated cholera vaccine consisting of *V. cholerae* LPS, crude fimbrial extract (CF) and P also showed a higher rate of antigen-specific antibody response in Thai volunteers compared with the vaccine consisted of the same antigens but in free form (Chaicumpa *et al.*, 1998). The liposome associated vaccine triggered intestinal antibodies of IgM and/or IgA isotypes, but not IgG antibody. This human study confirms the findings in animals that a liposome-based cholera vaccine induces a superior class-specific- and antigen-specific-antibody response than does a free antigen vaccine. Thus, liposome appears to be a promising oral adjuvant and delivery system for the development of vaccine against infection. However, liposomes probably have the greatest potential for success when used with other immunostimulatory agents.

Mucosal vaccines generally require the use of adjuvants. A new class of adjuvant is unmethylated bacterial CpG DNA. It has been found that the bacterial DNA has direct immunostimulatory effects on leukocytes *in vitro* due to unmethylated CpG dinucleotides, which are under-represented and methylated in vertebrate DNA (Moldoveanu *et al.*, 1998). CpG dinucleotides in particular base contexts (CpG motifs) provide broad adjuvant activities that are due to several different effects it has on innate and adaptive immune responses. First, it causes B cells to proliferate, differentiate, isotype switch and secrete immunoglobulin. As well, CpG DNA directly activate monocytes, macrophages and dendritic cells to secrete interferon-α/β (IFN-α/β), interleukins (IL-1, IL-6, IL-12), chemokines, and tumor necrosis factor-α (TNF-α), which in turn stimulate natural killer (NK) and T cells to secrete additional cytokines. A T-helper function provided by the strong type 1-like pattern of cytokine production that is dominated by IL-12 and IFN-γ, with little secretion of type-2

cytokines. CpG also appear to have significant potential as mucosally administered adjuvant that was found to induce both serum IgG and mucosal IgA and capable of promoting antigen-specific immune responses (McCluskie *et al.*, 2001). The adjuvant effects of CpG appear to be maximized by their conjugation to protein antigens or their formulation with delivery vehicle (O'Hagan *et al.*, 2001).

In this thesis, an oral cholera vaccine composed of three antigenic components, *i. e.* LPS, procholeragenoid, and toxin-coregulated pili (TcpA) of *V. cholerae* O1, was entrapped into a liposome made of sphingomyelin and cholesterol. The vaccine is added with unmethylated bacterial CpG DNA. Wistar rats were orally primed with the vaccine and were boosted at 14-day intervals. Antigen-specific antibody-producing cells in the blood circulation and in the intestinal lamina propria of individual experimental rats were enumerated by using the enzyme-linked immunospot (ELISPOT) assay and the double antibody sandwich method of immunofluorescence assay (IFA), respectively, at days 2, 3, 4, 5, 6, 13, and 21 after the booster immunization.