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Immunogenicity and protective role of three formulations of oral cholera vaccine

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Three formulations of oral cholera vaccine were compared with respect to their immunogenicity and protective ability in a rat ileal loop model. Eight-week-old Wistar rats were divided into five groups. The first group received orally vaccine A consisting of liposome-associated V. cholerae *lipopolysaccharide, fimbriae and procholeragenoid, whereas the rats of groups 2 and 3 received orally vaccines B and C consisting of heat-killed fimbriated and non-fimbriated whole cell* V. cholerae, *respectively. Rats of groups 4 and 5 were controls that received orally liposomes alone and normal saline solution, respectively. It was found that vaccine A elicited stronger immune responses to all three* V. cholerae *antigens. The antibody responses were detected in both serum and intestinal lavage samples. Vaccine B elicited only modest serum and intestinal responses to* V. cholerae *fimbriae (anti-F). No detectable immune response was found in rats of group 3 immunized with vaccine C. Rats immunized with vaccines A and B had a similar order of magnitude of numbers of vibrios adhered to their intestinal mucosa. These numbers were less than those associated with the intestinal tissues of control rats of groups 4 and 5 by about two orders of magnitude. Although without any detectable immune response, rats of group 3 that were immunized with vaccine C showed some reduction in numbers of vibrios associated with their intestinal mucosa. The numbers of vibrios recovered from the intestinal segments of rats of all treatment groups were in the order group 1 = 2 < 4 = 5. Electron micrography also revealed patches of vibrio colonization on the mucosa of rats of groups 3, 4 and 5. These features were not found in the groups vaccinated with vaccines A and B. The inhibition of vibrio colonization afforded by the vaccines was biotype- and serotype-non-specific. The results suggest that the heat-killed whole cell fimbriated* V. cholerae *may be an alternative vaccine preparation to the liposome-associated refined antigen vaccine at a lower cost.* © *1997 Elsevier Science Ltd. All rights reserved*

Keywords: *V. cholerae*; oral vaccine; fimbriae; procholeragenoid; cholera toxin; lipopolysaccharide; liposomes; electronmicroscopy

Oral immunization is the preferred route for eliciting host immune responses to pathogens or their products such as toxins, adhesins, etc. at mucosal surfaces. Beside directly stimulating the mucosal immune system to release immunological defense factor(s), e.g. secretory immunoglobulins into or near to the site of infection, oral vaccines offer several advantages over parenteral ones. The use of the oral route eliminates the need for needles and so reduces the risk of blood borne infections. Also, there is no need for specially trained personnel to administer the oral vaccine. However, despite these advantages, oral vaccines have a major constraint: their limited ability to survive the peristalsis and ciliary movement of the tract, the mechanical (epithelial cell) and chemical (e.g. mucin) barriers, the acid pH of the stomach, and the alkaline pH and degradative enzymes present in the small intestine, which can eliminate and destroy the immunogens prior to their uptake and presentation to the immune apparatus[1].

Studies have shown that particulate antigens, especially when presented as viable organisms, are more effective than soluble antigens in inducing local as well as generalized secretory and systemic immune responses[1–3]. At least three reasons can be given to explain this. First, the size and composition of particu-

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late antigens may allow them to survive the environment of the gastro-intestinal tract more effectively. Second, some particulates are more efficiently absorbed by the gut-associated lymphoid tissue (GALT), specifically, through M (microfold) cells into Peyer's patches[4]. Third, soluble antigens cross the epithelial barrier of the gut in the form of low-molecular weight molecules. The recognition of these molecules by lymphoid cells at sites other than in the Peyer's patches has been proposed as the stimulus that initiates systemic tolerance after antigen feeding[5] and may provide a negative signal to the mucosal immune system[1]. During the past decade, modern technology has facilitated the development of oral antigen delivery systems with immunopotentiating activity[1,2,6–9].

Cholera caused by *Vibrio cholerae* serogroup 01 represents novel local infection of the intestine. No viable vibrios invade the tissue beyond the intestine. Oral vaccination is believed to be an effective measure for prevention of the disease; thus, several oral vaccine formulations have been developed and tested. These include live attenuated mutants from which toxin genes have been deleted by genetic engineering, killed whole cells and/or refined antigens[10–15]. The live vaccines were highly immunogenic when given orally. However, most of them were associated with undesirable side effects, i.e. diarrhoea in the vaccinees. Moreover, back mutation of these mutants to the wild-type organism is still possible. The killed whole cells or refined antigens are apparently safe with no untoward reactions. However, most of them have low immunogenicity, and multiple, spaced doses are required for eliciting a significant degree of protection. In the light of these findings, the means to increase the magnitude and duration of the immune response evoked by the non-living vaccines is a perceived necessity. In 1990, a liposome-associated vaccine prepared from lipopolysaccharide (LPS), cell-bound haemagglutinin (CHA) and procholeragenoid (P) was tested in comparison with a vaccine prepared from the same antigens in free form. It was found that the liposome-associated formulation was more immunogenic than the free antigen formulation in a rat model[9]. Similar results were obtained when the two formulations were tested in human volunteers (data to be published).

In designing a vaccine, however, besides safety and immunogenicity, several other factors need to be considered, such as the convenience and cost of reproducibly generating the vaccine in large quantities. Our liposome-associated refined antigen oral cholera vaccine, though equipped with no untoward reactions, has high immunogenicity and delivery simplicity, but encounters the need of antigen extraction and vaccine entrapment into the liposome. These factors would contribute to a high cost of the vaccine, which is not suitable for developing countries where cholera epidemics and endemics are problems of public health concern.

We therefore carried out the experiments to compare the immunogenicity and protective efficacy of a liposome-associated refined antigen oral vaccine with a vaccine prepared from a heat-killed fimbriated *V. cholerae* mutant in the rat ileal loop model. If the latter is proven to be as immunogenic and protective, it might replace the liposome-associated refined antigen formulation at a much lower cost. In our experiments, the heat-killed non-fimbriated *V. cholerae* vaccine and the liposome without incorporated antigens were also included as controls.

MATERIALS AND METHODS

Bacterial strains

Vibrio cholerae biotype classical, serotype Inaba, strain Bdg 17, which is a fimbriated mutant, was kindly provided by Dr Masahiko Ehara, Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University, Japan. Details of this strain are provided elsewhere[16]. This bacterial strain was used for the preparation of the *V. cholerae* fimbriae and a heat-killed fimbriated vaccine. *V. cholerae* biotype El Tor, serotype Ogawa isolated from a cholera patient in Thailand in 1987 was used for the preparation of a heat-killed, non-fimbriated whole cell vaccine and lipopolysaccharide and for the challenge study.

Preparation of antigens

Crude and purified V. cholerae *fimbriae. V. cholerae* fimbriae were prepared from *V. cholerae* 01, biotype classical, serotype Inaba, non-flagellated strain Bgd 17 by the method described by Ehara *et al*[17] with modifications. Briefly, *V. cholerae* was cultured in 1-l Roux bottles, each containing 80 ml of alkaline tryptone (AT) under static condition at 37°C for 16–18 h. The bacteria were harvested by centrifugation at 10000*g* at 4°C for 15 min. The bacterial pellet was resuspended in a small volume of phosphate-bufferred saline (PBS, 20 mM, pH 7.4). The preparation was subjected to an ultraturrax mixer (Janke and Kunkel, Germany) at full speed, at 4°C for 5 min three times, in order to shear free the fimbriae. The preparation was then centrifuged as above, and the supernatant was collected. The cell pellet was resuspended in the PBS and re-treated as above two more times. All supernatants were pooled, and the crude fimbriae were obtained in the precipitate after adding solid ammonium sulphate to the preparation to a concentration of 0.2 M, stirred for 20 min and kept at 4°C overnight. Crude fimbriae was obtained by centrifuging the preparation at 10000*g* at 4°C for 30 min. The pellet was solubilized in PBS and dialysed against it at 4°C overnight (crude fimbriae; CF was prepared). Purified fimbriae were prepared by loading the crude fimbriae on to a linear sucrose gradients (20–50% saturation), which was prepared in a 10-ml volume plastic centrifuge tubes. The tubes were centrifuged at 100000*g* for 20 h in a Sorvall Supracentrifuge. Fractions of 1.5 ml were collected separately, starting from the top of the content in each tube. Each fraction, after dialysis against cold distilled water, was monitored by SDS–PAGE. The fractions which contained the 18-kDa protein were collected as purified fimbriae (F)[17].

Lipopolysaccharide (LPS). Lipopolysaccharide (LPS) was prepared from *V. cholerae* 01, biotype El Tor, serotype Ogawa by the phenol–water extraction method described by Westphal and Jann[18]. The LPS from the first extraction was re-extracted two more

times, using the same procedure, until no protein could be detected by the Lowry method[19] and SDS–PAGE and Coomassie brilliant blue staining.

Procholeragenoid (P). This high-molecular-weight, heat-treated cholera toxin was a kind gift from Dr E. Furer, Bacteriology Department, Swiss Serum and Vaccine Institute, Berne, Switzerland.

Cholera toxin (CT). This toxin was purchased from Sigma (St. Louis, MO). It was used as the antigen in the indirect ELISA for assessment of anti-CT antibodies in serum and intestinal lavage samples of all experimental rats.

Preparation of vaccines

Liposome-associated refined antigen formulation (vaccine A). Antigens of *V. cholerae*, namely LPS, CF and P, were entrapped in liposomes by the bath-sonication method to produce a water-in-oil emulsion as previously described[9]. Each dose of this vaccine consisted of 5 mg of LPS, 5 mg of CF and 200 μg of P. Briefly, 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. Cholera antigens of one dose of vaccine (5 mg LPS, 5 mg CF and 200 μg P) 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 in batch Y of the lipids. The preparations of both tubes were subjected separately to ultrasonication at 20 kHz for 10 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 was dried to a gel-like consistency, 3 ml of normal saline (NSS) were added to make a homogeneous preparation. One dose of vaccine A was prepared. The vaccine was used to immunize rats of group 1.

Heat-killed fimbriated V. cholerae *(vaccine B).* The fimbriated *V. cholerae* strain Bdg17 was grown in AT broth in 100 ml aliquots under static condition at 37°C for 16–18 h (about 10^8 cells ml^{-1}). The culture was centrifuged at 10000*g* at 4°C for 20 min to pellet bacterial cells. The bacteria collected were suspended in NSS to give approximately 10^9 cells ml^{-1}. The preparation was heated at 56°C for 20 min. The viability of the bacteria was checked by plating several 0.1-ml aliquots on to trypticase soy agar (TSA). Upon obtaining no bacterial growth, 1 ml of the preparation (10^9 cells) was made up to 3 ml of NSS, and this was used as one dose of vaccine B. The vaccine was used to immunize rats of group 2.

Heat-killed non-fimbriated V. cholerae (vaccine C). Vaccine C was prepared from a non-fimbriated *V. cholerae* biotype El Tor, serotype Ogawa. The procedure used for preparing vaccine B was followed throughout. The vaccine was used to immunize rats of group 3.

Experimental animals and immunization

Eight-week-old Wistar rats of both sexes were obtained from the National Laboratory Animal Centre, Mahidol University. They were starved for at least 15 h, and 1 ml of 5% $NaHCO_3$ was given orally to each rat prior to vaccination. The group 1 rats each received vaccine A three times at 14-day intervals, whereas rats of groups 2 and 3 received vaccine B and vaccine C given at the same immunization schedule. The group 4 rats each received orally empty liposomes (L), whereas each rat of group 5 received orally 3 ml of NSS at the same times as for the immunization with vaccines for groups 1, 2 and 3, and these animals served as controls. The animals were returned to the domesticated cages with adequate supply of food and water.

Sample collection and antibody assessment

Five days after the last immunization, food was withheld from all rats, whereas drinking water was supplied to them *ad libitum*. After starving for 24 h, a serum sample was collected from the blood of each rat before they were killed by ether euthanasia. The small intestine was removed, and intestinal lavage was collected individually by flushing inside the intestine with 20 ml of distilled water. The lavage was centrifuged; the faecal pellet was discarded, and the supernatant was lyophilized. The dried material was then re-suspended in 1 ml of distilled water. Isotype and antigen-specific antibodies (i.e. IgM, IgG and IgA) were determined against purified F, LPS and CT by indirect ELISA as previously described[20].

Antibody assessment of all specimens was performed on the same day using the same conditions and reagents to avoid any possible variations. Fifty microlitres of either LPS (50 μg dry weight ml^{-1}), F (20 μg ml^{-1}), and CT (2 μg ml^{-1}; Sigma) were added to wells in ELISA plates. The plates with LPS were placed in a 37°C incubator overnight, while the plates with F and CT were kept in a humid chamber at room temperature overnight. The antigen-coated plates were washed thoroughly with 0.01 M PBS, pH 7.4, containing 0.05% Tween-20 (PBST) solution to remove unbound materials, and 200 μl PBS-Tween 20 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 h. After another wash as above, 100 μl of samples (twofold serial dilutions; serum samples were started at 1:10, whereas intestinal lavages were started at neat) were added to appropriate wells (PBS, pH 7.4, was added to the blank wells to serve as negative controls). Control positive serum (serum of a rat immunized intraperitonally several times at 2-week intervals with 1 log increasing doses of live *V. cholerae* El Tor Ogawa strain 017 SR, starting at 1×10^4 ml^{-1}) was included in each plate. The antigen–antibody reaction was allowed to occur at room temperature for 2 h, and then the non-reacted materials were washed off by the PBS-Tween 20. Peroxidase-labelled rabbit anti-rat IgM, IgG and IgA (SEROTEC) diluted 1:1000 in PBST in 100-μl aliquots were applied to appropriate wells, and the plates were incubated at 37°C for 1 h. After washing as above, freshly prepared substrate was added (100 μl per well). The reaction was allowed to take place in the dark for 20 min, then stopped by adding

50 μl of 1 N NaOH to each well. The optical densities in all wells were determined at 425 nm against the blanks by an ELISA reader. The indirect ELISA titre of a sample was the highest dilution of the sample that gave an optical density of 0.05 or higher when the background optical densities of the blanks were removed.

Assessment of *V. cholerae* colonization to the intestine

Inhibition of *V. cholerae* colonization to the intestinal mucosa afforded by each vaccine formulation was determined using the rat ileal loop model[21]. Five days after the last ingestion of vaccines or placebos, rats of all groups were fed with 3 ml solution of $MgSO_4.7H_2O$ before being starved for 24 h. A laparotomy was performed through a mid-line incision under ether anaesthesia. Four blind small intestinal loops were made. The distal loop started 10 cm above the ileo-caecal junction; each loop was 5 cm in length, and each of the loops was separated from each other by a gap of 1 cm. *Vibrio cholerae* El Tor, Ogawa were inoculated into each loop (10^7 cells in 1 ml volume). The abdominal cavity was closed with silk sutures and Michel clips. The rats were returned to the cages with drinking water supplied for 4 h before they were killed by cervical dislocation under ether anaesthesia. Each intestinal loop was removed, cut open longitudinally and the mucoid luminal content drained off. The tissue was thoroughly washed with several changes of sterile NSS in order to remove all materials that were not firmly adhered to the mucosal surface. Each intestinal piece was homogenized in 10 ml of sterile NSS using an ultraturrax homogenizer. The number of mucosa-associated vibrios was then determined by plating 0.1 ml of each of the 10-fold serial dilutions on to taurocholate bile-salt sucrose (TCBS) agar for viable counts.

Electron microscopic study of *V. cholerae* colonization at the epithelium

The intestinal pieces from rats of each group after vibrio challenge were prepared as described above. After the loosely attached vibrios had been removed by thoroughly washing, the tissue was cut into small pieces and fixed with 2.5% glutaraldehyde in 0.1 M PBS, pH 7.2 overnight. Each piece of the tissue was washed with PBS three times and once with distilled water, then dehydrated through acetone serials. The tissue was dried in a critical-point drying apparatus (Hitachi, HCP-2, Japan), trimmed properly, mounted on a copper stub, coated with gold by using sputter coater (Emitech, K550, UK), then examined under a scanning electron microscope (Hitachi, S-2360N, Japan).

RESULTS

It was noticed at the time that the rats were sacrificed that the intestinal loops of rats of the control groups (groups 4 and 5) had fluid distension when compared to those of the three vaccinated groups, which appeared relatively normal.

Table 1 summarizes the serum antibody responses of all rats of the five treatment groups. All rats of group 1 that received liposome-associated refined antigen vaccine (vaccine A) had significant increased numbers of IgM antibodies to all antigens, i.e. F, CT and LPS. All of them also had serum IgG anti-fimbriae and anti-CT, whereas two rats and one rat, respectively, had serum IgA anti-fimbriae and anti-CT. None of the rats of this treatment group had serum anti-LPS in the form of IgG or IgA isotype. Significant levels of IgM anti-fimbriae were detected in serum samples of all group 2 rats that received heat-killed fimbriated *V. cholerae* (vaccine B). However, no other antigen-specific antibodies of any isotype could be otherwise detected (*Table 1*). No antibody response was detected in serum samples of rats of groups 3, 4 and 5.

Antibodies detected in lavages of all rats are shown in *Table 2*. Antibodies to fimbriae of IgM isotype were detected at significant levels in the intestinal lavages of all rats of groups 1 and 2 (*Table 2*). Group 1 rats also had significant levels of lavage IgM anti-CT and anti-LPS. Two rats from group 1 had lavage IgA antibodies to all antigens, whereas rats from group 2 had no antibodies to CT and LPS in their lavages. Rats from groups 3, 4 and 5 did not have detectable levels of antibodies in their intestinal lavages.

Table 3 illustrates the numbers of *V. cholerae* that adhered to the mucosa of intestinal loops of rats of all

Table 1 Number of rats with serum antibody responses to fimbriae (F), cholera toxin (CT) and lipopolysaccharide (LPS) in all treatment groups

Antigen	Treatment	Number of rats with class-specific antibody			Total responders (%)
		IgM	IgG	IgA	
F	Vaccine A	3/3	3/3	2/3	100
	Vaccine B	3/3	0	0	100
	Vaccine C	0	0	0	0
	Liposome alone	0	0	0	0
	NSS	0	0	0	0
CT	Vaccine A	3/3	3/3	1/3	100
	Vaccine B	0	0	0	0
	Vaccine C	0	0	0	0
	Liposome alone	0	0	0	0
	NSS	0	0	0	0
LPS	Vaccine A	3/3	0	0	100
	Vaccine B	0	0	0	0
	Vaccine C	0	0	0	0
	Liposome alone	0	0	0	0
	NSS	0	0	0	0

The ELISA titres of the responders ranged from 1:20 to 1:640 for anti-F; 1:40 to 1:5120 for anti-CT and 1:160 to 1:640 for anti-LPS.

Table 2 Number of rats that showed significant antibody levels to fimbriae (F), cholera toxin (CT) and lipopolysaccharide (LPS) in the intestinal lavages

Antigen	Treatment	Number of rats with class-specific antibody			Total responders (%)
		IgM	IgG	IgA	
F	Vaccine A	3/3	0	2/3	100
	Vaccine B	3/3	0	0	100
	Vaccine C	0	0	0	0
	Liposome alone	0	0	0	0
	NSS	0	0	0	0
CT	Vaccine A	3/3	0	2/3	100
	Vaccine B	0	0	0	0
	Vaccine C	0	0	0	0
	Liposome alone	0	0	0	0
	NSS	0	0	0	0
LPS	Vaccine A	3/3	0	2/3	100
	Vaccine B	0	0	0	0
	Vaccine C	0	0	0	0
	Liposome alone	0	0	0	0
	NSS	0	0	0	0

The ELISA titres of the responders ranged from 1:2 to 1:8.

groups. One-factor ANOVA analysis indicated that immune rats of groups 1 and 2 had significantly reduced numbers of *V. cholerae* adhered to the mucosa of their intestinal loops when compared with the numbers of the vibrios recovered from those of the control groups that received NSS (group 5). However, the numbers of vibrios recovered from the loops of the two vaccinated groups were not different. Unfortunately, experiments on vibrio recovery from the intestinal segments of groups 3 and 4 were incomplete, and only three segments of one rat of each group were examined. Nevertheless, the numbers of vibrios recovered from intestinal segments of rats of group 3 that received heat-killed, non-fimbriated vaccine (Vaccine C) were significantly less than those of groups 4 and 5, although the numbers were higher than those of groups 1 and 2. There was no significant difference between the numbers of vibrios recovered from the intestinal segments of rats of groups 4 and 5.

V. cholerae cells were not found on the intestinal villi of the immune rats of groups 1 and 2 when the intestinal tissue was examined by scanning electron microscopy. A representation of the appearance of the tissue is shown in *Figure 1*A; however, patches of vibrios were seen scattered on many intestinal villi of the rats from groups 3, 4 and 5; the appearance of the tissue from these rats is shown in *Figure 1*B. *Figure 1*(C and D) reveals higher magnifications of the indicated square areas in *Figure 1*(A and B, respectively).

DISCUSSION

The results of our previous work showed that liposomes were effective adjuvants for an oral cholera vaccine prepared from *V. cholerae* LPS, haemagglutinin and procholeragenoid. The mucosal immune response to the liposome-associated oral vaccine was higher than to the free antigen vaccine in terms of the number of antigen-specific antibody-producing cells in the lamina propria[9]. Whether the adjuvant quality of liposomes is due to the more effective uptake by M cells of Peyer's patches and/or their subsequent interaction with antigen-processing cells is not known. However, liposomes have been found in endocytic vesicles of M cells in close proximity to lymphocytes following intestinal administration[1,22]. Because liposomes are multilamellar vesicles that mimic cell membranes, the uptake and processing of antigen may be enhanced by enclosing the antigen in the lipid vesicle. Another proposed mechanism for the adjuvant properties of liposomes is their ability to protect the antigen from acids and digestive enzymes of the gastro-intestinal

Table 3 Number of *V. cholerae* firmly attached to the intestinal mucosa of rats of all treatment groups

Rat no.	Loop no.	Log number of *V. cholerae* recovered				
		Vaccine A	Vaccine B	Vaccine C	Liposome	NSS
1	1	4.00	6.77	6.54	7.92	7.69
	2	5.07	5.35	6.89	7.64	7.47
	3	5.94	5.29	6.35	6.97	8.69
	4	5.20	4.49	nd	nd	7.90
2	1	5.17	5.29	nd	nd	6.81
	2	5.71	5.13	nd	nd	7.24
	3	4.00	5.06	nd	nd	7.84
	4	5.30	6.65	nd	nd	6.07
3	1	5.50	5.35	nd	nd	7.32
	2	4.47	6.50	nd	nd	6.60
	3	4.60	5.05	nd	nd	7.20
	4	4.80	5.11	nd	nd	7.20
Mean ± SD		4.98 ± 0.62^a	5.50 ± 0.72^a	6.59 ± 0.27^b	7.51 ± 0.49^c	7.33 ± 0.68^c

Means ± SD with the same superscript are not different at 95% ($P < 0.05$), whereas those with different superscripts are statistically different at $P > 0.05$ (one-factor ANOVA). nd, not done.

system. Although they are not completely resistant to lipases and bile salts found in the small intestine, cholesterol-containing liposome (like the one that we used) can provide at least partial resistance[1,23,24].

As mentioned earlier, in designing a vaccine, several factors need to be taken into consideration, i.e. not only vaccine safety and immunogenicity, but also the convenience and cost when produced on an industrial scale. Our liposome-associated oral vaccine, although proven to be highly immunogenic, consists of refined antigens derived from extraction and the preparative liposomes that may be expensive if the vaccine has to be produced for mass vaccination. As such, the heat-killed, highly fimbriated strain of *V. cholerae* was tested as an alternative in this study. The strain Bgd 17 was chosen because it could be grown under conditions that would promote a high degree of expression of the hydrophobic fimbriae. These fimbriae render a firm adherence of the vibrios to the intestinal mucosa[17], which would facilitate the antigen uptake and presentation to the antigen processing cells. Moreover, this strain was highly immunogenic and protective when the formalin-fixed whole cells were used to immunize rabbits subcutaneously and intramuscularly[17]. Oral immunization with the heat-killed Bgd 17 whole cells in rats elicited significant levels of serum as well as intestinal anti-fimbrial antibodies but only in the form of IgM isotype (*Tables 1* and *2*). No other antigen-specific antibodies were elicited by the heat killed, highly fimbriated whole cell vaccine (vaccine B). The rats immunized with heat-killed, non-fimbriated whole cell vaccine (vaccine C), liposome and normal saline solution did not have any detectable response. In contrast, the liposome-associated refined antigen vaccine evoked not only the high response of all antigen-specific antibodies (anti-fimbrial, anti-CT, and anti-LPS) in serum but also a significant level of all in the intestinal lavages. Beside IgM isotype, the antibodies elicited by the vaccine A were also in the form of serum IgG and IgA and intestinal IgA. The level of serum anti-CT was exceptionally high (1:5120). This may also provide immunity to cholera by passive leakage into the intestine. The anti-fimbrial and anti-LPS antibodies in the intestinal lavage would confer anti-bacterial and/or inhibition of vibrio colonization, while the anti-CT would function in inhibiting Gm_1 binding of the CT-B subunit. The antibodies of different isotypes were found to be equally protective against *V. cholerae* infection upon the same antibody weight basis[25].

Despite the fact that no significant amount of other antibodies except anti-fimbrial antibodies was found in the intestinal lavages of rats orally immunized with heat-killed, fimbriated Bgd 17 (Vaccine B), these rats showed a marked reduction in the number of *V. cholerae* O1 El Tor, Ogawa (heterologous strain) that adhered to the mucosa of intestinal sections when compared to those of the controls (groups 4 and 5) and

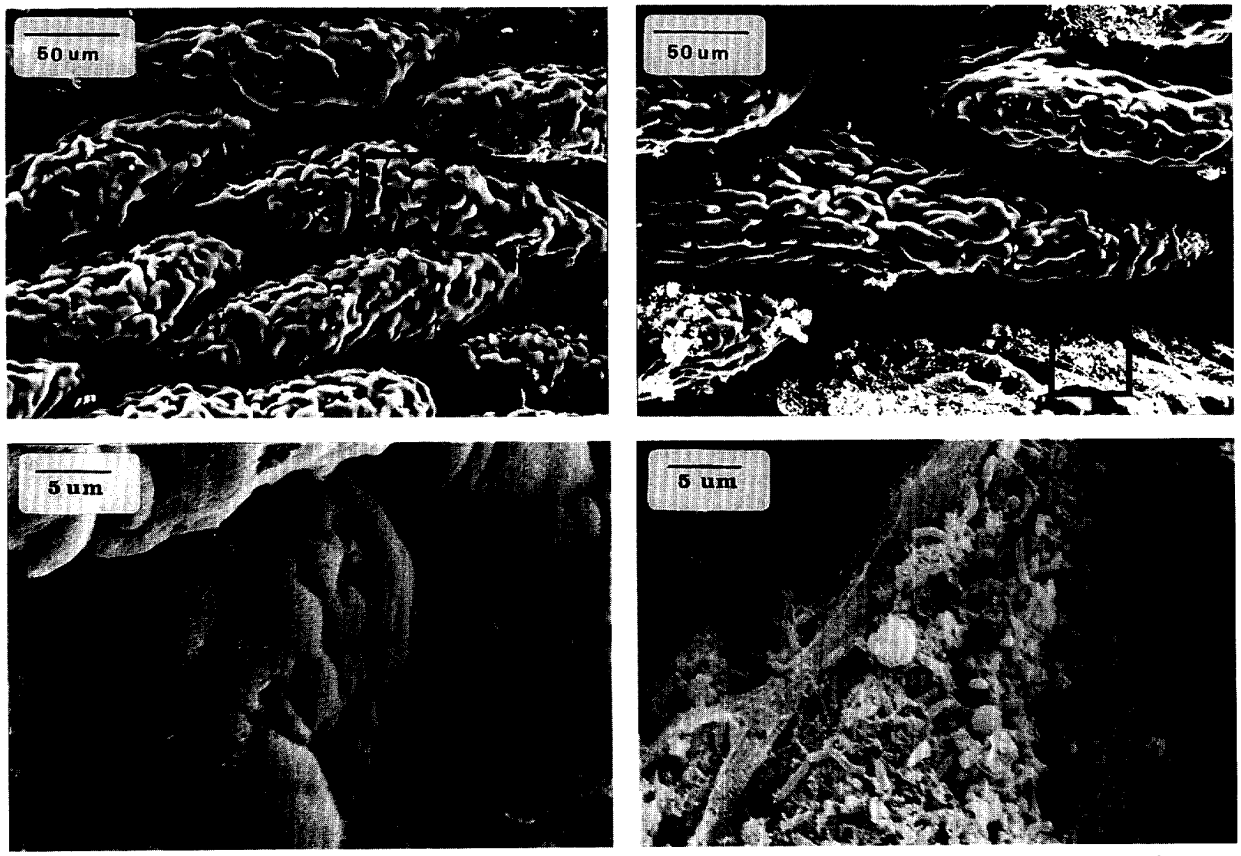

Figure 1 Representative electron micrographs showing the mucosal surfaces of rat small intestines. (A) The mucosal surface of a vaccinated rat (group 1); the mucosal surface is intact with no adherent vibrios. (B) The mucosal surface of control rat (group 5); patches of adherent vibrios are seen. (C) and (D) Higher magnification of the indicated square areas of (A) and (B), respectively.

also to those of group 3 that received heat-killed, non-fimbriated vibrios. The reduction in the number of adhered vibrios was in the order of two logs, which was a similar order of magnitude as found in rats immunized with the liposome vaccine. Moreover, the inhibition of the vibrio adherence was found to be independent of biotype and serotype. These findings confirmed the findings previously reported by Ehara and colleagues of which the rabbit model was used[17].

Examination of the intestinal segments of rats immunized with either liposome vaccine or heat-killed fimbriated whole cell vaccine revealed an intact feature of the intestinal epithelium without colonization of the challenge vibrios. However, localized colonization of the bacteria was seen in all specimens taken from the rats immunized with heat-killed, non-fimbriated vibrios, liposomes and NSS. Representative pictures taken from the rats of group 1 (immunized) and control rats of group 5 are shown in *Figure 1*(A–D). Despite the low intestinal antibody titres, the intestinal segments of the vaccinated rats seemed to be completely protected from colonization by the challenge vibrios. The vibrios recovered from the immune intestinal segments (*Table 3*) might represent those entrapped in the mucous and glycocalyces covering the epithelial surface and not really attached to the brush border membrane of the epithelial cells. The mucous and glycocalyces were not preserved in the process for electron microscopy, and thus no bacteria were seen.

Our experiments indicate that although the heat-killed whole cell vaccine prepared from the fimbriated *V. cholerae* strain Bgd 17 was less immunogenic than the liposome vaccine, it could also confer protection against *V. cholerae*. An expected increase in the anti-bacterial responses (anti-LPS and anti-fimbrial) might be achieved by increasing the amount of heat-killed, fimbriated vibrios in each dose of the vaccine, while anti-CT would be elicited by adding procholeragenoid or B subunits of CT to the vaccine B formulation.

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