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Immunogenicity of Liposome-Associated Oral Cholera Vaccine Prepared from Combined *Vibrio cholerae* Antigens

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SUMMARY **Liposomes were prepared from bovine brain sphingomyelin and cholesterol. They were reinforced by incorporation of osmium tetroxide to prevent their immediate degradation inside the host. Combined *Vibrio cholerae* antigens (lipopolysaccharide, crude cell-bound hemagglutinin and procholeragenoid) were orally administered to experimental rats either as free or liposome-associated. A total of 70 experimental rats was utilized in experiments comparing the immune responses of rats to liposome-associated vaccine, free vaccine, liposomes, or placebo, and to vaccines where the lipid or antigen levels were reduced. Immediately after feeding with sodium bicarbonate to lower the gastric acidity, they were fed either cholera vaccines or placebo. Results from serum ELISA revealed that the liposomes localized the immune response to the intestinal mucosa. They displayed an adjuvant property in terms of evoking a higher immune response to *V. cholerae* antigens, as measured by the appearance of specific antibody-producing cells in the intestinal mucosa, than when the antigens were fed alone. The adjuvanticity was found to be lipid dose dependent. Liposomes prepared with high lipid content enhanced immunogenicity of the admixture antigens to a greater degree.**

Although the oral route has been established as the most efficient means of stimulating the gut mucosal immune response which accounts for protection in cholera,[1,2] there are still no oral vaccines available for cholera immunization programmes. Live attenuated *Vibrio cholerae* strains in which the cholera toxin (CT) gene has been deleted by either chemical treatment or genetic engineering have been prepared and tested in volunteers.[3] These strains had high immunogenicity when given orally. However, most of them were associated with undesirable side effects i.e. diarrhoea in the vaccinees. Concurrently, an oral vaccine prepared from killed whole *V.cholerae* plus B subunit of CT has been developed and tested both in volunteers and in the field in cholera endemic areas.[4-6] At the same time, refined *V.cholerae* antigens which have immunogenic, synergistic and inherent adjuvant properties have been defined, isolated and evaluated as a combined oral vaccine in both animals and humans.[7,8]

The oral vaccines prepared from killed whole *V.cholerae* or refined antigens are apparently safe, with no untoward reactions but low immunogenicity. None of them can be delivered in a single dose which is sufficiently protective to be of public health significance. Multiple, spaced-doses were required for eliciting certain degrees of immune response in the hosts. In the light of these findings, the means to increase the magnitude and duration of the protective immunity evoked by a killed whole cell/refined antigen vaccine is a perceived necessity. Thus, the need of an adjuvant and/or a safe delivery vehicle for oral vaccines has been emphasized by the World Health Organization since 1979.[9] This would be expected to lessen the number and size of doses and enhance efficiency of an oral vaccine especially in the low responder group, i.e. small children who are at greatest risk to cholera.

Liposomes, the concentric spheres of phospholipid bilayers, can serve as delivery vehicles for

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substances which are associated with them. Earlier studies have shown that molecules such as drugs [10] or antigens [11,12] can be entrapped in spaces in liposomes, incorporated into lipid bilayers, [13-15] or adsorbed to the liposomal surfaces. [16,17]

In this study, experiments were conducted to investigate the potential of liposomes as adjuvants for oral cholera vaccines prepared from combined antigens of *V.cholerae.*

MATERIALS AND METHODS

Preparation of *V.cholerae* antigens.

The antigens used as vaccine components were lipopolysaccharide (LPS), crude cell-bound hemagglutinin (crude CHA) and procholeragenoid (P). The LPS was extracted from the bulk of smooth variant of strain 017SR which is an El Tor biotype-, Ogawa serotype- *V.cholerae* by the phenol-water method described by Westphal and Jann. [18] The extracted-LPS was reextracted until no protein could be detected in the preparation. The antigen was kept frozen at -20° C until used.

Crude CHA was prepared from a culture of rough variant of the 017SR *V.cholerae.* The bacteria grown on trypticase soy agar in Roux bottles at 37° C for 48 hours were harvested in a potassium phosphate buffer, *p*H 6.8 (PPB) containing 0.05 mM $MgCl_2$ and 1 mM EGTA, washed and adjusted to 50 mg/ml with the same solution. The preparation was subjected to the full speed of an ultraturrax mixer (Janke and Kunkel, West Germany) for 10-20 minutes, then centrifuged at 12,000 rpm for 20 minutes. The bacterial cell deposit was resuspended in PPB to the original volume and resubjected to the mixer. The process was repeated several more times. All of the supernates were pooled, concentrated and recentrifuged to remove cell debris and flagella at 17,000 rpm for 20 minutes. The supernate was collected and the dry weight and hemagglutinating activity (v.s. 2.5% sheep red blood cells) were determined.

Procholeragenoid (P), which is a 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) was purchased from Sigma Chemical Company, USA.

Preparation of liposomes.

Liposomes were prepared from sphingomyelin and cholesterol following the method of Szoka and Papahadjopoulos [19] with modifications. [12]

Preparation of liposome-associated vaccine.

Two batches of lipid solution (batches X and Y) were prepared from bovine brain sphingomyelin and cholesterol (Sigma Chemical Company, USA). Each batch contained 50 mg of sphingomyelin and 20 mg of cholesterol dissolved in 7.5 ml of chloroform/7.5 ml of ether. A combined vaccine was prepared by mixing 2 ml each of the LPS (7 mg/ml) and crude CHA (3 mg/ml) with 240 μl of procholeragenoid (2 mg). After thorough mixing, the antigenic mixture was divided into two equal aliquots (A and B). Aliquot A was mixed with 0.5 ml of 2% osmium tetroxide (OsO_4) and kept at room temperature for 5 minutes. Batch X of the organic lipid solution was then poured into the tube of OsO_4 treated aliquot A of the antigen (tube 1). Batch Y of the lipid solution was mixed with 2.5 ml distilled water in another tube (tube 2). After thorough mixing, the two phase mixtures of tubes 1 and 2 were subjected to ultrasonication separately until each formed a homogeneous single phase emulsion. The two emulsions 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, 15 ml of phosphate-buffered saline, *p*H 7.4 (PBS) were gradually added to the preparation and the material was brought to a homogeneous suspension by mechanical mixing. To the suspension was added 0.5 ml OsO_4 solution and the mixture was kept for 30 minutes at room temperature. The preparation was dialysed against PBS, *p*H 7.4 overnight at 4° C. This liposome-associated vaccine was used to orally immunize the rats of group 1 of the first experiment (Table 1).

Another batch of liposome-associated vaccine was prepared for the second experiment. However, the content of the lipids in the liposomes was reduced by half of that in the first batch, while the concentrations of the *V.cholerae* antigens, i.e. LPS, crude CHA and P in the vaccine were equal to those of the first experiment. The vaccine was used to immunize rats of group 1 in the second experiment.

Three more batches of liposome-associated vaccines were prepared for immunizing the rats of groups 1, 2 and 3 of the third experiment. The lipid levels used in the second experiment were maintained but the antigen levels were gradually reduced as follows: In the first group, the lipid and antigen levels used in the second experiment were maintained. However, lower levels of antigen consisting of 500 μg of LPS, 500 μg of crude CHA and 50 μg of P [6] were used in group 2. These levels of antigen were further reduced to half for rats in group 3 (Table 1).

Preparation of free antigen vaccine.

Aliquot B of the antigen mixture was diluted with PBS to 15 ml. The preparation was used to orally immunize rats of group 2 of the first experiment. Rats of group 2 of the second experiment and group 4 of the third experiment received similar amounts to their counterparts in the first experiment. Thus each rat of these groups received a trivalent vaccine in free form which contained

1.4 mg of LPS, 0.6 mg of crude CHA and 0.2 mg of P. In the third experiment, each rat of group 5 received free antigen vaccine containing 0.5 mg of LPS, 0.5 mg of crude CHA and 0.05 mg of P, while those in group 6 received 0.25 mg of LPS, 0.25 mg of crude CHA and 0.025 mg of P. The specific variations among experiments and groups are summarized in Table 1.

After immunization, food and water were withheld for another hour to facilitate the passage of the inoculum to the intestinal tract, after which the rats were subjected to uniform conditions of care and management.

Table 1. Lipids and antigens received by each rat in the experiment groups (5 rats per group).

Experiment No.	Group	Lipid (mg)		Antigen (mg)		
		Sphingo-myelin	Choles-terol	LPS	crude CHA	P
I	1	20	8	1.4	0.6	0.2
	2	–	–	1.4	0.6	0.2
	3	20	8	–	–	–
	4	–	–	–	–	–
II	1	10	4	1.4	0.6	0.2
	2	–	–	1.4	0.6	0.2
	3	10	4	–	–	–
	4	–	–	–	–	–
III	1	10	4	1.4	0.6	0.2
	2	10	4	0.5	0.5	0.05
	3	10	4	0.25	0.25	0.025
	4	–	–	1.4	0.6	0.2
	5	–	–	0.5	0.5	0.05
	6	–	–	0.25	0.25	0.025

Preparation of fluorescein-labelled antibodies.

Rabbits were intravenously immunized with either live *V.cholerae* 017SR smooth variant or CT to produce anti-live *V.cholerae* (anti-VC) or anti-cholera toxin (anti-CT), respectively. For the anti-VC preparation, a rabbit was injected six times at three day intervals with 1 ml of normal saline solution (NSS) containing 10^6, 10^7, 10^8, 10^9, 2×10^9 and 2×10^9 live 017SR, respectively. For the anti-CT production, another rabbit was injected with six doses of CT in 1 ml of NSS at one week intervals i.e. 4, 10, 20, 40, 40 and 40 μg, respectively. The rabbits were bled by cardiac puncture two weeks thereafter. Total serum immunoglobulins were isolated from the sera by ammonium sulphate precipitation. The IgG fractions were then obtained from the immunoglobulin pools by protein A-Sepharose 4B column chromatography. They were conjugated with fluorescein isothiocyanate (FITC) dye by the method of Cherry [20] and standardized by method of The and Feltkamp, [21] respectively. The anti-VC-FITC and anti-CT-FITC were thus prepared.

Collection of sera and intestinal tissues.

Fifteen days after immunization, all rats were bled individually from the heart and followed by ether euthanasia. The abdominal wall and peritoneum of each rat were cut open. The middle part of the jejunum (28-32 cm from pylorus) was cut into 1 cm pieces. Each piece was kept in a sealed Eppendorf vial at -70° C. Blocks of the tissues for cryostat sectioning were prepared a day later or at the latest within one week and processed for antibody producing cell enumeration.

Indirect enzyme-linked immunosorbent assay (ELISA).

Indirect ELISA was performed on serum specimens of rats for determining specific anti-LPS, anti-crude CHA and anti-CT activity. Wells in the ELISA plates were appropriately coated with antigens (10 μg/ml) and incubated at 37° C for 2 hours and overnight at 4° C. Excess antigens were washed off with PBS-Tween 20 (PBST) and the empty sites on the plates were blocked with 1% BSA in PBST. After washing to remove unbound BSA, appropriately diluted serum samples were delivered to the antigen wells. The last wells of all rows in the plates received only PBS which served as blanks. The plates were incubated at 37° C for 2 hours, the excess materials were discarded by washing. Peroxidase conjugated rabbit anti-rat immunoglobulins (Dako, Denmark) at a dilution of 1:1,000 was added to the appropriate wells. After incubation for one hour at 37° C, unbound conjugate was removed by washing. The substrate was delivered to each well; the reaction was allowed to occur for 30 minutes in the dark then it was stopped by adding 50 μl/well of 1 N NaOH. The optical density (OD) of each well was determined against the blanks by an ELISA reader (Uniskan II, Labsystems) at 492 nm.

Enumeration of antibody producing cells (AbPC) in the intestinal tissues.

Cells producing antibodies to LPS, crude CHA and CT in the intestinal lamina propria of the experimental rats were enumerated by a double antibody sandwich immunofluorescence method. The cryostat section of jejunum was applied to a microscopic slide and fixed with

90% methanol, then with absolute methanol. The section was then covered with appropriate antigen at appropriate concentration (10 μg/ml of LPS for enumerating anti-LPS producing cells, 10 μg/ml of crude CHA for anti-crude CHA producing cells and 2 μg/ml of CT for anti-CT producing cells). The reaction was allowed to take place for 30 minutes at 26° C, then the non-reacted materials were washed off with PBS, *p*H 7.2. The section was overlayed with either anti-VC-FITC conjugate (1:200) for anti-LPS or anti-crude CHA producing cell enumerations or with anti-CT-FITC conjugate (1:100) (optimum dilutions obtained from previous titrations) for detection of anti-CT cells. The preparation was kept in a humidified chamber for another 30 minutes then washed extensively with PBS. The slide was air-dried and mounted with mounting fluid (Microrak, Syva, USA).

The mucosal immune response of the rats was determined by enumeration of positive antibody producing cells in the jejunal lamina propria with a Zeiss Universal fluorescence microscope. Two intestinal sections from each rat were used for counting the positive cells of each antibody specificity. A total of 25 microscopic fields (25x objective) per section was counted with consideration of whether cells were found in the villous projections (12 microscopic fields) or in the basal portions (13 microscopic fields) of the mucosa. One intestinal section of each experimental rat was stained with hematoxylin and eosin. The number of plasma cells which appeared as a cell with extended cytoplasm and eccentric nucleus per one microscopic field was also determined.

RESULTS

Serum antibodies.

The serum antibody responses of rats which received oral antigens and placebo in the first experiment were evaluated through indirect ELISA. The cut-off limit for a positive titre was at the optical density 0.05 (at this OD, positive and negative reactions could be differentiated also by color difference). In the placebo group (group 4), three out of five rats (Nos. 3, 4, 5) showed positive ELISA titres to CT, crude CHA, and crude CHA and LPS, respectively. However, the titres did not exceed 1:320. None of the rats which received liposomes alone (group 3) gave detectable antibody titres to any of the antigens. Among the rats given osmicated liposome-associated vaccine (group 1), one rat had background antibody titre to LPS (1:40), while the other four rats were not positive to any of the antigens. However, rats which received free vaccine (group 2) gave a very strong antibody response to CT (> 1:2,560); among these, one of them had anti-LPS at a dilution of 1:160 and another one had an anti-crude CHA response at a dilution of 1:320. However, these anti-LPS and anti-crude CHA titres were equal to the background titres found in rat No. 5 of group 4.

Osmicated liposome adjuvanticity for oral cholera vaccine.

In the first experiment, rats which received osmicated liposome-associated vaccine (group 1) gave higher anti-LPS, anti-crude CHA

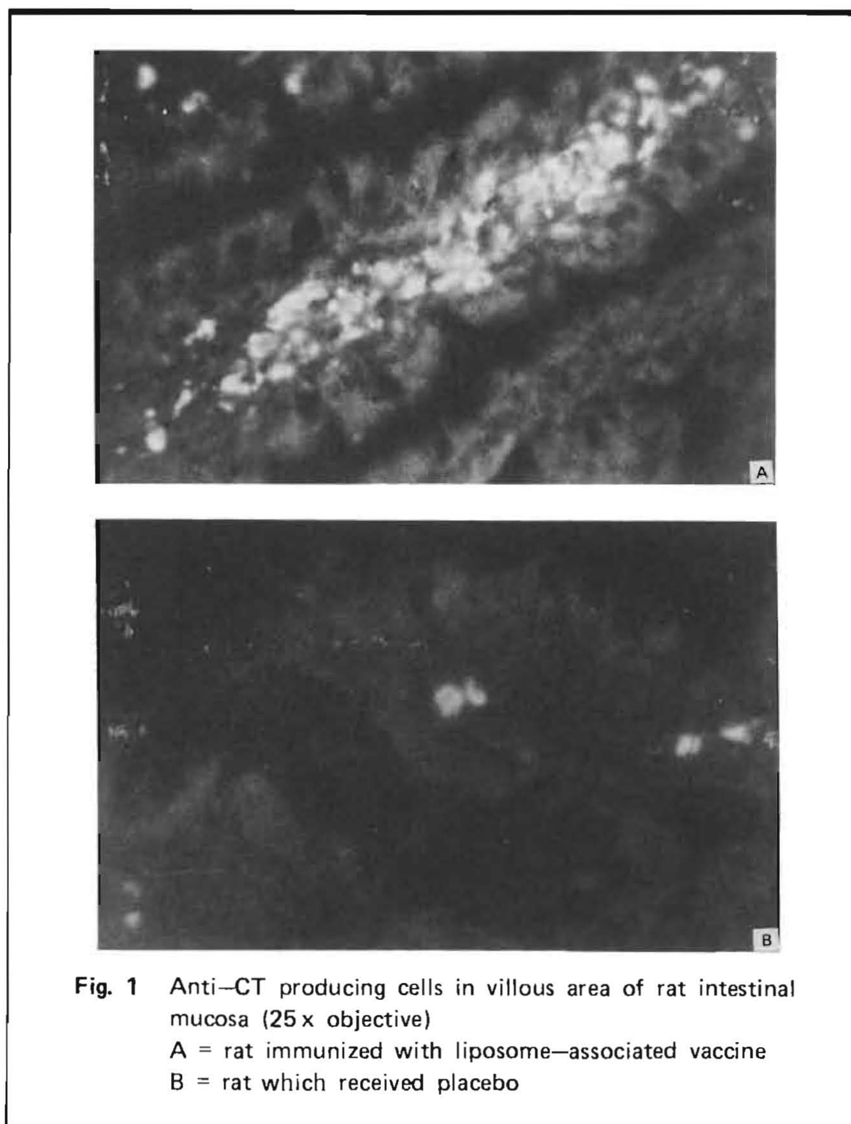

Fig. 1 Anti–CT producing cells in villous area of rat intestinal mucosa (25 x objective)
A = rat immunized with liposome–associated vaccine
B = rat which received placebo

and anti-CT cell counts than rats given free antigens in similar doses (group 2). The average cell counts for anti-LPS, anti-crude CHA and anti-CT cells for group 1 were 71.02, 66.06 and 59.82, respectively, while for group 2 they were 41.54, 48.84 and 41.81, respectively ($p < 0.01$). Rats of groups 3 and 4 had statistically similar AbPC average even if rats of group 3 received osmicated liposomes alone while rats of group 4 only received placebo. The average cell counts of these 2 groups were far lower than those of groups 1 and 2 i.e. the anti-LPS, anti-crude CHA and anti-CT counts of rats which received liposomes alone were 8.98, 9.70 and 9.07, respectively, while rats which received placebo had average counts of 2.51, 1.83 and 0.70, respectively (Table 2). Figure 1 illustrates anti-CT cells in a villous area of the jejunum of a rat immunized with liposome-associated vaccine (A) and placebo (B).

Rats in the second experiment received antigens or placebo in similar regimens as did their counterparts in the first experiment, except that the levels of the lipids used in groups 1 and 3 of the first experiment were reduced to half in the corresponding groups of the second experiment. The results shown in Table 3 reveal that there was a reduction in AbPC counts in group 1 of the second experiment. The anti-LPS, anti-crude CHA and anti-CT cells were 56.83, 54.88 and 51.06, respectively. The numbers were significantly lower ($p < 0.01$, Wilcoxon's signed rank test) than those of group 1 of the first experiment. Other results of the second experiment paralleled those of the first. Although the magnitude of response in rats which received osmicated liposomes - associated vaccine (group 1) was reduced, it was still higher than that of rats which received free antigens (group 2). The average numbers of anti-LPS, anti-crude CHA and anti-CT cells of rats of group 2 were 41.97, 40.69 and 38.92, respectively (Table 3) which were not different from their counterparts in the first experiment (group 2; Table 2). Likewise the response of rats which received liposomes alone (group 3) did not significantly differ from those of the control group (anti-LPS, anti-crude CHA and anti-CT cells averaged 6.34, 5.45 and 5.22, respectively for group 3 against 3.52, 4.14 and 1.60, respectively for group 4) (Table 3).

In the third experiment, the doses of cholera antigens were reduced and given either as osmicated liposome-associated (groups 1, 2 and 3) or free vaccines (groups 4, 5 and 6) to corresponding groups. The corresponding groups which received similar levels of antigens were groups 1 and 4 (1.4 mg of LPS, 0.6 mg of crude CHA, and 0.2 mg of P per individual rat); groups 2 and 5 (0.5 mg of LPS, 0.5 mg of crude CHA and 0.05 μg of P); and groups 3 and 6 (0.25 mg of LPS, 0.25 mg of crude CHA, and 0.025 mg of P). Com-

Table 2. Mucosal immune response of rats in the first experiment expressed as a mean of AbPC per microscopic field (25 x objective).

Group No.	Specificity of Ab		
	Anti–LPS	Anti–crude CHA	Anti–CT
1	71.02*	66.06*	59.82*
2	41.54**	41.84**	41.18**
3	8.98+	9.7+	9.07+
4	2.51+	1.83+	0.7+

Means of different superscripts (*, ** v.s. +) are significantly different at $p < 0.01$ (Wilcoxon's signed rank test).

Table 3. Mucosal immune response of rats in the second experiment expressed as a mean of AbPC per microscopic field (25 x objective).

Group No.	Specificity of AbPC		
	Anti–LPS	Anti–crude CHA	Anti–CT
1	56.83*	54.88*	51.06*
2	41.97**	40.69**	38.92**
3	6.34+	5.45+	5.22+
4	3.52+	4.14+	1.60+

Means with the different superscripts (*, ** v.s. +) are significantly different at $p < 0.01$ (Wilcoxon's signed rank test).

parisons between the experimental groups revealed that rats which received liposome-associated vaccines gave significantly better responses than those which were given free antigens ($p < 0.01$). Among the groups which received the highest doses of antigens (groups 1 and 4), there were significant differences in responses in terms of AbPC counts. Liposome-associated antigens (group 1) were more immunogenic than free antigen vaccines (group 4). The mean AbPC counts of group 1 for anti-LPS, anti-crude CHA, and anti-CT cells were 52.70, 54.77 and 53.65, respectively, while the corresponding mean counts for group 4 were 41.77, 40.50 and 40.27, respectively (Table 4).

Rats of group 2 (which received antigens associated with liposomes) had better responses than those which received free antigens (group 5). The average anti-LPS, anti-crude CHA and anti-CT responses of group 2, were 61.20, 59.83 and 57.26; while the corresponding mean AbPC counts for group 5 were 38.54, 39.23 and 39.06, respectively. Rats which received the lowest doses of antigens (groups 3 and 6) also differed significantly in their responses to the antigens. Those with the liposomes (group 3) gave higher AbPC counts than those with the free vaccine (group 6). Liposome-associated antigens induced better anti-LPS, anti-crude CHA and anti-CT responses at 57.39, 64.02 and 57.28, respectively; in contrast the corresponding responses in free vaccine group were 37.99, 40.58 and 39.98 cells respectively per microscopic field. Rats which received vaccines associated with liposomes (groups 1, 2 and 3) responded similarly regardless of the antigen doses that they received; likewise, rats which received free antigen vaccines (groups 4, 5 and 6) had no statistically different response levels.

Table 4 Mucosal immune response of rats to varying levels of oral cholera antigens expressed as a mean of AbPC per microscopic field (25x objective).

Group No.	Specificity of AbPC		
	Anti–LPS	Anti–crude CHA	Anti–CT
1	52.70*	54.77*	53.65*
2	61.20*	59.83*	57.26*
3	57.39*	64.02*	57.28*
4	41.77+	40.50+	40.27+
5	38.54+	39.23+	39.06+
6	37.99+	40.58+	39.98+

Means with the different superscripts (* v.s. +) are statistically different at $p < 0.01$ (Willcoxon's signed rank test).

DISCUSSION

In this study, liposomes with large internal aqueous space (200-500 nm), high stability and high captured volume were prepared for experiments based on the procedure of Szoka and Papahadjopoulos.[19] The liposomes were osmicated following the method of New *et al.*[12] in order to retard their degradation in the body.

After 15 days of oral immunization of rats with the liposome-associated vaccine, free antigen vaccine, liposomes and placebo, both serum and local (intestinal mucosa) immune responses were evaluated. Results from serum ELISA revealed that CT in the free antigen vaccine was able to generate a serum antibody response. In contrast, CT in the liposome associated vaccine did not generate any significant serum response at an equivalent dose of antigen. Orally-administered CT has been reported to elicit strong serum and mucosal antibody responses,[22-24] but liposome-associated oral CT did not do so in our experiment. This latter finding suggests that the antigen has been firmly associated with (if not entrapped in) the liposomes. The effect did not seem to be due to the osmication procedure, as deliberate experiments showed no difference in either serum or mucosal responses between rats immunized with free antigens and rats immunized with OsO_4 treated antigens (data not shown). The osmication procedure might merely have enhanced stronger interactions between the liposomes and the antigenic components, since the same procedure was able to sustain powerful serum responses in mice, even a year after a single parenteral dose of antigen.[12] Furthermore, a close association between liposomes and vaccine components enhanced by the osmium treatment is a necessary condition in the adjuvant action of liposomes.[17,25] Liposomes without associated *V.cholerae* antigens did not evoke any significant antibody titres to LPS, crude CHA or CT, a response similar only to those of rats which received placebo.

The mucosal immune response was measured by the appearance of specific antibody producing cells (anti-LPS, anti-crude CHA and anti-CT) in the lamina propria of the intestines using a double antibody sandwich immunofluorescence method. Morphology of the antibody containing cells was examined by phase

contrast and 40 × objective of the microscope before the number of cells per one 25 × microscopic field was determined. This was done to ensure that the cell was a real antibody containing cell. Whole IgG fractions of rabbit immune sera to live *V. cholerae* and CT were used in preparing the anti-VC-FITC and anti-CT-FITC conjugates, respectively. The working dilutions of the conjugates were 1:200 and 1:100, respectively. These dilutions were rather high, while the antigen concentrations used were rather low, sufficiently so to eliminate the background levels of fluorescence in the tissue due to interactions other than the ones sought. This was confirmed by the finding that the jejunal sections of the non-immune rats gave negligible AbPC counts when compared to the immune rats. Also it can be seen from figure 1 that the fluorescence was confined only to the lamina propria and did not appear anywhere else. These findings imply that 1) the antigens, at the concentrations used in our assay, did not adhere nonspecifically to the tissue and 2) leukocytes other than the plasma cells (e.g. macrophages, null cells, polymorphonuclear cells which were located not only in the lamina propria but also in the intra-epithelial compartments, and which might have had taken up the antigens) did not fluoresce, otherwise the fluorescence would appear in the intra-epithelial area as well.

Fifteen days after immunization, increases in antibody producing cells were found in the intestinal lamina propria of rats which received *V.cholerae* antigens. A homogeneous distribution of AbPC has been noted in the lamina propria comprising the middle parts of the jejunum (28-32 cm from the pylorus), indicating that any portion of this area could be used in the study.

The results of the first experiment showed that liposomes are effective adjuvants for the oral cholera vaccine. The mucosal response to the liposome-associated vaccine was higher than to the free antigen vaccine. Since the use of liposomes in our experiment also prevented the serum response to CT, the result indicates that perhaps the liposomes could confine the response to the intestinal mucosa. In contrast, rats which received free antigen vaccine had a significantly lower mucosal response but a good serum response. These results confirmed that the antigenic stimulation by orally applied free-antigen was divided between the serum and mucosal compartments.[23] The responses of rats receiving the liposomes alone did not differ from those of rats which received placebo. Liposomes did not cause any nonspecific B cell stimulation in the absence of other antigens.

In the second experiment, the reduction of lipids resulted in a reduced mucosal response in terms of the AbPC counts. The significant reduction in the response implies that the adjuvant effect of the liposomes was lipid dose dependent. Moreover, even though the response was reduced, the AbPC counts of rats which received the liposome-associated vaccine were still significantly higher than those which received the free vaccine, thereby confirming the adjuvanticity of the liposomes.

Results of the third experiment showed that even when the antigen levels were reduced, liposome-associated antigens were consistently more immunogenic than the corresponding levels of free antigens. These findings agree with the results of the first and second experiments and further demonstrate that liposomes enhance the immunogenicity of the associated antigens. Although the free antigens were synergistically immunogenic when given in combination, possibly relying on the adjuvant properties of LPS and P,[7,8] the immune responses they generated were lower than the responses to the liposome-associated antigens.

In the third experiment, the reduction of antigen levels was not sufficient to evoke statistically significant differences in the immune responses among groups of rats receiving similar preparations of vaccines, i.e. in the groups which received liposome-associated vaccines, similar responses were elicited regardless of the antigen levels used and among groups given different levels of free antigens, the differences in AbPC responses were, likewise, insignificant. Experiments should be designed to find out the lowest levels of all antigens which would still give the maximum responses.

In the case of adjuvants other than liposomes, the reported immunogenicity of the antigens depended on the adjuvant dose, e.g. adjuvanticity of *Bordetella pertussis* improved as the number of the organism was increased.[26] In our study, the adjuvanticity appears to be influenced by the lipid levels of the liposomes (experiment 1 v.s. experiment 2).

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REFERENCES

1. Newby JJ, Stokes CR. Local Immune Responses of the Gut. CRC Press, Inc.: USA. 1984 : 143-98.
2. WHO Memorandum. Recent advances in cholera research: memorandum from a WHO meeting. Bull WHO 1985; 63 : 841-84.
3. Levine MM, Kaper JB, Black RE, Clements ML. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiol Rev 1983; 47 : 510-50.
4. Black RE, Levine MM, Clements ML, *et al.* Oral immunization with killed whole vibrio and B subunit or procholeragenoid combination vaccines: immune responses and protection from *V.cholerae* challenge. In: Proceedings of the 19th

Joint US-Japan Cholera Conference. Martinus Nijhoff. 1983.

5. Clemens JD, Harris JR, Khan MR, *et al.* Field trial of oral cholera vaccines in Bangladesh. Lancet 1986; 2 : 124-7.
6. Clemens JD, Stanton BF, Chakraborty J, *et al.* B subunit-whole cell and whole cell-only oral vaccines against cholera: studies on reactogenicity and immunogenicity. J Infect Dis 1987; 155 : 79-84.
7. Chaicumpa W, Chaisri U, Tapchaisri P, Chongsa-nguan M, Pongponratn E. Oral vaccine against cholera prepared from *V.cholerae* antigens. Southeast Asian J Trop Med Public Health 1987; 18 : 142-8.
8. Pierce NF, Cray WC Jr, Sacci JB Jr. Oral immunization of dogs with purified cholera toxin, crude cholera toxin, or B subunit: evidence for synergistic protection by antitoxic and anti-bacterial mechanisms. Infect Immun 1982; 37 : 687-94.
9. WHO Memorandum. Intestinal immunity and vaccine development. Bull WHO 1979; 57 : 719-34.
10. Gregoriadis G, Swain CP, Willis EJ, Tavill AS. Drug-carrier potential of liposomes in cancer chemotherapy. Lancet 1974; i : 1313-6.
11. Allison AC, Gregoriadis G. Liposomes as immunological adjuvants. Nature 1974; 252 : 252.
12. New RRC, Theakston DG, Zumbuehl O, Iddon D, Friend J. Liposomal immunization against snake venoms. Toxicon 1985; 23 : 215-9.
13. Banerji B, Alving CR. Anti-liposome antibodies induced by lipid A. I. Influence of ceramides, glycosphingolipids, and phosphocholine on complement damage. J Immunol 1981; 126 : 1080-4.
14. Dancey GF, Yasuda T, Kinsky SC. Enhancement of liposomal membrane immunogenicity by incorporation of lipid A. J Immunol 1977; 119 : 1868-73.
15. Nicolotti RA, Kochibe N, Kinsky SC. Comparative immunogenic properties of N-substituted phosphatidylethanolamine derivatives and liposomal model membranes. J Immunol 1976; 117 : 1898-902.
16. Bakouche O, Gerlier D. Enhancement of immunogenicity of tumour virus antigen by liposomes: the effect of lipid composition. Immunology 1986; 58 : 507-13.
17. Shek PN, Sabiston BH. Immune response mediated by liposome-associated protein antigens II. Comparison of the effectiveness of vesicle-entrapped and surface-associated antigen in immunopotentiation. Immunology 1982; 47 : 627-32.
18. Westphal O, Jann K. Bacterial lipopolysaccharide extraction with phenol-water and further application of the procedure. Methods Carbohydr Chem 1965; 5 : 83-91.
19. Szoka F Jr, Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc Natl Acad Sci USA 1978; 75 : 4194-8.
20. Cherry WB. Fluorescent antibody techniques. In: Manual of Clinical Microbiology. Blair JE, Lenette EH, Truant JP, eds. American Society for Microbiology. 1970 : 639-704.
21. The TH, Feltkamp TEW. Conjugation of fluorescein isothiocyanate to antibodies. I. Experiments on the conditions of conjugation. Immunology 1970; 18 : 865-73.
22. Elson CO, Ealding W. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. J Immunol 1984; 132 : 2736-41.
23. Clancy RL, Bienenstock J. Enteric infection and immunization. In: Immunological Aspects of Liver and Gastrointestinal Tract. Ferguson A, Macsween RNM, eds. MTP Press: Lancaster, England. 1976 : 121-52.
24. Pierce NF, Koster FT. Stimulation of intestinal immunity. In Acute enteric infections in children: New prospects for treatment and prevention. Holme, Holmgren, Merson, Mollby eds. 1981 : 413-23.
25. Shek PN, Sabiston BH. Immune response mediated by liposome-associated protein antigens. I. Potentiation of the plaque-forming cell response. Immunology 1982; 45 : 349-56.
26. Unanue ER, Askonas BA, Allison AC. The role of macrophages in the stimulation of immune responses by adjuvants. J Immunol 1969; 103 : 71-8.