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Cholera toxin B subunit conjugated bile salt stabilized vesicles (bilosomes) for oral immunization

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Abstract

Bile salt stabilized vesicles, bilosomes appear to be a promising and potential carrier system for oral delivery of peptides and proteins. Bilosomes containing bovine serum albumin (BSA), a model antigen, were prepared and conjugated with cholera toxin B subunit (CTB) in order to enhance their affinity towards M cells of Peyer's patches. Stability studies were undertaken to ascertain the effect of simulated gastric fluid (SGF, pH 1.2), simulated intestinal fluid (SIF, pH 7.5) and different concentrations of bile salts. Intactness and biological activity of CTB were checked by hemagglutination test. A single oral dose of CTB-conjugated bilosomes produced almost equivalent response compared to parenteral administration of antigen with Freund's complete adjuvant (FCA). However, in contrast to FCA, oral administration of bilosomes is convenient and devoid of any adverse effects that are observed with parenteral administration of FCA. Serum IgG titers after single administration were significantly better ($P < 0.05$) than oral administration of antigen with other systems for 3 consecutive days, suggesting an effective stimulation of systemic immune response. Mucosal IgA titers obtained advocated a possible application of CTB-conjugated bilosomes as oral vaccine delivery system.

Keywords: Bilosomes; Bile salts; CTB; BSA; Oral immunization; M cell targeting

1. Introduction

Intestinal lumen is a portal of entry for numerous pathogens that range from viruses to bacteria or protista. Although antibiotic therapy has been frequently employed to treat infections, increasing number of resistant strains and inefficiency of anti-viral drugs to resolve viral infections has accelerated the development of other strategies to prevent infections. Immunoprophylaxis using vaccines represents a feasible tool since targeted immune protection can be attained and health care cost can be reduced. In principle, vaccination is a simple and effective means of achieving disease control in human and animal populations. However, the efficiently regulated mucosal immune system presents a number of practical problems (Simmons et al., 2001). Most antigens are apparently poorly immunogenic, when processed through mucosal surfaces stimulating no or weak serum immunoglobulin (IgG) or secretory IgA responses. Mucosal immune response can be successfully elicited with judicial use of adjuvants or novel carrier systems. Studies revealed

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that co-administration of mucosal adjuvants such as cholera toxin (CT) and heat labile enterotoxin from *E. coli* effectively support antigen specific immune responses (Tochikubo et al., 1998; Dickinson and Clements, 1996). Among all carrier systems developed during last two decades, polymeric biodegradable microparticles and mainly PLA or PLGA microparticles are probably the most extensively studied (Gutierro et al., 2002a; Katz et al., 2003; Yeh et al., 2002). Among other delivery systems liposomes, which are bilayered phospholipid membrane vesicles offer a number of potential advantages and have attracted considerable interest as mucosal delivery systems (Childers and Michalek, 1994; Jackson et al., 1990; Michalek et al., 1992). However, the susceptibility of conventional liposomes to bile salts and enzymatic degradation in gastrointestinal tract led to the development of coated versions of vesicles. In our laboratory polysaccharide coated liposomes have been developed for oral immunization (Venkatesan and Vyas, 2000).

Nonionic surfactant based vesicles (niosomes) that are assemblages of non-ionic amphiphiles into closed bilayer structures have also been reported to possess strong adjuvanticity (Brewer et al., 1998; Hassan et al., 1996). Studies revealed that incorporation of bile salts in niosomal formulation could stabilize the membrane against the detrimental effects of bile acids in GI tract (Schubert et al., 1983). These bile salt stabilized vesicles referred to as 'bilosomes' demonstrated appreciable immune response after oral administration suggesting an enormous potential as an effective carrier system for oral immunization (Conacher et al., 2001). Furthermore, it has been envisaged that anchoring specific ligand to the surface of these vesicles enable them to bind with M cells of Peyer's patches for efficient uptake and elicitation of immune response.

Harokopakis et al. (1995, 1998) successfully conjugated cholera toxin B subunit (CTB) to the surface of liposomes by employing heterobifunctional reagent. Studies revealed that CTB was stable and retained the biological activity after tagging with liposomal surface.

The aim of present work was to develop M cell specific target oriented vesicular system, stable in GI tract. Bovine serum albumin (BSA) was selected as a model antigen. CTB anchored bilosomes were characterized for size, shape, entrapment efficiency and stability. Systemic as well as mucosal antibody response were measured in order to assess the suitability of system for oral vaccination.

2. Materials and Methods

2.1. Chemical and immunological reagents

Sorbitan tristearate and cholesterol were obtained from Fluka chemika, India. Dipalmitoyl phosphatidyl ethanolamine (DPPE), Succinimidyl (4-*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), *N*-succinimidyl-3-(2-pyridyl dithio) propionate (SPDP), Cholera toxin B subunit (CTB), Freund's complete adjuvant (FCA), triethanolamine, anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG peroxide conjugate, anti-mouse and anti-cholera toxin from rabbit were purchased from Sigma, USA. Bovine serum albumin (BSA) was obtained from Himedia, India. G_{M1} protein was a kind gift from Panacea Biotech, India. Tetramethyl benzidine (TMB), Hepes buffer, Tris buffer (pH 6.8) and L-cysteine buffer were purchased from Genei, Bangalore, India.

2.2. Preparation and characterization of CTB-conjugated bilosomes

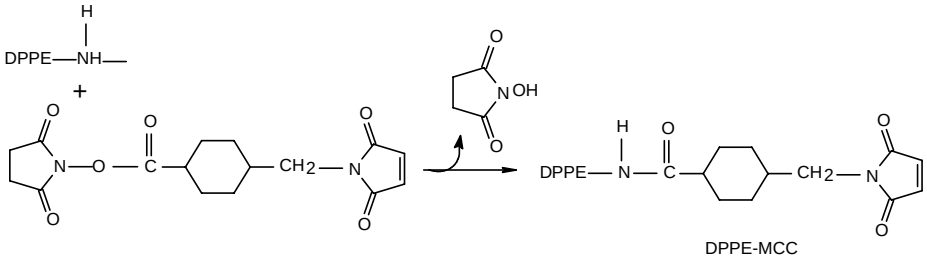
2.2.1. Derivatization of DPPE

DPPE was modified by using the heterobifunctional reagent SMCC following a method reported by Harokopakis et al. (1995). Briefly, 50 mg of DPPE was incubated with 35.8 mg SMCC in 3.6 ml chloroform/methanol mixture (9:1) containing 19 μl freshly distilled triethanolamine overnight at room temperature (Fig. 1). The solution was diluted with 5 ml chloroform and extracted twice with 1 ml NaCl (1%, w/v) in order to remove excess SMCC and other water soluble byproducts. Preparation was lyophilized, resuspended in 1 ml chloroform and ultimately passed through silica column to obtain purified product (DPPE-MCC).

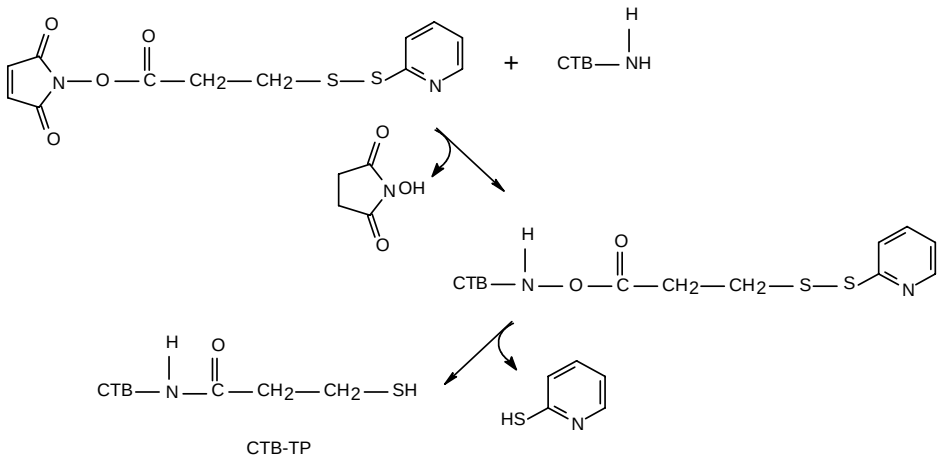
2.2.2. Preparation of antigen loaded bilosomes

Sorbitan tristearate, cholesterol and DPPE-MCC in a molar ratio of 7:3:1 were dissolved in minimum amount of chloroform in a round bottom flask.

Step I: DPPE + SMCC



Step II: CTB + SPDP



Step III: DPPE-MCC + CTB-TP

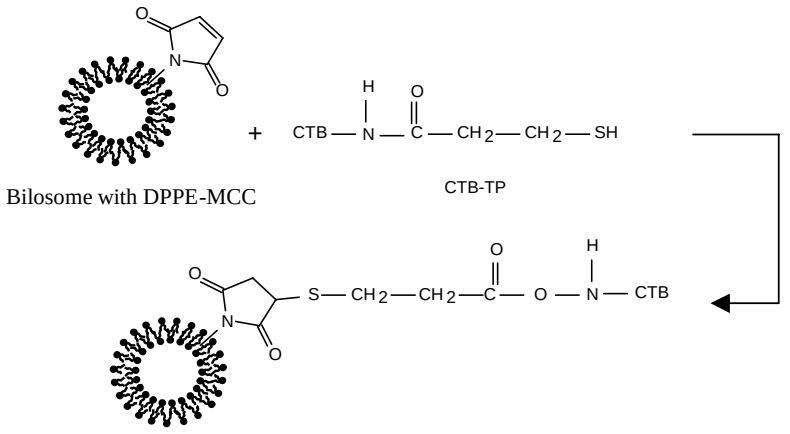


Fig. 1. Conjugation of CTB to bilosomes. Abbreviations: DPPE: dipalmitoyl phosphatidyl ethanoloamine; SMCC: succinimidyl-4-*N*-maleimidomethyl-cyclohexane-1-carboxylate; CTB: cholera toxin B subunit; SPDP: *N*-hydroxysuccinimidyl-3(2-pyridyldithio) propionate; DPPE-MCC: dipalmitoyl phosphatidyl ethanolamine-4-*N*-maleimidomethyl cyclohexane-1-carboxylate; CTB-TP: Cholera toxin B subunit-thiopropionate; DTT: dithiothreitol.

Solvent was removed under reduced pressure to form a thin film on the side of flask. The film was subsequently dispersed with the addition of 5 ml BSA solution (500 μg/ml) in PBS (pH 7.4) in presence of bile salt (Nadeoxycholate, 20 mg) resulting in formation of large multilamellar vesicles (MLVs). Small unilamellar bilosomes were prepared by extruding MLVs through 400 nm followed by 100 nm pore membrane (Millipore, USA). Unentrapped BSA and Nadeoxycholate were removed by minicolumn centrifugation method using sephadex G-100 column (Fry et al., 1978). Fractions were collected and challenged with TritonX-100 (0.2%, v/v). After suitable dilution antigen was measured using BCA method. Experiment was repeated thrice using a fresh column each time. Size of bilosomes before and after extrusion was measured using Malvern zeta sizer, UK. Results are shown in Table 1.

2.2.3. Synthesis of CTB-DP

Lysine residues of CTB were modified by using amine reactive reagent SPDP following a method reported by Harokopakis et al. (1995). Thiol groups are necessary for reaction with the maleimide group of DPPE-MCC bilosomes. Typically, CTB was dissolved in Hepes buffer and incubated with SPDP (1:10 molar ratio) in dark at room temperature for 30 min. The reaction was quenched with 10 μl of 20 mM L-lysine (20 mM Tris buffer, pH 6.8) and then the reaction product was reduced with the addition of 5 μl of 7.7 mg/ml dithiothreitol in deionized water. Unreacted material and byproducts were removed using size exclusion chromatography column.

2.2.4. Test for intactness of CTB after conjugation

ELISA assay was performed to check ability of modified CTB to bind G_{M1} ganglioside receptors. Briefly, G_{M1} ganglioside protein coated ELISA plates were incubated with modified and unmodified CTB for 2 h at room temperature followed by treatment with rabbit anti-CT antibodies after washing with PBS-Tween 20 (0.05%, v/v). Subsequently, goat anti-IgG conjugated horseradish peroxide was incubated for 2 h at room temperature. Tetramethyl benzidine (TMB) substrate was added followed by addition of H_2SO_4 (2 N) after 90 min to produce color, which was measure at 450 nm within 15 min.

2.2.5. Coupling of CTB to bilosomes

One milligram reduced CTB-DP was incubated with 1 ml of DPPE-MCC bearing bilosomes at 4 °C overnight for conjugation of CTB to bilosome surface. The coupling reaction was stopped by addition of 10 μl of L-cysteine buffer (pH 6.8). Bilosomes were separated from unconjugated proteins by using minicolumn centrifugation (Sephadex G-75 packed column) and washed thrice with Hepes buffer.

2.2.6. Hemagglutination assay

CTB binding efficiency with G_{M1} ganglioside was assessed by hemagglutination assay, where human erythrocytes were incubated with dried film of G_{M1} ganglioside with occasional stirring for 10 min at 37 °C followed by 1 h at room temperature. Unincorporated G_{M1} was separated by centrifugation at 1000 × g for 12 min with washing for two times with PBS (pH 7.4). CTB-conjugated bilosomes were added (100 μl) in twofold dilutions to wells of V bottom PVC plates (Tarsons, India) followed by addition of G_{M1} enriched erythrocytes (0.25%, v/v) in wells. Mixture was incubated for 4 h at 4 °C and any sign of hemagglutination was visually evaluated.

Table 1
Composition of various formulations with % encapsulation efficiency and mean vesicle size

Vesicular system	Composition (molar ratio)	Encapsulation efficiency (%)	Mean vesicle size (nm)	
			Before extrusion (μm)	After extrusion (nm)
Bilosomes	Span65:Chol:DPPE-MCC (7:3:1) + 20 mg Na deoxycholate	28.2 ± 3.4	1.9 ± 0.4	108.6 ± 11.4
Niosomes	Span65:Chol:DPPE-MCC (7:3:1)	30.4 ± 3.7	1.7 ± 0.3	104.3 ± 9.2

2.3. Stability in simulated fluids and bile salt solutions

Stability of bilosomes tagged with CTB was determined in simulated gastric fluid (SGF, pH 1.2), simulated intestinal fluid (SIF, pH 7.5) and different concentrations of bile salts (5 and 20 mM) by addition of 1.8 ml of different solutions to 0.2 ml of vesicular dispersion. After 2 h samples were withdrawn and unentrapped BSA was removed by minicolumn centrifugation method (Fry et al., 1978). Unentrapped BSA free vesicles were challenged with Triton X 100 (0.2%, v/v) and BSA was estimated using BCA assay kit. Intactness of surface CTB was also assessed by hemagglutination assay after bile salt treatment.

2.4. Immunization protocol

2.4.1. Animals and inoculations

Female Balb/c mice aged 6–8 weeks, weighing 15–20 g were used for in vivo studies. Animals were housed in groups of five with free access to food and water. They were deprived of food 3 h prior to oral administration. The mice were immunized intrasophageally with preparations equivalent to 100 μg of BSA following two protocols. Protocol 1: single primary inoculation on day 0 followed by booster dose after 3 weeks. Protocol 2: three primary inoculations for 3 consecutive days. Booster immunization was done after 3 weeks. Single subcutaneous immunization with booster dose after 3 weeks was also carried out for antigen solution to serve as standard.

2.4.2. Collection of fluids

Preimmune samples of serum and secretions (saliva, vaginal fluid and intestinal lavage) were obtained on day 0 before immunization. Subsequent to immunization, collections were made at days 14 , 28, 42 and 56. Serum was obtained by centrifugation of blood samples collected from retroorbital plexus of mice under ether anesthesia. For collection of saliva, mice were injected 0.2 ml sterile solution of pilocarpine (10 mg/ml) intraperitoneally (IP). The mice began to salivate after approximately 2 min and the saliva was collected by using capillary tube. Intestinal lavage was performed using the technique reported by Elson et al. (1984). Briefly, four doses of 0.5 ml lavage solution (NaCl 25 mM, Na_2SO_4 40 mM, KCl 10 mM, $NaHCO_3$ 20 mM and polyethylene glycol-MW 3350; 48.5 mM) were administered intragastrically at 15 min intervals using a blunt tipped feeding needle. Thirty minutes after the last dose of lavage solution the mice were given 0.2 ml pilocarpine (10 mg/ml) IP. A discharge of intestinal contents occurs regularly over next 20 min, which was collected carefully. Vaginal secretions were collected by using a pipettor to douche the mice with 0.1 ml of PBS (pH 7.4), which was then aspirated back into the pipette tip and used for determination of antibody levels. In order to increase the volume of material available for assay, wherever necessary, samples from two mice of identical immunized groups were pooled.

2.5. Anti-BSA antibody detection

Antibody responses in immunized animals were monitored using a microplate ELISA procedure. Microtiter plates (Nunc-Immuno Plate® Fb 96 Mexisorp, NUNC) were coated with 100 μl/well of 10 μg/ml BSA in PBS (pH 7.4) and incubated overnight at 4 °C. In order to detect total IgA, wells were coated with anti-mouse IgA. The plates were thoroughly washed with PBS-Tween 20 (0.05%, v/v) (PBST). The serum samples were serially diluted 1:1 with PBS and 100 μl of each sample was added to each well of coated ELISA plates. The plates were incubated for 1 h at room temperature and washed three times with PBST. One hundred microliters of peroxidase labeled goat anti-mouse IgG, IgG2a or IgG1 was added to each well. The plates were covered and after incubation for 1 h at room temperature washing was repeated. One hundred microliters of tetramethyl benzidine (TMB) solution was added to each well followed by addition of 50 μl of H_2SO_4 after 90 min. After 15 min incubation developed color was measured at 450 nm using plate reader (Labsystems, Finland). End point titers were expressed as the log 2 of the reciprocal of last dilution, which gave an optical density (OD) at 450 nm above the OD of negative controls. IgG2a/IgG1 fraction was calculated from respective log 2 values of end point titers.

2.6. Determination of specific IgA

Specific IgA antibodies were measured with a method reported by Elson et al. (1984) with slight modifications. The same sequence was followed as

described for total IgA estimation except that wells were coated with BSA. As a standard for anti-BSA, a reference hyperimmune serum was used and the antibody concentration expressed in units of standard activity: one unit of anti-BSA antibody is defined as that amount, which gives an absorbance at 450 nm equivalent to a $1{:}10^6$ dilution of the reference antiserum. Since the reference serum is an IgG antibody, goat anti-mouse IgG was used for the standards and goat anti-mouse IgA used for the unknowns. In order to ensure that readings with these two goat antisera are comparable, each antiserum was titered against equivalent amounts of either IgG or IgA coated to wells. The titer of each goat antiserum, which gave an OD 450 reading of 1.0, when the wells were coated with 10 ng of the relevant immunoglobulin isotype was determined and these titers were used for further assays.

2.7. Statistics

Analysis of antibody titers was performed on logarithmically transformed data and standard deviation (S.D.) was calculated. Student's *t*-test was used to compare mean values of different groups. Statistical significance was designated as $P < 0.05$.

3. Results and discussion

3.1. Preparation and characterization of CTB-conjugated bilosomes

Bilosomes were prepared by cast film method, which is a fairly good method for preparation of multilamellar vesicles. Span65 (Sorbitan tristearate), a non-ionic surfactant was used as a major ingredient along with cholesterol and derivatized DPPE. DPPE was modified using a heterobifunctional linker, SMCC following a method reported by Harokopakis et al. (1995). The reaction of DPPE with SMCC involves nucleophilic attack of the nitrogen atom of amino group present on DPPE with carboxyl carbon of SMCC. SMCC possesses a maleimide group, which can be easily transferred to molecules having free amino groups. Studies revealed that spacers using maleimide derivatives (thioether bonding) function as better crosslinking reagents than others leading to

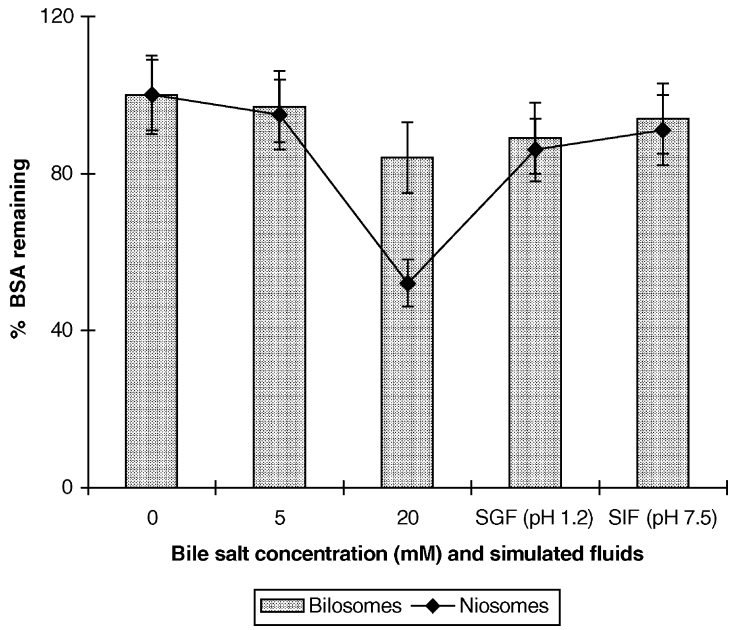


Fig. 2. Stability of bilosomes and niosomes at different bile salt concentrations, SGF (pH 1.2) and SIF (pH 7.5). Results are expressed as mean ± S.D.

different types of bridging (i.e. disulfide) between molecules (Loughrey et al., 1990, 1993). It has been proposed that long spacer arm of these linkers allows linked protein molecules to assume a conformation similar to the native form and also stability of the thioether bond formed by nucleophilic addition of thiol groups to double bond of the maleimide group adds to the applicability of SMCC linker. In addition, the long spacer between CTB and the surface of the vesicles reduces steric hindrance and allow the vesicle linked CTB molecule to interact with G_{M1} receptor on M cell. The CTB-conjugated bilosomes were thus expected to gain access to the GALT to produce efficient mucosal immune response.

Major surfactant used for preparation of vesicles, sorbitan tristearate was selected because it is a saturated surfactant and forms more stable bilayer (low fluidity) compared to unsaturated phospholipids used for preparation of liposomes (Gregoriadis, 1990). Moreover, these saturated surfactants are less sensitive than unsaturated lipids toward oxidative attacks (Halliwell and Gutteridge, 1989) and generation of free radicals, which could adversely affect the conjugation of CTB to DPPE-MCC.

Dertzbaugh and Elson (1993) demonstrated that adjuvanticity of CT and its B subunit is partially due to their high affinity to glycolipid G_{M1} receptor. All intestinal cells possess G_{M1} on their lumenal surface with varying density, however, it has been reported that CTB complexed to colloidal gold particles preferentially bind to M cell surface than that of adjacent enterocytes (Owen et al., 1986). Exquisitely designed studies have exhibited that the glycocalyx, which coats the M cells, although thinner than that of neighboring enterocytes, can still pose problem for targeting to these cells (Frey et al., 1996). In fact, these investigators concluded that particles of average diameter 1 μm or larger coated with CTB could not adhere to M cells, unlike smaller CTB coated particles, which could readily bind. Therefore, size of MLVs, prepared by cast film method was reduced to around 100 nm by

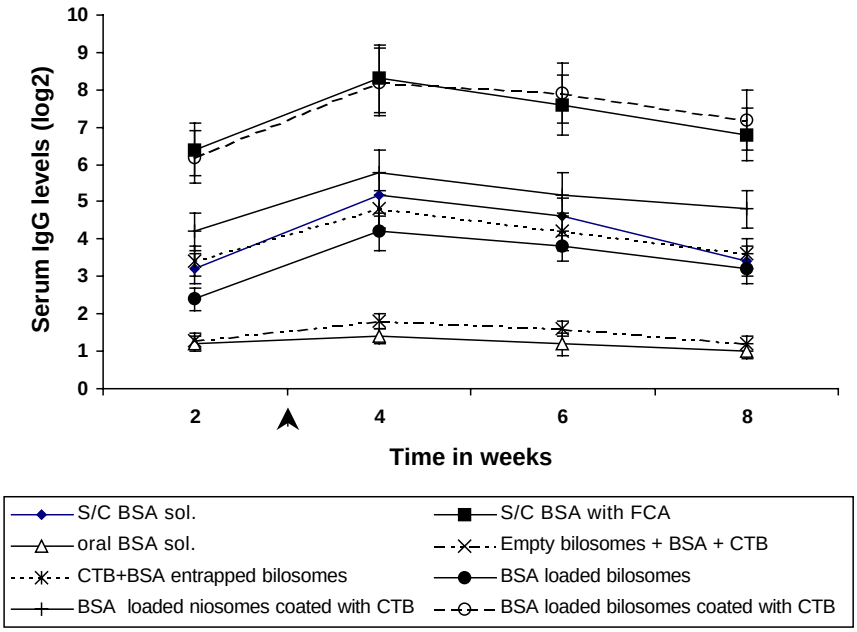


Fig. 3. Induction of serum IgG response elicited by the subcutaneous administration of BSA solution (group 1), BSA with FCA (group 2) and oral administration of BSA solution (group 3), empty bilosomes with CTB and BSA (group 4), CTB and BSA loaded bilosomes (group 5), BSA loaded bilosomes (group 6), BSA loaded niosomes conjugated to CTB (group 7) and BSA loaded bilosomes conjugated to CTB (group 8) at a constant dose of 100 μg BSA. Immunization was done following protocol 1 with booster dosing (⮝) after 3 weeks. Values represent log 2 of endpoint dilutions ± S.D.

extrusion through 400 and 100 nm membrane filters (Table 1).

The reaction of CTB with amine group of SPDP is also a nucleophilic addition to carbonyl carbon of SPDP. It has been reported that reaction of 1:10 molar ratio CTB:SPDP for 30 min produced best results with negligible effect on G_{M1} binding efficiency of CTB (Harokopakis et al., 1995). The biological activity of modified CTB (cholera toxin B subunit-thiopropionate) to bind G_{M1} ganglioside receptor was determined by ELISA. Results showed that modified CTB retained the affinity for G_{M1} protein. Furthermore, to determine the biological activity of CTB after coupling to bilosome surface, hemagglutination assay was performed. It was observed that CTB-conjugated unilamellar vesicles exhibited agglutination property compared to controls and CTB unconjugated bilosomes gave negative agglutination results.

Bilosomes (MLVs) were prepared according to the method reported by Conacher et al. (2001). However, size of vesicles was reduced by extrusion in order to make them suitable for M cell targeting. Concept of bilosome preparation is based on the rationale that though initially bile salts destabilize lipid vesicular membranes, however after inclusion as an integral part of the system, bile salts would stabilize the lipid vesicles on future bile salt exposure (Lee, 1991; Schubert et al., 1983). This hypothesis was checked by challenging the prepared systems with bile salt solutions.

3.2. Stability studies

Stability of prepared vesicles was checked in simulated gastric fluid, SGF (pH 1.2) and simulated intestinal fluid, SIF (pH 7.5). Results suggest that bilosomes are comparably more stable in simulated fluids than niosomes, however difference was insignificant ($P > 0.05$). It has been reported that bile salt concentration in healthy humans have been in a range between 5 and 20 mM (Westergaard, 1977; Coleman et al., 1979), therefore stability of formulations was also tested in these two extreme concentrations. It was observed that at 5 mM concentrations around

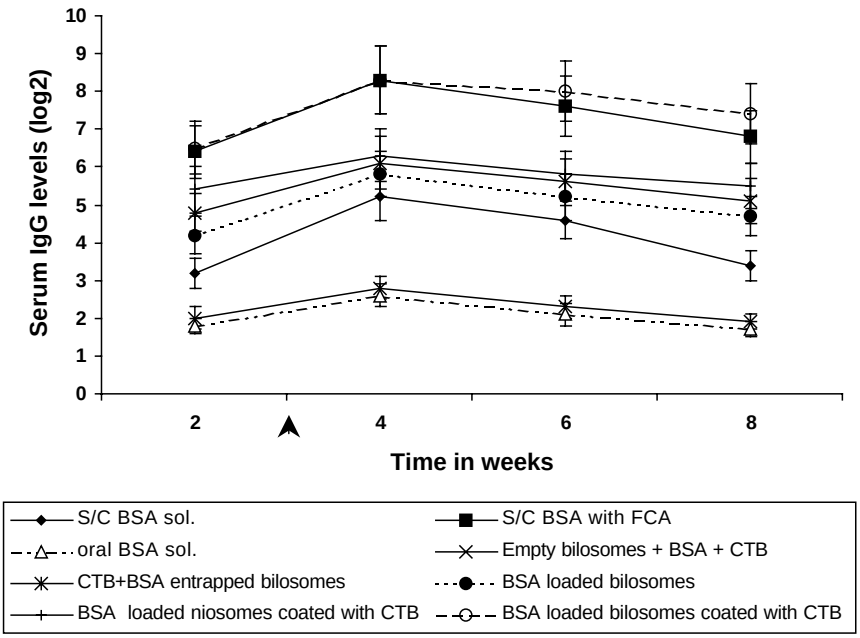


Fig. 4. Induction of serum IgG response elicited by the subcutaneous administration (protocol 1) of BSA solution (group 1), BSA with FCA (group 2) and oral administration (protocol 2) of BSA solution (group A), empty bilosomes with CTB and BSA (group B), CTB and BSA loaded bilosomes (group C), BSA loaded bilosomes (group D), BSA loaded niosomes conjugated to CTB (group E) and BSA loaded bilosomes conjugated to CTB (group F) at a constant dose of 100 μg BSA. Secondary immunization (⮝) was done after 3 weeks. Values represent log 2 of endpoint dilutions ± S.D.

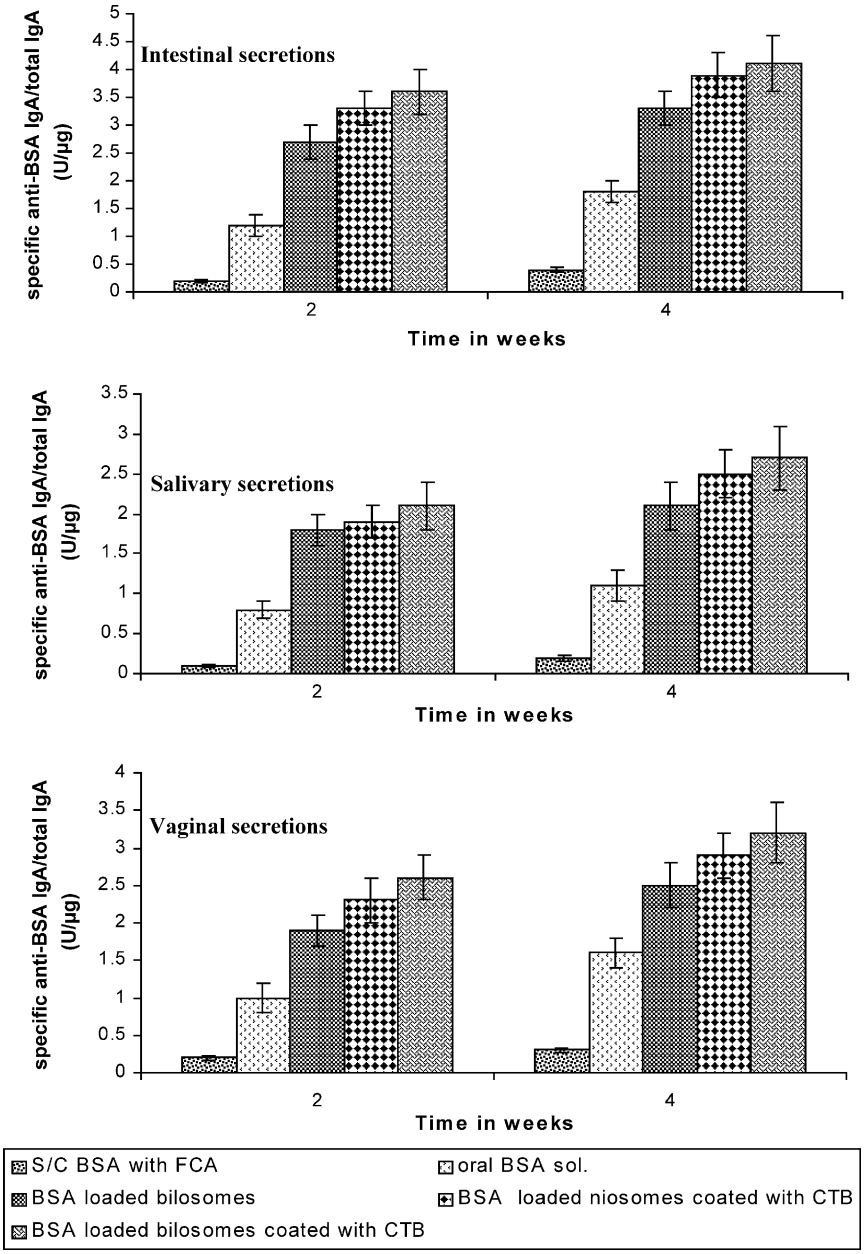


Fig. 5. IgA antibody response in secretions after immunization with subcutaneous administration (protocol 1) of BSA with FCA (group 2), oral administration (protocol 2) of BSA solution (group A), BSA loaded bilosomes (group D), BSA loaded niosomes conjugated with CTB (group E), BSA loaded bilosomes conjugated with CTB (group 8).

92–95% of BSA was retained in vesicles, whereas at higher bile salt concentration only bilosomes retained 84% of BSA compared to niosomes (∼52%) (Fig. 2).

It can be inferred from results that bilosomes exhibit relatively better stability compared to niosomes. Intactness of surface anchored CTB was also checked by agglutination assay, where negligible reduction in

biological activity of CTB was noticed after treatment with SGF (pH 1.2), SIF (pH 7.5) and bile salt solutions (5 and 20 mM).

3.3. In vivo antibody response

The level of anti-BSA antibodies (IgG) was determined for all experimental groups after 2, 4, 6 and 8 weeks. Figs. 3 and 4 show serum IgG response obtained with two protocols, single inoculum (protocol I) and three inoculations in consecutive days (protocol II), respectively. Booster immunization was performed for all groups after 3 weeks. Results suggest that oral immunization with bilosomes conjugated with CTB significantly increased IgG levels ($P < 0.05$) compared to other formulations administered orally. It has been observed that BSA emulsified with FCA also produced almost equivalent IgG titer, when administered subcutaneously. At the other extreme, when three doses of BSA were orally administered (protocol II) to produce primary immune response, almost all formulations exhibited an increase in IgG response except CTB-conjugated bilosomes. Probably this may be due to saturation of IgG levels achieved through oral route. This is concordant with the findings of Gutierro et al. (2002b) that through oral route only a particular level of serum IgG titer can be attained. For IgA estimation subcutaneous injection of BSA with FCA, orally administered BSA solution, BSA loaded bilosomes, BSA loaded niosomes conjugated with CTB and BSA bearing bilosomes anchored with CTB were selected. IgA levels were measured after 2 and 4 weeks. Fig. 5 depicts the fractions of specific IgA against BSA to total IgA levels in intestinal, salivary and vaginal secretions. It can be inferred from the results that CTB anchored bilosomes efficiently elicit mucosal immune system, even with single dose priming following protocol I. Other formulations administered following protocol II produced relatively low IgA titers. It was observed that negligible IgA response was observed with subcutaneous injection of BSA along with FCA. It may be attributed to the fact that parenterally administered antigen lacks the ability to stimulate mucosal immune system.

It has been reported that the nature of antigen presenting cell (APC), which presents the antigen to the specific naïve T cells, will favor the isotype and magnitude of the B cell antibody response (Morokata

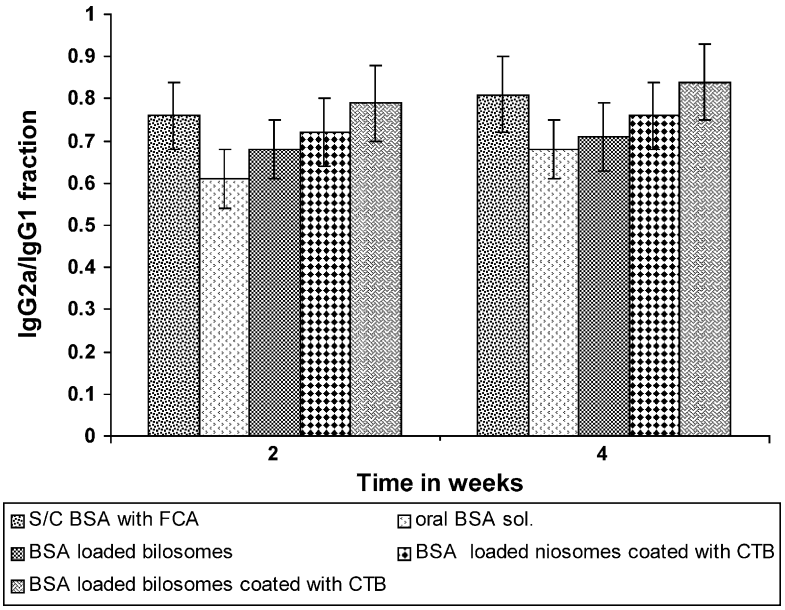


Fig. 6. Serum IgG2a/IgG1 ratio elicited after the immunization with subcutaneous administration (protocol 1) of BSA with FCA (group 2), oral administration (protocol 2) of BSA solution (group A), BSA loaded bilosomes (group D), BSA loaded niosomes conjugated with CTB (group E), BSA loaded bilosomes conjugated with CTB (group 8).

et al., 2000; Constant and Bottomly, 1997). Neutra et al. (1996) reported different body distribution of dendritic cells on the epithelial barriers at different sites of the body. Dendritic cells are considered as initiators and modulators of the immune response and are capable to process the antigen by both the major histocompatibility complex (MHC) class I and MHC class II pathways, depending upon the nature of the observed antigen (Banchereau et al., 2000). Thus different formulations loaded with antigen could be differently processed and presented to a specific T helper lymphocyte subpopulation, which could originate differences in the specific serum IgG2a/IgG1 antibody production. IgG1 is an antibody associated to inflammatory and mast cells or eosinophil responses, while IgG2a is able to act as an opsonin, activates the compliment and binds to macrophages enhancing the phagocytic response (Lubeck et al., 1985). It has been determined that switching to IgG2a is induced by IL-12 and IFN-γ (secreted by Th1 CD4+ cell subset) and inhibited by IL-4, IL-5 and IL-10 (secreted by Th2 CD4+ cell subset) (Constant and Bottomly, 1997). Fig. 6 shows that formulations are capable of eliciting a combined serum IgG2a/IgG1 response, with predominance of the IgG1 response. Results are consistent with those reported by Banchereau et al. (2000). They suggested that particulate antigens can be processed and presented either by MHC class I or MHC class II by dendritic cells and macrophages, which stimulates Th1 and Th2 lymphocyte subpopulations, while soluble antigens are exclusively presented by class II MHC, stimulating the Th2 response. This reflects that oral administration of BSA in bilosomes conjugated with CTB can elicit both Th1 and Th2 immune response, however more studies concerning T lymphocyte proliferative assays and cytokine production should be conducted in order to completely characterize the immune response elicited by this system.

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