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M-cell targeted delivery of recombinant hepatitis B surface antigen using cholera toxin B subunit conjugated bilosomes

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ABSTRACT

The present study aims to improve upon our earlier findings with bilosomes as potential delivery vehicle through oral route for recombinant hepatitis B surface antigen (HBsAg). The work entails the conjugation of bilosomal system with cholera toxin B subunit (CTB) to increase transmucosal uptake via M-cell specific delivery approach. The study encompasses the development and characterization of HBsAg-loaded CTB-conjugated system for percent antigen entrapment, size, shape, and stability in SGF (USP, pH 1.2), SIF (USP, pH 7.5) and in bile salt solutions. Biological activity of CTB, subsequent to conjugation, was verified by hemagglutination test. Anti-HBsAg IgG response in serum and anti-HBsAg sIgA in various body secretions were estimated using ELISA, following oral immunization with 10 μg dose-loaded CTB-conjugated bilosomes (CTB2) and 20 μg dose-loaded CTB-conjugated bilosomes (CTB1) in BALB/c mice. The results showed that CTB1 produced anti-HBsAg IgG antibody titre response comparable to that of the intramuscular (IM) injection of 10 μg of alum-adsorbed HBsAg. Moreover, all the bilosomal preparations elicited measurable sIgA vis-à-vis negligible response with IM administered HBsAg. Thus, HBsAg-loaded CTB-conjugated bilosomes provide a promising potential for targeted oral immunization against hepatitis B.

1. Introduction

Oral immunization offers several important advantages over conventional parenteral immunization (Walker, 1994; Shalaby, 1995; Simerska et al., 2009). Oral intake has been the most sought-after route of drug administration owing to the wide acceptability of this "natural" route, improved patient compliance, low cost of therapy and ease of administration. Particularly, this obviates the need of trained personnel and sterile needles needed for injectables, thus facilitating repeated administration of vaccine doses.

Parenteral as well as mucosal exposure to hepatitis B antigen positive body fluids is known to be responsible for transmission of hepatitis B virus (Hilleman, 2003; Chen et al., 2009). The antibodies produced in response to the parenteral immunization are unable to provide protection at the mucosal level, a major site for entry of most infectious agents, including hepatitis B virus (Jaganathan and Vyas, 2006). Owing to the failure to induce mucosal response, the need for improvement in the existing approach of using injectable vaccines has been emphasized by several authors (Shalaby, 1995; Clark et al., 2001a,b; Ryan et al., 2001; Magistris, 2006; Mann et al., 2009; Myschik et al., 2009). However, the presence of acid and enzymes in the gastrointestinal (GI) tract poses adverse environment for the immunologicals, leading to the destruction of antigen and consequently, inefficient uptake by the gut associated lymphoid tissue (GALT). This, in turn, requires larger and more frequent dosing of the antigen, resulting in systemic non-responsiveness or oral tolerance (Shalaby, 1995). It is a major impediment in the path of successful targeted oral mucosal immunization. Therefore, the M-cell targeted oral mucosal immunization using appropriate ligand anchored vaccine delivery systems has been postulated to be an efficient alternative to the conventional approach of parenteral vaccination (Gupta et al., 2007).

Several experimental approaches have been put forward and used to protect the orally administered antigens from the hostile environment of the gut, including the use of synthetic particulate and live microbial delivery vehicles (Lehr, 1994; Wilding et al., 1994; DiBiase and Morrel, 1997; Brayden and O'Mahony, 1998; Hillery, 1998; McClean et al., 1998; Singh and O'Hagan, 1998). For the development of oral hepatitis B vaccine alone, numerous strategies have been described which include genetic alteration for the production and delivery of the hepatitis B vaccine in edible plants like lettuce (Kapusta et al., 1999, 2001), potato tubers (Kong et al., 2001; Thanavala et al., 2005; Thanavala and Lugade, 2009) and cherry tomatillo (Gao et al., 2003). Some other vital techniques encompass, encapsulation of immunogenic peptide of HBsAg and of the plasmid DNA encoding hepatitis B virus pro-

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tein each in poly(D,L-lactide-co-glycolic acid) (PLGA) (Rajkannan et al., 2006; He et al., 2005). Further, HBsAg encapsulated alginate-coated chitosan nanoparticles (Borges et al., 2007), lectin-anchored biodegradable PLGA nanoparticles (Gupta et al., 2007), and non-conjugated bilosomes (Shukla et al., 2008) have also been employed for oral immunization against hepatitis B.

For its effective delivery, the administered antigen should target to and exhibit high levels of binding to M-cell apical membranes, and subsequently be internalized and transported to the M-cells (Clark et al., 2001a,b). Targeting of synthetic delivery vehicles to M-cell receptors can be achieved by conjugation of the latter to ligands like lectins, microbial adhesions and immunoglobulins (Ermak and Giannasca, 1998; Clark et al., 2001a,b). Polymerized liposomes of approximately 200 nm diameter have been targeted to M-cells by coating with UEA1 (Chen et al., 1996). Cholera toxin (CT) as well as its B subunit (CTB) is a potent mucosal adjuvant, when administered together with an antigen by oral or nasal route (Holmgren et al., 1993). Toxicity of the CT holotoxin in humans limits its usefulness as an adjuvant; the CTB subunit, therefore, offers safer perspective as a constituent of the mucosal vaccines (Bernard et al., 1997). And in this regard, CTB has high affinity for cell surface ganglioside G_{M1} receptor present in the apical membranes of the M-cells. Thus, it has the potential to act as a ligand for M-cell targeting delivery vehicles. Harokopakis et al. (1995) demonstrated that conjugation of recombinant CTB with liposomes greatly enhance their effectiveness as an antigen delivery system following oral administration. Earlier, oral administration of antigens by means of bilosomes have showed successful stimulation of both systemic and mucosal immune responses suggesting bilosomes to be a promising carrier system for oral immunization (Conacher et al., 2001; Mann et al., 2004; Singh et al., 2004; Mann et al., 2006; Shukla et al., 2008; Bennett et al., 2009).

Recently, our group has demonstrated that five times higher (50 μg) entrapped dose of HBsAg is required to produce serum antibody titres against hepatitis B via the oral route using non-conjugated bilosomes as compared to that of the intramuscular immunization (10 μg of HBsAg) (Shukla et al., 2008). In the present work, we have attempted to improve our strategy further by CTB-mediated M-cell receptor-specific targeted active delivery using CTB-conjugated bilosomes vis-à-vis non-targeted passive delivery by preferential uptake of negatively charged non-conjugated bilosomes by GALT, employed in our previous work. The present work has further been extended by including hemagglutination assay to ascertain biological activity of CTB following conjugation to the bilosomal surface. Other objective of this study has been substantial dose reduction to the minimum possible level in order to match with the IM dose, i.e., 10 μg of HBsAg, which would be more economical and also eliminate any chances of systemic non-responsiveness (phenomenon generally observed at larger antigen dose levels).

2. Materials and methods

2.1. Materials

2.1.1. Chemical and immunological reagents

Sorbitan tristearate and cholesterol were procured from M/s Fluka Chemika, Mumbai, 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), triethanolamine, goat anti-mouse IgG/IgA peroxide conjugate, anti-mouse and anti-cholera toxin from rabbit, sodium deoxycholate, FITC-BSA and Sephadex G-100 were purchased from M/s Sigma Chemical Co., St. Louis, MO, USA. Recombinant hepatitis B surface antigen was obtained as a gift sample from M/s Shantha Biotechnics Ltd., Hyderabad, India. AUSZYME® monoclonal kit was obtained from M/s Abbott Laboratories, Chicago, IL, USA. G_{M1} protein was a kind gift from M/s Panacea Biotech, Lalru, Punjab, India. Tetramethyl benzidine (TMB), Hepes buffer, Tris buffer (pH 6.8), L-cysteine buffer and micro-BCA protein estimation kit were purchased from M/s Genei, Bangalore, India. All other chemicals and reagents were of analytical grade and purchased from local suppliers unless otherwise mentioned.

2.2. Methods

2.2.1. Preparation and characterization of CTB-conjugated bilosomes

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

2.2.1.2. Preparation of HBsAg-loaded bilosomes. Sorbitan tristearate, cholesterol and DPPE-MCC were dissolved in a molar ratio of 7:3:1 in 10 ml of chloroform in a round-bottom flask. Solvent was removed under reduced pressure by rotary evaporator to form a thin film on the glass surface. The film was then hydrated with 3.5 ml of phosphate buffer saline (PBS, pH 7.4), containing 100 mg of sodium deoxycholate along with 800 μg (20 μg/dose, CTB1) and 400 μg (10 μg/dose, CTB2) of HBsAg, to produce HBsAg-loaded bilosomes. The entire preparation volume was then made up to 4 ml with PBS. The bilosomes were then sized by extrusion through 200 nm pore membrane (Millipore, USA). The unentrapped HBsAg and sodium deoxycholate were removed by mini-column centrifugation using Sephadex G-100 (Fry et al., 1978). Eluent fractions were periodically collected and challenged with Triton X-100 (0.2%, v/v). The samples were so diluted that they fall within the concentration limits of the standard calibration curve between 10 and 100 μg/ml. HBsAg was then measured using micro-BCA protein estimation kit.

2.2.1.3. Production of CTB-DP. Lysine residues of CTB were modified using amine reactive reagent, SPDP, as described by Harokopakis et al. (1995). CTB was dissolved in Hepes buffer and incubated with SPDP (1:10 molar ratio) for 30 min in dark at room temperature. 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 subsequently with the addition of 5 μl of 7.7 mg/ml dithiothreitol. Size exclusion chromatography column was used to remove byproducts and unreacted substances.

2.2.1.4. Pairing of CTB to bilosomes. In order to conjugate CTB to bilosomal surface, 1 ml of DPPE-MCC bearing bilosomes were incubated with 1 mg of reduced CTB-DP at 4 °C for overnight. This was followed by addition of 10 μl of L-cysteine buffer (pH 6.8), to stop the coupling reaction. Bilosomes were separated from non-conjugated proteins by means of mini-column centrifugation (Sephadex G-100 packed column), and washed thrice with Hepes buffer.

2.2.1.5. Hemagglutination assay. Hemagglutination assay was performed in order to determine the CTB binding efficiency with G_{M1}

ganglioside. Here, a dried film of G_{M1} ganglioside was incubated with erythrocytes with occasional stirring at 37 °C for 10 min, followed by at room temperature for 1 h. Unincorporated G_{M1} was separated by centrifugation at 1000 × *g* (15 min). One hundred microlitres of CTB-conjugated bilosomes were added to the wells of V bottom PVC plates, followed by addition of G_{M1}-enriched erythrocytes (0.25%, v/v). Mixture was incubated for 4 h at 4 °C. Indication of hemagglutination, if any, was evaluated by visual examination.

2.2.1.6. Characterization of CTB-conjugated bilosomes. The morphology of the CTB-conjugated bilosomes was assessed by transmission electron microscopy (TEM) using a transmission electron microscope (Philips CM-10, Netherlands) after negative staining of the samples with 2% (w/v) phosphotungustic acid solution. The particle size was determined by photon correlation spectroscopy using a Zetasizer Nano ZS 90 (Malvern Instruments Co., U.K.).

2.2.2. Stability in simulated fluids and bile salt solutions

Stability of CTB-conjugated HBsAg-loaded bilosomes was determined at room temperature in simulated gastric fluid (SGF USP, pH 1.2), simulated intestinal fluid (SIF USP, pH 7.5) and aqueous solutions of bile salt (5 and 20 mM). A volume of 1.8 ml of different solutions was added to 0.2 ml of bilosomal formulation. Aliquots of samples were withdrawn after 2 h, and free or unentrapped HBsAg was removed by mini-column centrifugation by Sephadex G-100 column (Fry et al., 1978). Unentrapped HBsAg-free bilosomes were challenged with Triton X-100 (0.2%, v/v) and HBsAg was determined using AUSZYME® monoclonal kit.

2.2.3. Fluorescence microscopy

For verification of efficient targeting and localization of CTB-conjugated bilosomes in the GALT, fluorescence microscopy was performed. FITC-BSA was loaded as a fluorescent marker into the CTB-conjugated and non-conjugated bilosomes. The animals were sacrificed, the small intestine removed, cut, and microtomy carried out after 5 h of oral administration of bilosomal formulation (Shalaby, 1995). Sections of around 3 μm thickness were then examined ($n = 3$) under a fluorescence microscope (Nikon Eclipse E 600). Control animals were administered with an equivalent amount of unentrapped FITC-BSA orally, and microtomy was carried out.

2.2.4. Immunization and sample collection

Female BALB/c mice of 6–8 weeks age, weighing 15–20 g were employed for *in vivo* studies. Animals with free access to food and water were housed in groups of five. They were deprived of any food intake for 3 h prior to immunization. The study protocols followed as approved by the Institutional Animals Ethical Committee of Dr. Hari Singh Gour University, Sagar, India. The studies were carried out according to the guidelines of the Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. The mice were immunized by intragastric administration, following the protocol of three primary inoculations for three successive days and boosting after 3 weeks. Mice were divided in four groups comprising of 5 animals each. Group 1 received 0.5 ml of 10 μg dose of CTB-conjugated HBsAg bilosomes (CTB2), group 2 received 0.5 ml of 20 μg dose of CTB-conjugated HBsAg bilosomes (CTB1), and group 3 received 0.5 ml of 10 μg dose of unentrapped HBsAg for three successive days. Booster immunization was carried out after 3 weeks. Group 4, i.e., the control group received 10 μg of alum-adsorbed HBsAg IM on day 0 and a booster dose 3 weeks following primary immunization.

Samples of serum and endogenous secretions, i.e., saliva, vaginal fluid and intestinal lavage were collected from the immunized animals on day 0 before immunization. Blood was drawn periodically from the retro-orbital plexus of mice under light ether anesthesia after 14, 28, 42 and 56 days of booster dosing. Sera were stored at −40 °C until analyzed using ELISA. The intestinal lavage, vaginal and salivary secretions were also collected after 5 weeks of booster immunization. Mice were administered with an intraperitoneal (IP) injection 0.2 ml of pilocarpine (10 mg/ml) and 20 min later, saliva samples was drawn from the buccal cavity using a capillary tube. Intestinal lavage was collected using a 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 regular intervals of 15 min using a blunt tipped feeding needle. Thirty minutes subsequent to the last dose, the mice were administered with 0.2 ml of pilocarpine (10 mg/ml) IP, and the released of intestinal contents were collected for the next 20 min. Vaginal secretions were collected 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 the antibody levels. These fluids were stored with 100 mM phenylmethyl sulfonyl fluoride (PMSF) at −40 °C until analyzed using ELISA for secretory antibody (sIgA) levels.

2.2.5. Measurement of specific IgG and IgA responses

ELISA was used to monitor antibody responses in the immunized animals. The 96-well microtitre plates (Nunc-Immuno Plate® Fb 96 Mexisorp, NUNC) were coated with 100 μl/well of 10 μg/ml HBsAg in PBS (pH 7.4), and incubated at 4 °C for overnight. The plates were then washed thrice with PBS-Tween 20 (0.05%, v/v) (PBST) and blocked with PBS-BSA (3%, w/v) for 2 h at 37 °C, followed by washing with PBST. Serum/body fluids were serially diluted 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 thrice with PBST. A volume of 100 μl of peroxidase-labeled goat anti-mouse IgG/IgA (1:1000 dilution) was added to each well. The plates were covered and washing was repeated after incubation for 1 h at room temperature. One hundred microparticles of tetramethyl benzidine (TMB-H_2O_2) solution was added to each well, followed by addition of 50 μl of H_2SO_4 after 90 min. After 15 min of incubation, the plate was read at 450 nm using an ELISA plate reader (Biorad, USA). End point titres were expressed as the logarithm of the reciprocal of the end dilution, which gave an optical density (OD) at 450 nm.

2.2.6. Statistical analysis

Analysis of antibody titres was performed on logarithmically transformed data and the data were presented along with the magnitudes of standard deviations (S.D.). Student's *t*-test was performed to compare mean values of different groups. Multiple comparisons were made using a one-way analysis of variance (ANOVA) followed by Dunnett's post-ANOVA-test using GraphPad InStat™ software (GraphPad Software, Inc., CA, USA). Statistical significance was considered at a level of <5%.

3. Results

3.1. Preparation and characterization

Hemagglutination assay, performed to determine the biological activity of CTB after conjugation to bilosome surface, exhibited that CTB-conjugated bilosomes exhibited agglutination property, whereas CTB non-conjugated bilosomes did not show the same.

The TEM photomicrographs (Fig. 1) indicate that vesicles were spherical in shape. The mean particle size, as determined by photon correlation spectroscopy using a Malvern Zetasizer, Nano ZS 90 (Malvern Instruments Co., U.K.), was found to be 202 ± 20 nm,

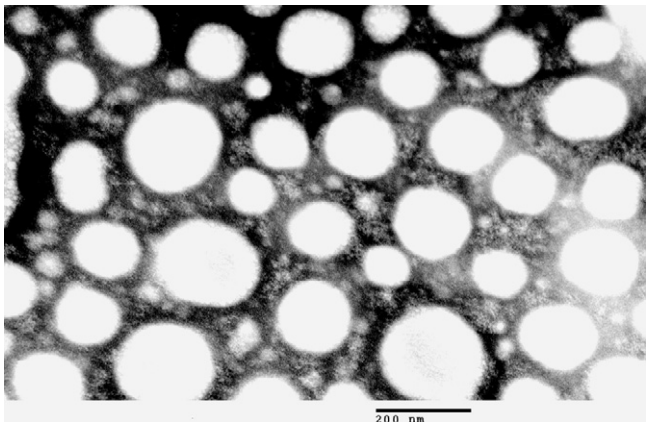

Fig. 1. TEM photomicrograph of CTB-conjugated bilosomes.

representing nano-size range of bilosomes. Percent entrapment of HBsAg in the bilosomes was found to be about 20–24%.

3.2. Stability studies

The stability studies on the formulation indicate that about 86% and 91% of HBsAg were retained in the vesicles in SGF (USP, pH 1.2) and SIF (USP, pH 7.5), respectively. Assessment of stability carried out in the bile salt solutions showed that about 91% and 80% of HBsAg was retained in the vesicles at 5 and 20 mM concentrations of bile salt, respectively.

3.3. Fluorescence microscopy

As shown in Fig. 2C, the localized fluorescence in the GALT region was much higher after the administration of FITC-BSA loaded CTB-conjugated bilosomes, as compared to that of the sections in which unentrapped FITC-BSA (Fig. 2A) and FITC-BSA loaded non-conjugated bilosomes (Fig. 2B) were administered orally. Further, FITC-BSA loaded CTB-conjugated bilosomes showed higher localized fluorescence vis-à-vis that of the non-conjugated bilosomes, ratifying higher levels of binding to M-cell apical membranes of the former, vouching their efficient targeting.

3.4. Immunological studies

Fig. 3 depicts the systemic immune responses graphically. The serum anti-HBsAg titre obtained following oral administration of CTB1 (20 μg dose) was comparable with that of the titres of IM administration of 10 μg of alum-adsorbed HBsAg, i.e., control group. However, the responses obtained following oral administration of CTB1 (20 μg dose) were statistically significantly different ($p < 0.01$), when compared with orally administered CTB2 (10 μg dose) and unentrapped HBsAg (10 μg).

The mucosal immune response measured as IgA titres is graphically shown in Fig. 4. Orally administered CTB-conjugated bilosomal formulations (CTB1 and CTB2) produced significantly higher sIgA responses ($p < 0.05$) in mucosal secretions vis-à-vis the IM injection of 10 μg alum-adsorbed HBsAg, i.e., control group. The IM administered alum-adsorbed HBsAg and orally administered unentrapped HBsAg could not even bring about measurable sIgA in the mucosal secretions.

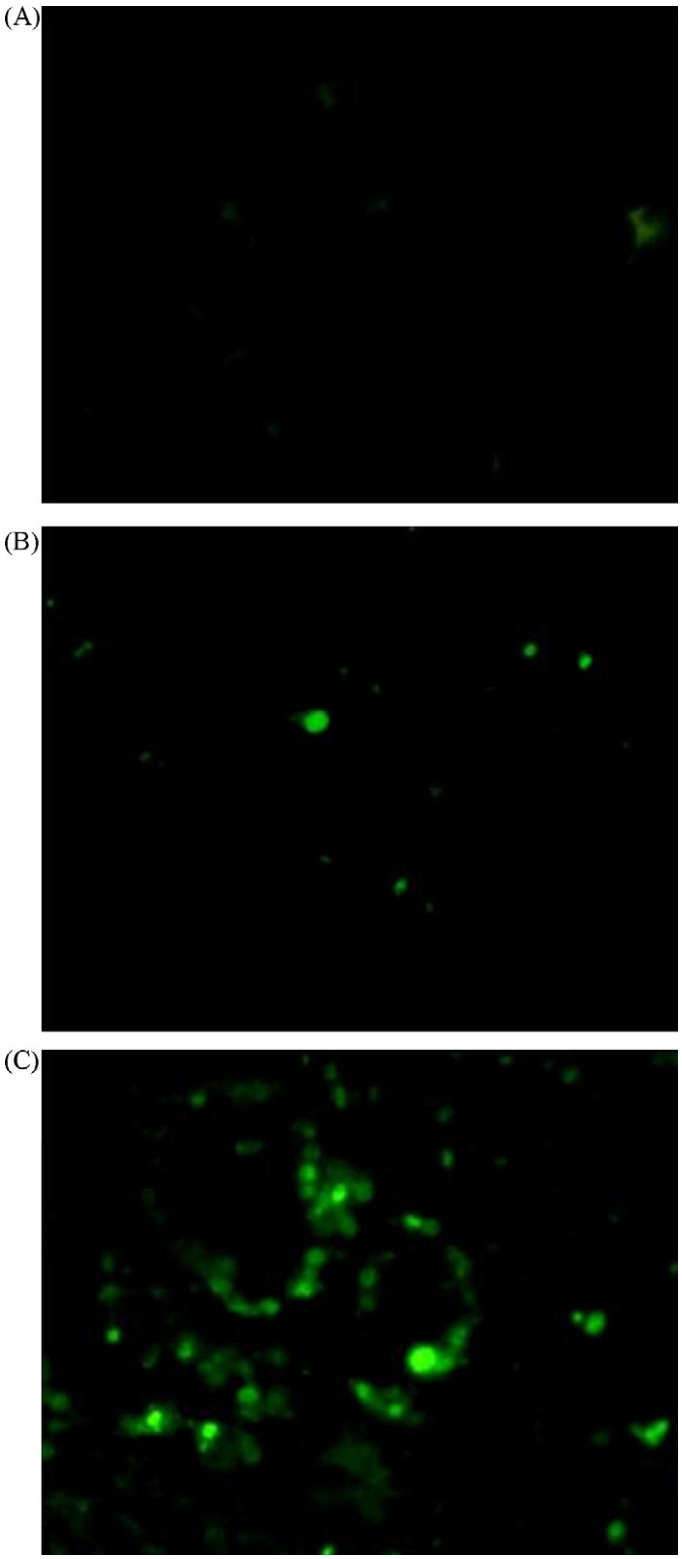

Fig. 2. Fluorescent image of small intestine (GALT) following oral uptake of (A) unentrapped FITC-BSA administered orally, (B) FITC-BSA entrapped in non-conjugated bilosomes administered orally, (C) FITC-BSA entrapped in CTB-conjugated bilosomes administered orally.

4. Discussion

Sorbitan tristearate was used as a key ingredient along with cholesterol and modified DPPE for the preparation of the bilo-

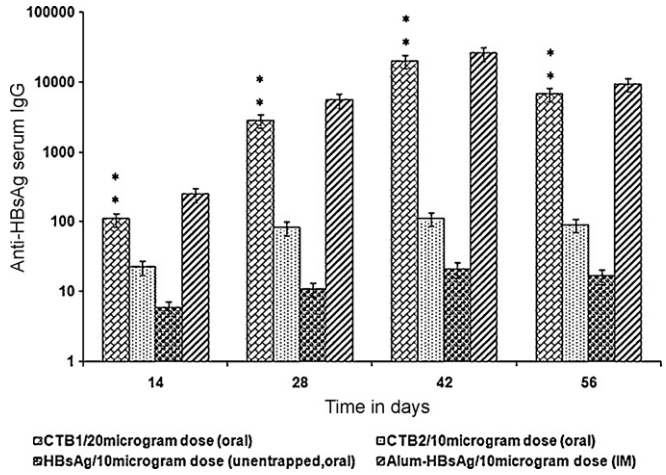

Fig. 3. Mean (±S.D.) of serum anti-HBsAg IgG profile of mice immunized orally with different formulations. The serum was collected after 14, 28, 42 and 56 days of boosting ($n = 5$). Statistical significance was considered at $p < 0.05$.

somes. DPPE was modified using SMCC as a heterobifunctional linker molecule to act as a spacer between CTB and the bilosomal surface. Also, it causes reduction in the steric hindrance and subsequently allows the CTB-conjugated bilosomes to interact with G_{M1} receptor on M-cells. G_{M1} is found in abundance on the luminal surface of all intestinal epithelial cells. However, adherence studies using CTB complexed to colloidal gold, by Owen et al. (1986), demonstrated a preferential association to the surface of M-cells rather than that of adjoining enterocytes. Thus, indicating that G_{M1} ganglioside molecules on M-cells are more accessible or numerous than those on the surface of other absorptive enterocytes. Coupling of the CTB molecules to the bilosomal surface allows them to bind specifically to M-cell membrane receptors. The hemagglutination assay confirmed that CTB retained its binding activity following conjugation to bilosomes. CTB-conjugated bilosomes agglutinated G_{M1}-enriched erythrocytes. The adjuvanticity of CT and its B subunit can be ascribed to their high affinity to G_{M1} ganglioside receptors (Dertzbaugh and Elson, 1993). However, it has been reported by Frey et al. (1996) that CTB-coated particles, with an average diameter of 1 μm or less, tend to readily bind with M-cells. Moreover, Clark et al. (2001a,b) used lectin Ulex europaeus agglutinin 1 (UEA1) to target polymerized liposomes, i.e., Orasomes, which were approximately 200 nm in diameter (oral vaccine delivery vehicles), to mouse M-cells. Therefore, the

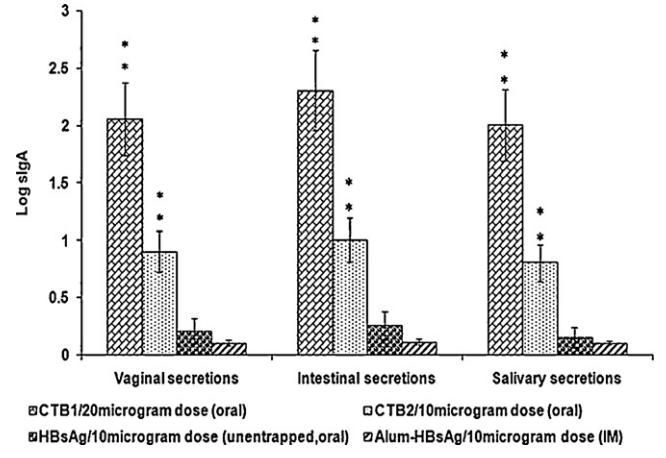

Fig. 4. Mean (±S.D.) secretory IgA level in vaginal, intestinal and salivary secretions of mice immunized orally with different formulations following 5 weeks of boosting ($n = 5$). Alum HBsAg vs. CTB1 ($^{**}p < 0.01$), alum HBsAg vs. CTB2 ($^{**}p < 0.01$).

size of bilosomes was reduced to 200 nm by extrusion. The TEM studies (Fig. 1) demonstrated that the vesicles were spherical in shape. The amount of antigen entrapped in the bilosomes was analogous to that obtained in the other vesicular formulation of HBsAg-entrapped liposomes (Sanchez et al., 1980).

Oral immunization with antigens often leads to weak immune responses probably due to limited adsorption of antigens by the Peyer's patches, a major inductive site for mucosal response in the GI tract (Dertzbaugh and Elson, 1993). Therefore, the protection of vaccine antigens from the hostile GI environment containing acid and enzymes is essential in case of oral delivery of vaccines. Thus, the studies indicated significant capacity of the CTB-conjugated bilosomal formulation to tolerate different bioenvironmental stresses as well as to retain the stability of the antigen.

In the present study, the fluorescence microscopy demonstrated effective CTB-mediated M-cell receptor-specific localization and efficient targeting to the M-cells of the GALT. Comparative assessment of FITC-BSA loaded CTB-conjugated bilosomes with that of unentrapped FITC-BSA and FITC-BSA loaded non-conjugated bilosomes, revealed remarkable differences in fluorescence intensity (Fig. 2).

Peyer's patches are vital for induction of mucosal immune responses. M-cells overlying Peyer's patches take up the gut luminal antigens by endocytosis and transport them to underlying lymphoid cells in the dome region containing functional T, B and antigen presenting cells (McGhee et al., 1992). The present study demonstrated specific and efficient targeting to the M-cells. Orally administered antigens have resulted in enhanced immunogenicity when they are either mixed with CT or conjugated to CTB (Dertzbaugh and Elson, 1993). The CTB-mediated M-cell receptor-specific localization, thus, resulted in significant stimulation of both systemic and mucosal antibody responses after oral administration. Moreover, the CTB-conjugated bilosomes with only twice higher entrapped dose of HBsAg (CTB1, 20 μg/dose, oral) produced comparable anti-HBsAg IgG antibody titre response to that of IM injection (10 μg alum-adsorbed HBsAg). Production of HBsAg-specific mucosal IgA antibodies is essential for protection from mucosally transferred hepatitis B virus (Isaka et al., 2001). In this regard, the much higher levels of sIgA in the CTB-conjugated bilosomal preparations vis-à-vis IM injection (10 μg alum-absorbed HBsAg) vividly demonstrate significantly better mucosal responses in case of the CTB-conjugated bilosomes.

5. Conclusions

In conclusion, the CTB-conjugated bilosomes can be used for efficient M-cell targeted oral immunization against hepatitis B. Also, the formulation of CTB-conjugated bilosomes achieved substantial dose reduction, i.e., from the erstwhile 50 μg to current 20 μg via oral route. Also, only 20 μg of entrapped dose was required using CTB-conjugated bilosomes to produce a comparable systemic antibody response, with an extra advantage of defense at the mucosal level. Moreover, the CTB-conjugated bilosomes employing less dose of 20 μg will not only be more economically viable, but would also eliminate any chances of oral tolerance (i.e., systemic non-responsiveness), a phenomenon usually observed at larger dose levels of the antigen. Thus, improved delivery and target orientation of CTB-conjugated HBsAg-loaded bilosomes can become one of the promising strategies for improving patient compliance and consequently, augmenting immunization potential against hepatitis B viral infection.

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