04-3	**Bioencapsulated antigen induces significant immune response on oral immunization**	

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INTRODUCTION AND OBJECTIVES

Hepatitis B virus (HBV) is transmitted on parenteral as well as on mucosal exposure to hepatitis B surface antigen (HBsAg) positive body fluids generally from HBV infected persons (Hilleman 2003). Presently available hepatitis B vaccine is administered parenterally and induces only systemic immune response. The systemic immune response is unable to provide protection at the level of mucosa, which is the major entry site for most infectious agents including HBV.

Bioencapsulation of recombinant HBsAg and mucosal immunization via the oral route utilizing appropriate delivery systems can be an effective and attractive alternative to the conventional parenteral immunization. Oral administration of antigens induces production of sIgA, not only antigen application site, but also in other external secretions due to the dissemination of antigen-sensitized cells to other tissues (Shalaby 1995). However, the problem of degradation of antigen in the hostile environment of the gut consequently requires larger doses and more frequent dosing of antigen. Furthermore, much larger doses can induce antigen tolerance. Therefore the purpose of the present study was to overcome these problems by the use stabilized nano-vesicles (nano-bilosomes) which could provide both protection to the antigen as well as enable transmucosal uptake and subsequent comprehensive immunization.

MATERIALS AND METHODS

In the present study nano-bilosomes containing recombinant hepatitis B surface antigen were prepared, using sorbitan tristearate, cholesterol and dicetyl phosphate in 7:3:1 molar ratio, by thin film hydration method. HBsAg loaded nano-bilosomes were characterized in vitro for their shape, size, percent antigen entrapment and stability in various simulated fluids and bile salt solutions. Fluorescence microscopy was carried out to verify the uptake of nano-bilosomes by gut associated lymphoid tissues (GALT). The in vivo part of the study comprised estimation of anti-HBsAg IgG response in serum and anti-HBsAg sIgA in various body secretions following oral immunization with low dose loaded bilosomes (B1, 10 µg), intermediate dose loaded bilosomes (B2, 20 µg) and high dose loaded bilosomes (B3, 50 µg) in BALB/c mice.

RESULTS AND DISCUSSION

Characterization

The transmission electron photomicrographs (Figure 1) clearly indicated that vesicles were unilamellar and 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 210±20 nm. The amount of HBsAg entrapped in the bilosomes was found to be around 20-23% of the amount added. The amount of antigen entrapped in the nano-bilosomes was comparable with the data for other vesicular formulation such as liposomes incorporating HBsAg (Sanchez 1980).

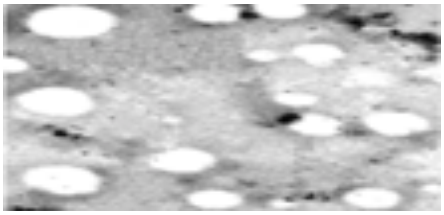

Figure 1 Transmission electron photomicrographs

Stability studies

Stability of the formulations was assessed in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.5). It was found that in around 90% and 95% of HBsAg was retained in the vesicles in SGF and SIF respectively. The formulations were also tested in 5 mM and 20 mM bile salt solutions. It was found that around 95% and 85% of HBsAg was retained in the vesicles concentration at 5mM and 20mM concentration respectively.

The stability determination was carried out in order to assess the ability of the nano-bilosomes to withstand various bioenvironmental stresses as well as to retain the stability of antigen. The studies demonstrated significant stability in simulated fluids as well as in different bile salt concentrations.

Fluorescence microscopy

Fluorescence microscopy revealed that after administration of FITC-BSA loaded nano-bilosomes the localized fluoresce in the GALT region was much higher (Figure 2 B) compared to sections in which unentrapped FITC-BSA was administered orally (Figure 2 A). This indicated effective and efficient uptake of nano-bilosomes by the GALT and therefore it can be concluded that nano-

bilosomes are efficient in transporting vaccines to the Peyer's patches, resulting in both mucosal and systemic immune responses.

Immunological studies

The serum anti-HBsAg titre obtained after oral administration of B3 (i.e. high dose, 50 µg/dose) was comparable with titres obtained after intramuscular administration of 10 µg alum adsorbed HBsAg (control group) ($p>0.05$) but the responses obtained following oral administration of B3 (i.e. high dose, 50 µg/dose) were significant ($p<0.01$) when compared with B1 (i.e. low dose, 10 µg/dose). The systemic immune responses are graphically shown in Figure 3.

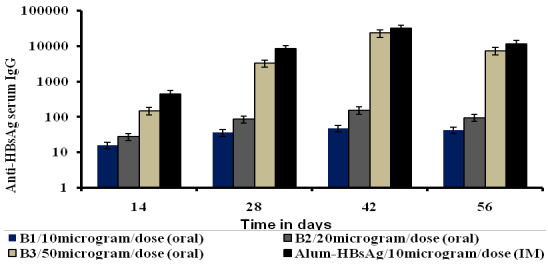

Figure 3. Serum anti-HBsAg IgG profile of mice immunized orally with different formulations. Serum collected after 14, 28, 42 and 56 days of boosting. Values are expressed as mean ± S.D. (*n*=5). Statistical significance was considered at $p<0.05$.

All the orally administered nano-bilosomal formulations produced significant sIgA responses ($p<0.01$) in mucosal secretions when compared with alum adsorbed HBsAg (control group), which was administered intramuscularly. Alum adsorbed formulations did not elicit detectable sIgA in mucosal secretions. The mucosal immune response measured as IgA titres is graphically presented in Figure 4.

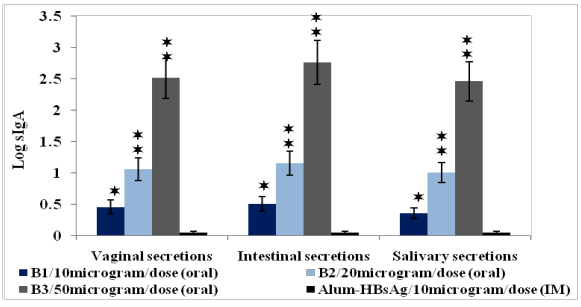

Figure 4. Secretory IgA level in secretions of mice immunized orally after 5 weeks of boosting. Values are expressed as mean ± S.D. (*n*=5). Alum HBsAg vs B1, ($p<0.05$ *), Alum HBsAg vs B2, ($p<0.01$ **), Alum HBsAg vs B3, ($p<0.01$ **)

Oral tolerance induction is a key feature of intestinal immunity (Worbs 2006). However, a fine balance is necessary between the mucosal delivery of antigens and induction of tolerance. This study demonstrated effective and significant stimulation of both systemic and mucosal immune responses after oral administration of HBsAg-loaded nano-bilosomes without the induction of oral tolerance. Furthermore, nano-bilosomes with higher dose of HBsAg (B3, 50 µg/dose, oral) produced comparable anti-HBsAg IgG antibody titre responses vis-à-vis intramuscular injection of 10 µg alum adsorbed HBsAg (control group) in addition to a mucosal antibody response. Production of HBsAg-specific mucosal IgA antibodies is important for protection from mucosally transferred hepatitis B virus and this study successfully demonstrated that all the nano-bilosomal preparations elicited mucosal immune responses, with the highest response elicited in the case of nano-bilosomes with a higher dose of HBsAg (B3, 50 µg/dose, oral). The intramuscular injection of 10 µg of alum absorbed HBsAg (control group) was unable to produce detectable mucosal sIgA antibody response.

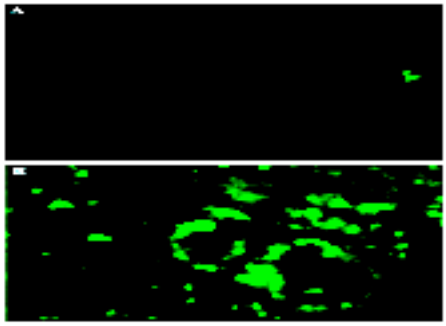

Figure 2: Fluorescent image of small intestine (GALT) after the uptake of orally administered
(A) Unentrapped FITC-BSA (B) FITC-BSA-loaded nano-bilosomes.

CONCLUSIONS

In conclusion, the nano-bilosomes are a promising carrier for bioencapsulation and oral delivery of HBsAg. Thus, HBsAg loaded nano-bilosomes can provide a needle free, painless approach for immunization against hepatitis B, thereby increasing patient compliance.

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