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Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B

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Abstract

DNA vaccines are capable of eliciting both humoral as well as cellular immune responses. Liposomes have been widely employed for DNA delivery through topical route; however, they suffer from certain drawbacks like higher cost and instability. In present study, non-ionic surfactant based vesicles (niosomes) for topical DNA delivery have been developed. DNA encoding hepatitis B surface antigen (HBsAg) was encapsulated in niosomes. Niosomes composed of span 85 and cholesterol as constitutive lipids were prepared by reverse phase evaporation method. Prepared niosomes were characterized for their size, shape and entrapment efficiency. The immune stimulating activity was studied by measuring serum anti-HBsAg titer and cyokines level (IL-2 and IFN-γ) following topical application of niosomes in Balb/c mice and results were compared with naked DNA and liposomes encapsulated DNA applied topically as well as naked DNA and pure recombinant HBsAg administered intramuscularly. It was observed that topical niosomes elicited a comparable serum antibody titer and endogenous cytokines levels as compared to intramuscular recombinant HBsAg and topical liposomes. The study signifies the potential of niosomes as DNA vaccine carriers for effective topical immunization. The proposed system is simple, stable and cost effective compared to liposomes.

Keywords: Topical immunization; DNA vaccines; Hepatitis B; Niosomes; Liposomes

1. Introduction

Hepatitis B virus (HBV) remains an important worldwide health problem. Infection with HBV causes acute as well as chronic necroinflammatory liver disease and many HBV carriers eventually develop serious complications such as liver cirrhosis or hepatocellular carcinoma. There are about 300 million chronic HBV carriers worldwide and these carriers represent a permanent source of HBV infection. The prospects for control of infection and disease depend on the availability of safe, effective and affordable vaccines. Cellular

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and humoral immune responses to HBV antigens are believed to play an essential role in the elimination of virus by the host. It is well-established that the humoral immune response to HBV envelop antigens lead to protection against infection. In contrast, broad based cellular immune response has been shown to be one of the most important factors contributing to virus elimination from infected hepatocytes and may play an important role in the pathogenesis of the severity of hepatitis and the subsequent development of chronic liver disease (Geissler et al., 1995; Chisari and Ferrari, 1995; Davis, 1996).

Current protein based vaccines contain HBV surface antigen (HBsAg) and are safe and generally efficacious. However, they are expensive, do not induce adequate immunity in all individuals and are ineffective for treatment of chronic HBV carriers. Recently, much attention has been focused on DNA vaccines. In addition to their ability to induce both cellular and humoral immune responses, DNA vaccines are generally regarded as being potentially safer, relatively cheap and easy to produce, no special storage requirements because they are extremely stable and allow for potential simultaneous immunization against multiple antigens or pathogens via co-expression of multiple epitopes on single plasmid (Hassett and Whitton, 1996; Tuteja, 1999).

Topical immunization, i.e. non-invasive vaccination onto the skin (NIVS) provides a robust and novel approach of vaccination. Classical vaccination methods that use needles or require multiple dosage using an invasive route suffer from the problems of administration, expenses and patient compliance. The skin is exploited as a route of immunization because it shows specific (immunity) and non-specific (inflammation) response against foreign substances. These responses are the result of presence of immunocompetent cells within the skin layers, e.g. Langerhan's cells (LCs), dendritic cells (DCs) and epidermotropic lymphocytes. Other cells present are mast cells in dermis, resident antigen presenting cells and transient inflammatory lymphocytes. Apart from these epidermal antigen presenting cells and migratory T-lymphocytes, these all cells are collectively known as skin-associated lymphoid tissue (SALT) and constitute the skin immune system (SIS). These cells altogether function in association with lymph nodes and are responsible for generation of both cellular and humoral immune responses (Singh et al., 2002; Gupta et al., 2004).

The feasibility of topical immunization using DNA vaccines was first demonstrated by Tang and co-workers (Tang et al., 1997; Shi et al., 1999). The non-invasive vaccine onto the skin can be achieved by means of suitable carriers such as liposomes, niosomes, virosomes, etc., or application of naked DNA in solution. Topical application of both naked and liposome-entrapped plasmid vector for hepatitis B surface antigen (HBsAg) resulted in antigen specific immune response (Fan et al., 1999).

Vesicular carrier systems liposomes and niosomes have been advocated for topical delivery of bioactives (Schreier and Bouwstra, 1994; Fang et al., 2001; Bouwstra and Honeywell-Nguyen, 2002). The low cost, high purity, content uniformity, greater stability and ease of storage of non-ionic surfactants have presented niosomes as better alternatives to liposomes. These vesicles appear to be similar in terms of their physical properties to liposomes, being prepared in same way and under a variety of conditions forming unilamellar or multilamellar structures (Yoshioka et al., 1994; Vora et al., 1998; Uchegbu and Vyas, 1998).

Aim of the present study was to establish potential of niosomes as topical DNA vaccine carriers. Niosomes encapsulating plasmid DNA encoding for HBsAg were prepared and characterized. The specific immunological response elicited by niosomes was compared with that induced by administration of liposomes and naked plasmid DNA through topical route and intramuscular administration of naked DNA and pure recombinant HBsAg.

2. Materials and methods

2.1. Materials

L-α-Soya phosphatidyl choline (Soya PC), cholesterol and Triton X-100 were purchased from Sigma Chemicals Co. (USA). Span 85 was procured from Fluka, Switzerland. Plasmid pRc/CMV HBS expressing sequence coding for the small proteins of the hepatitis B virus (subtype ayw) was a gift sample from Aldevron (USA) and amplified in *Escherichia coli* DH-5α. Recombinant HBsAg protein was a kind gift from M/s. Panacea Biotech, India. Anti-HBsAg

antibody estimation kit was obtained from Abbott (Abbott Labs, USA). Mouse interleukin (IL-2) and interferon (IFN-γ) estimation kits (ELISA based) used were of Amerasham Life Sciences (UK). All other chemicals used were purchased from local suppliers and of analytical grade unless mentioned.

2.2. Preparation of vesicular carrier systems

Niosomes and liposomes were prepared by reverse phase evaporation method (Szoka and Papahadjopou los, 1978; Kiwada et al., 1985). Soya PC and span 85 were used for preparation of liposomes and niosomes, respectively. Phospholipid/surfactant and cholesterol in a molar ratio 7:3 were dissolved in diethyl ether, followed by emulsification with aqueous solution of plasmid DNA by probe sonication (Soniweld, India) for 5 min at 40 Kc/s. The organic solvent was evaporated in a rotary flash evaporator at 37 °C under reduced pressure (260–400 mm Hg). The lipid gel so formed was collapsed and transformed into a fluid with continual vigorous mechanical agitation using a vortex mixer. Unentrapped plasmid DNA was removed by Ficoll floatation technique (New, 1990). Vesicles were lysed using minimum amount of Triton X-100 (0.5%, w/v) and the liberated DNA was precipitated using absolute ethanol. The pellet of DNA was removed and estimated spectrophotometrically at 260 nm using Shimadzu 1601 double beam UV/Vis spectrophotometer. Vesicle size and size distribution studies were carried out using particle size analyzer (CILAS, 1604, France).

2.3. Agarose gel electrophoresis for in process stability of plasmid DNA

Agarose gel electrophoresis was performed in order to check the integrity of plasmid DNA in the formulations. Reagents of the electrophoresis and gel apparatus used were of Bangalore Genei, India. 0.8% gel of agarose was prepared according to manufacturer's instructions provided with kit. Ethidium bromide was added to gel before solidification. Vesicles (liposomes and niosomes) were disrupted using the minimum amount of Triton X-100 (0.5%, w/v) and the mixture was centrifuged at 6000 rpm for 10 min at 4 °C in a refrigerated centrifuge (Remi India Ltd., Mumbai). The samples of the supernatant and pure plasmid DNA were applied after mixing with gel loading dye (bromophenol blue) and gel was run at 100 mV. After running, gel was illuminated in the ultraviolet light and bands were visualized.

2.4. Immunization experiments

Male Balb/c mice 4–8 weeks old (four mice in each experimental group) were selected for the study. Hairs of the mice were shaved and skin was wiped with an alcohol swab and allowed to air dry. Aqueous solution of DNA (in PBS, pH 7.4), niosomes and liposomes in a dose equivalent to 100 μg plasmid DNA per animal were applied topically on day 1st and 14th with gentle rubbing. Mice were also immunized by single intramuscular injection of naked plasmid DNA as well as pure recombinant HBsAg for comparison. Blood samples were collected from retro orbital plexus of mice at weekly interval for 8 weeks and sera were kept at −40 °C until they were tested by ELISA for anti-HBsAg. Another groups of animals were sacrificed after 4 weeks of first application and spleens were removed for the determination of endogenous levels of IFN-γ and IL-2.

2.5. Analysis of humoral and cellular immune responses

Specific antibodies to HBsAg were analyzed by commercially available HBsAg ELISA (Abott Labs, USA). Titer values were expressed in milli international units per ml (mIU/ml). Endogenous levels of IL-2 and IFN-γ in mouse spleen homogenates were determined with two separate ELISA kits for these cytokines following the instructions given by the manufacturer. Spleen homogenates were prepared by the method reported by Nakane et al. (1992) with slight modifications. Briefly, spleens were weighed and homogenized in ice-cold PBS containing 1% CHAPS (Sigma, USA) and 10% (w/v) homogenates were prepared using tissue homogenizer (York, India). Homogenates were incubated on ice-bath for 1–2 h at temperature less than 0 °C so that insoluble matters settled down. Supernatant was centrifuged at 2000 × *g* for 20 min and the clear supernatant was used for cytokines estimation by ELISA.

2.6. Statistical analysis

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 considered at $P < 0.05$. For the comparative analysis of niosomes and liposomes with respect to end point titer as well as cytokines levels; Kruskal–Wallis test was performed and differences were considered statistical significant at $P < 0.05$.

3. Results and discussion

In this study, non-ionic surfactant based vesicles (niosomes) were developed for efficient delivery of plasmid DNA encoding small subunit proteins of Hepatitis B virus through topical route. Vesicular constructs (liposomes and niosomes) were prepared using reverse phase evaporation technique. The method is reported to encapsulate large hydrophilic macromolecules with relatively high entrapment efficiency (Szoka and Papahadjopoulos, 1978). Percent entrapment efficiency obtained was $48.7 \pm 3.2\%$ for liposomes, while it was $45.4 \pm 2.8\%$ for niosomes. Mean vesicle size of liposomes and niosomes was found to be 2.8 ± 0.17 and 2.3 ± 0.15 μm, respectively.

In-process stability of the encapsulated plasmid DNA was assessed using agarose gel electrophoresis. Agarose gel electrophoresis is widely used for analysis and characterization of DNA samples. The gel was run with spots of pure plasmid DNA and DNA extracted from vesicular formulations and only a single visible band for pure as well as extracted DNA was observed in the gel (Fig. 1). This reveals that the preparation conditions did not cause any degradation of DNA.

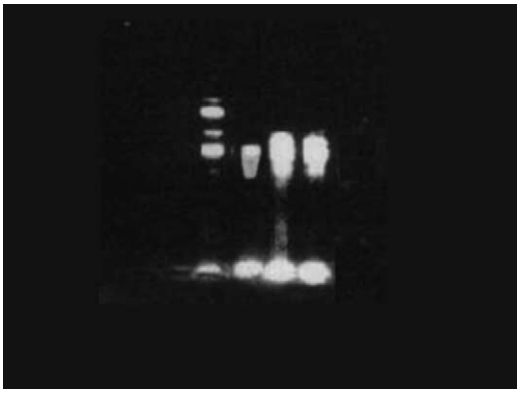

Fig. 1. Photograph of agarose gel electrophoresis of DNA formulations. Lane 1: marker; lane 2: native plasmid DNA; lane 3: DNA extracted from liposomal formulation; lane 4: DNA extracted from niosomal formulation.

3.1. Immunological response after topical immunization

The cellular and humoral response obtained after administration of DNA by its de novo expression was measured using specific ELISA techniques. The specific antibody titer (anti-HBsAg) in serum obtained after topical application of niosomes, liposomes, naked plasmid DNA and with the intramuscular HBsAg are shown in Fig. 2. Results showed that intramuscular injection of recombinant HBsAg produced initial higher response as compared to DNA vaccine groups but the titer value could not be sustained for longer time period and after achieving the maximum value after 4 weeks, it started declining. In contrast, naked plasmid DNA administered intramuscularly showed highest and well-sustained titer value than intramuscular recombinant HBsAg and topically administered vesicular formulations after an initial time lag. DNA vaccines produce the encoded antigen in vivo but it requires transfection and translation steps before protein (antigen) synthesis. This may be accounted for the time delay for the initiation of humoral response but DNA once taken up by myocytes synthesizes the required antigen for longer duration and leads to production of better humoral response as compared to pure antigen (Davis, 1996).

It was observed from the results that although topical immunization using vesicular formulations exhibited satisfactory anti-HBsAg titer but the response was lower when compared with intramuscular injection of naked DNA. However, encapsulation of plasmid DNA within vesicular carriers (liposomes and niosomes) resulted in better immunological response in comparison to topically applied naked plasmid DNA as a result of enhanced permeation of encapsulated plasmid across the intact skin. Results showed that a clinically protective levels of antibodies (>10 mIU/ml) were detected in serum of mice immunized with topical liposomes and niosomes after 2 weeks and the titer value was comparable to that elicited by intramuscular injection of pure HBsAg after 6 weeks (no statistical significant difference $P > 0.05$; Kruskal–Wallis test).

Several mechanisms have been proposed for topical delivery of bioactives via liposomes or niosomes.

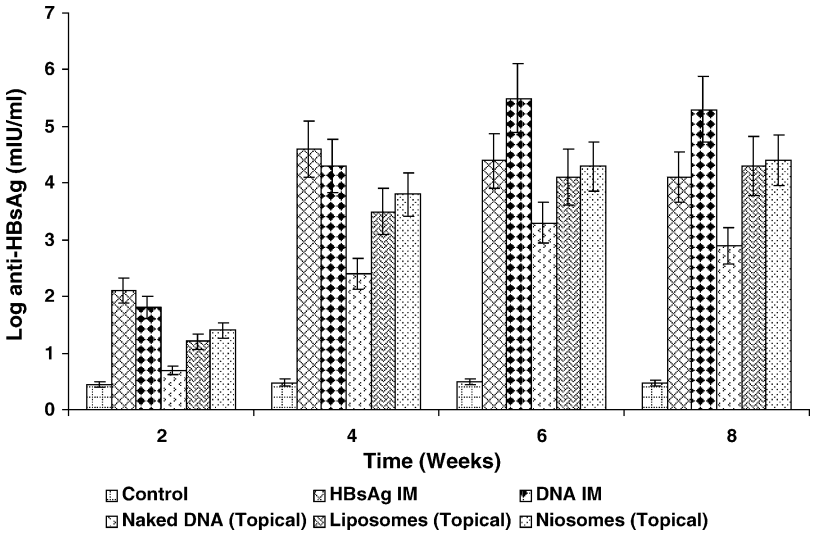

Fig. 2. Serum anti-HBsAg profile of mice immunized with different formulations.

Vesicle adsorption and fusion onto the surface of skin leads to a high thermodynamic activity gradient of bioactive-stratum corneum interface (Schreier and Bouwstra, 1994). Vesicles (liposomes and niosomes) are also reported to follow pilosebaceous route for entry of biomolecules including, interferon, monoclonal antibodies and DNA thus bypassing stratum corneum barrier (Touitou et al., 1994; Weiner, 1998). The results are in accordance with the previous findings (Fan et al., 1999; Hoffman, 2000) who suggested follicular transport of DNA and the hair follicles to be a promising target for gene therapy. Thus, the enhanced transport of plasmid DNA across skin leads to better presentation to APCs infiltrating at site (LCs, DCs and those of SALT). APCs contain both MHC-I and MHC-II molecules leading to processing and presentation of antigen via both endocytic and cytosolic pathway leading to elicit both humoral and cellular response. The uptake of naked DNA by immunologically relevant cells is reported to be minimal and involves only a minor fraction of these cells. Naked DNA is also susceptible to hydrolysis by deoxyribonuclease enzymes present in interstitial space (Perrie and Gregoriadis, 2000). The vesicles in addition to their inherent ability to be better taken up by the APCs also protect DNA from degradation by deoxyribonuclease attack. Moreover, vesicles could also act as rate-limiting membrane barrier and serve as a local depot for the sustained release of encapsulated plasmid (Schreier and Bouwstra, 1994). These might be the possible reason for a well-sustained titer value using the vesicular carriers.

Niosomes were found to be slightly better carrier system for topical DNA delivery compared to liposomes. Results are in accordance with the findings of Fang et al. (2001), who studied skin permeation and partitioning of a fluorinated quinolone antibacterial agent, enoxacin in liposomes and niosomes after topical application and elucidated better skin permeability of drug through niosomal formulations. Surfactants in formulations serve as penetration enhancer by raising the fluidity and reducing the barrier property of stratum corneum (Sarpotdar and Zatz, 1986; Valjakka-Koskela et al., 1998). Moreover, the principle constitutive lipid of niosomes (span 85) is composed of unsaturated fatty acids (trioleate chains) and the presence of unsaturated fatty acids in the lipids facilitated the skin permeation of bioactives due to change in fluidity of stratum corneum lipid structure caused by the packing nature of unsaturated fatty acids (Valjakka-Koskela et al., 1998; Valenta et al., 2000).

Endogenous cytokine levels (IL-2 and IFN-γ) were estimated in spleen homogenate after 4 weeks following administration of different formulations (Figs. 3 and 4). A significantly higher level of both IL-2 and IFN-γ ($P < 0.05$) was observed in mice immunized with DNA vaccine than those of control and

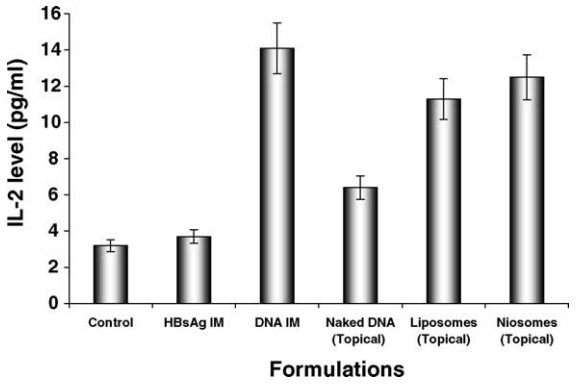

Fig. 3. Interleukin-2 level in spleen homogenate of mice immunized with different formulations after 4 weeks.

protein vaccine group. Furthermore, the levels of both cytokines in the mice immunized with DNA vaccine formulated in topical liposomes and niosomes was comparable to those of intramuscular DNA (no statistical significant difference, $P > 0.05$; Kruskal–Wallis test) and significantly higher than the topical application of naked DNA. IFN-γ production is a well-known property of the cells after antigenic stimulation. Both IL-2 and IFN-γ are Th1-dependent cytokines and their higher levels are evidenced for the strong cell mediated immune response that is equally important to eliminate virus from infected cells (Constant and Bottomly, 1997). It is clear from the results that although conventional recombinant protein based vaccine elicited higher humoral response (antibody production), it fails to elicit cell-mediated immune response, thus ineffective for treatment of chronic hepatitis B infection. The results are in accordance with findings of Leclere et al. (1997) and Geissler et al. (1997). When mice are immunized with recombinant HBsAg, the main type of immune response generated is the antibody response, since the exogenous antigen is mainly presented by B cells through the MHC class II pathway to Th2 cells. In the case of DNA vaccines, part of the HBsAg generated in vivo are secreted and presented by B cells through the MHC class II pathway leading to a good antibody response, while part of the antigen is cleaved within the antigen presenting cells and presented through MHC class I pathway, leading to a Th1/CTL response (Woo et al., 2001). The generation of a dominant Th1 cytokine profile is important to facilitate eradication of HBV infection (Geissler et al., 1995; Chisari and Ferrari, 1995; Davis, 1996). Thus, the skin is proved to be an attractive site for the effective expression of antigen followed by production of both antibody titer and Th1 cytokines.

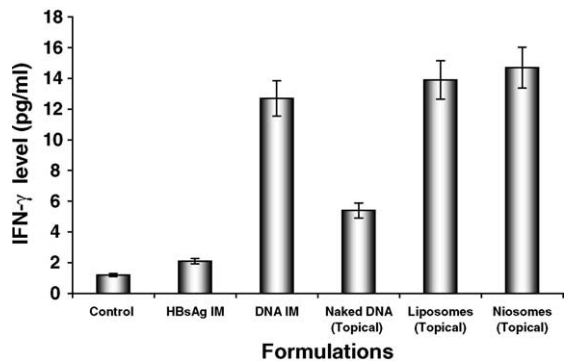

Fig. 4. Interferon-γ level in spleen homogenate of mice immunized with different formulations after 4 weeks.

4. Conclusion

DNA encapsulated niosomes prepared in present study were capable of inducing strong humoral and cellular immune response after topical application. Results are comparable to topical liposomes. Topical immunization using niosomes based DNA vaccine delivery offers all the advantages of DNA vaccines, and in addition overcome the disadvantages of classical invasive methods of vaccination and high cost as well as stability problems of liposomes. The proposed system is simple, economical, stable, painless and potentially safe. This may be able to boost vaccine coverage worldwide.

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