Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 340 (2007) 13–19
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Cationic transfersomes based topical genetic vaccine against hepatitis B

Sunil Mahor, Amit Rawat, Praveen K. Dubey, Prem N. Gupta, Kapil Khatri, Amit K. Goyal, S.P. Vyas *

Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences, Dr. Harisingh Gour Vishwavidyalaya, Sagar, MP 470003, India

Received 28 March 2006; received in revised form 2 March 2007; accepted 6 March 2007
Available online 12 March 2007

Abstract

DNA vaccines have been shown to elicit both cellular and humoral immune responses and to be effective in a variety of preclinical bacterial, viral, and parasitic animal models. We have recently described a needle-free method of vaccination, transcutaneous immunization, based on topical application of vaccine antigens on intact skin using a novel carrier system, namely transfersomes. In the present study, a novel modified version of transfersomes, i.e., cationic transfersomes for topical DNA vaccine delivery has been developed. Cationic transfersomes composed of cationic lipid DOTMA and sodium deoxycholate as constitutive lipids were prepared and optimized for their size, shape, zeta potentials, deformability and loading efficiency. Plasmid DNA encoding hepatitis B surface antigen (HBsAg) was loaded in the cationic transfersomes using charge neutralization method. The immune stimulating activity was studied by measuring serum anti-HBsAg titer and cytokines level (IL-2 and IFN-γ) following topical applications of plasmid DNA loaded cationic transfersomes in Balb/c mice and results were compared with naked DNA applied topically as well as naked DNA and pure recombinant HBsAg administered intramuscularly. Results revealed that DNA loaded cationic transfersomes elicited significantly ($^*P < 0.05$) higher anti-HBsAg antibody titer and cytokines level as compared to naked DNA. It was also observed that topical application of DNA loaded cationic transfersomes elicited a comparable serum antibody titer and endogenous cytokines levels as produced after intramuscular recombinant HBsAg administration. The study signifies the potential of cationic transfersomes as DNA vaccine carriers for effective topical immunization.

Keywords: DNA vaccine; Non-invasive immunization; Cationic transfersomes; Topical vaccine; Hepatitis B

1. Introduction

Hepatitis B virus infection is a major global health concern and is the most common cause of chronic liver disease. 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. Currently, there are several vaccines commercially available such as Engerix-B (yeast recombinant HBsAg) Recombivax or plasma derived vaccines with very good immunogenicity and tolerability that have contributed significantly on the reduction of the disease world-wide (Rich et al., 2003).

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. DNA immunization is a vaccination strategy that involves the direct introduction of plasmid DNA encoding pathogen or tumor-derived antigens into the host, with the benefit that both MHC Class I and Class II mediated responses are induced (Donnelly et al., 2005). DNA vaccines are potentially safer, relatively cheap, easy to produce and 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).

Non-invasive topical immunization via the skin may allow for the vaccination by individuals without any trained medical

* Corresponding author. Tel.: +91 7582 265525; fax: +91 7582 265525.
E-mail address: vyas_sp@rediffmail.com (S.P. Vyas).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.03.006

personnel and make wide spread vaccination more cost effective and feasible. Rapid and significant advances demonstrate that topical immunization may be a viable strategy for both prophylactic and therapeutic vaccines. Skin is an active immune surveillance site and is rich in very potent antigen presenting dendritic cells (DCs) such as Langerhan's cells (LCs) in the epidermis and the immature dendritic cells in the dermis (Banchereau and Steinman, 1998). 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 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 non-invasive vaccination through the skin can be affected by means of suitable carriers such as liposomes, niosomes, virosomes, etc. or application of naked DNA in solution (Mishra et al., 2006). Topical application of both naked and niosomes entrapped plasmid vector for hepatitis B surface antigen (HBsAg) resulted in antigen specific immune response (Vyas et al., 2005). A formulation known as transfersomes, however have characteristics that allow them to penetrate intact skin if applied topically in a non-occlusive manner (Cevc et al., 1996). High deformable and flexible characteristics of transfersomes allow them to deliver biomolecule(s) across skin barrier (Cevc et al., 1995). Sodium deoxycholate, an edge activator is responsible for rendering the structural flexibility to the transfersomes. With the sodium deoxycholate, cationic lipids can also be used for preparing a novel vesicular construct that is capable of penetrating intact skin. Moreover, the DOTMA being positively charged, tend them to form a composite complexation with DNA (anionic).

Our group have previously developed and reported on the application of transfersomes for topical immunization (Gupta et al., 2005a). A comparative study of different vesicular carriers, i.e., transfersomes, niosomes, liposomes was studied and established for topical immunization (Gupta et al., 2005b). Aim of the present study was to establish potential of cationic transfersomes as topical DNA vaccine carriers. Plasmid DNA encoding for HBsAg antigen loaded cationic transfersomes were prepared and characterized. The specific immunological response elicited by transfersomes was compared with that induced by administration of liposomes and naked plasmid DNA on topical or intramuscular administration of naked DNA and pure recombinant HBsAg.

2. Materials and methods

2.1. Materials

N-[1-(2,3-dioleyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), sodium deoxycholate (SDC) and Triton X-100 were purchased from Sigma Chemicals Co. (U.S.A.). Plasmid pRc/CMV HBS expressing sequence coding for the small proteins of the hepatitis B virus (subtype ayw) was a kind gift from Aldevron (Fargo, USA) and amplified in *E. coli* DH-5α. Recombinant HBsAg was a kind gift from M/s, Panacea Biotech. India. Anti-HBsAg antibody estimation kit was obtained from 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 cationic transfersomes

Cationic transfersomes were prepared by the method as described by Gupta et al. (2005a,b) with slight modifications. Cationic lipid DOTMA was mixed with PBS to make a concentration of 10 mg/ml (w/v). Then sodium deoxycholate was added to the mixture at different Cationic lipid to SDC ratio (95:5; 90:10; 85:15; 80:20 and 75:25%, w/w). Then the mixture was vortexed and sonicated in a bath type sonicator (Branson Ultrasonic Co., USA) for 1 h at room temperature. Solutions were then extruded through a 100 nm polycarbonate filters (Nucleopore, The Netherlands).

2.3. Plasmid DNA loading

Plasmid DNA was adsorbed and formed charge induced complex on the surface of the cationic transfersomes by gently mixing 1 ml of the preformed cationic transfersomes in a suspension containing pDNA to obtain a final pDNA concentration of 40–400 μg/ml. This system was kept for 30 min at room temperature to allow complete adsorption of negatively charged pDNA on to the surface of the cationic transfersomes.

2.4. Transmission electron microscopy

Prepared cationic transfersomes were characterized for their vesicle shape using transmission electron microscopy (JEM-200 CX, JEOL, Tokyo, Japan). Phosphotungustic acid 1% (PTA) was used as a negative stain for the transmission electron microscopy.

2.5. Particle size and Zeta potential of plain and DNA loaded cationic transfersomes

Average vesicle size, size distribution and zeta potentials of different plain and DNA loaded cationic transfersomes formulations were measured by photon correlation spectroscopy using Malvern zetasizer 3000HS (Malvern Instruments Co., UK) and shown in Table 1.

2.6. Plasmid loading efficiency

Plasmid loaded cationic transfersomes were taken and separated from the free (uncomplexed) DNA by using a Sephadex G-150 minicolumn and centrifugation technique (Fry et al., 1978). The method was repeated thrice with a fresh syringe packed with gel each time. The fraction that was finally collected was free from unassociated DNA. The vesicular fraction was added with a minimum amount of Triton X-100 (0.5%,

Table 1
Physicochemical properties of various plain (without DNA loaded) and DNA loaded transfersomal formulations

Formulations	DOTMA:SDC ratio (%, w/w)	Plain (without DNA) cationic transfersomes		DNA loaded cationic transfersomes			
		Avg. particle size (nm)	Zeta potential (mV)	Avg. particle size (nm)	Zeta potential (mV)	% Loading efficiency	Deformability index
CT1	95:5	91.54 ± 6.39	58.35 ± 4.52	198.23 ± 14.45	8.26 ± 0.73	88.62 ± 5.17	76.82 ± 4.81
CT2	90:10	88.39 ± 6.11	54.72 ± 4.16	172.97 ± 12.25	2.39 ± 0.26	85.45 ± 4.81	94.67 ± 6.34
CT3	**85:15**	**85.64 ± 5.93**	**49.61 ± 3.97**	**157.52 ± 10.83**	**−1.88 ± 0.21**	**81.39 ± 4.68**	**114.53 ± 7.67**
CT4	80:20	87.81 ± 6.27	21.16 ± 1.56	168.61 ± 11.89	−1.26 ± 0.15	75.28 ± 4.43	108.95 ± 7.06
CT5	75:25	90.08 ± 6.34	17.53 ± 1.33	185.18 ± 12.51	−1.43 ± 0.13	69.08 ± 3.59	101.44 ± 6.24

All values are expressed as mean ± S.D. ($N = 3$). DOTMA, *N*-[1-(2,3-dioleyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium chloride; SDC, sodium deoxycholate.
Bold values indicate the optimum formulations and its characterization parameters.

w/v) to disrupt the vesicles. The liberated DNA was estimated spectrophotometrically at 260 nm using Shimadzu 1601 double beam UV–vis spectrophotometer and percent loading efficiency was determined (Table 1).

2.7. Deformability index measurements

Elasticity of the bilayers of DNA loaded cationic transfersomes was evaluated by extrusion measurements (Bergh et al., 2001). Briefly, the vesicles were extruded through polycarbonate membrane filter with a pore size of 50 nm (Nucleopore, The Netherlands) at constant pressure (1.2 Mpa, 10 min). The elasticity of vesicle was expressed in terms of deformability index which is proportional to $j(r_v/r_p)^2$ where, j is the weight of suspension, which was extruded in 10 min through a polycarbonate filter of 50 nm pore size, r_v the size of vesicle and r_p the pore size of membrane.

2.8. Long-term storage stability of cationic transfersomes

Two sets of optimized cationic transfersomes (CT-3) were stored at two different temperatures, i.e., refrigerated temperature (4 ± 1 °C) and room temperature (28 ± 1 °C) for 1-month period. The effect of storage on the vesicle size and percent residual vesicles number was measured. The percent residual vesicle number was defined as the number of vesicle remained intact after storage in each mm^3 counted by optical microscopy using a hemocytometer (Feinoptik, Blakenburg, Germany).

2.9. Agarose gel electrophoresis

Agarose gel electrophoresis was performed to check the integrity of plasmid DNA in the formulations. Reagents of the electrophoresis and gel apparatus used were from 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. Vesicular preparations 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, the gel was illuminated in the ultraviolet light and bands were visualized.

2.10. MTT assay

For the cytotoxicity assay, the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed (Mosmann, 1983). MTT was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue. Briefly, HepG2 cells were seeded at a density of 1×10^4 cells/well in a 96-well plate flat bottom tissue culture Plate 24 h prior to assay. After 4 h of incubation at 37 °C under 5% CO_2 with different DNA loaded cationic transfersomes, 20 μl of MTT solution was added to each well and incubated for 3 h. The unreduced MTT and medium were then discarded. Each well was washed with 100 μl of PBS and added with 20 μl of PBS and 180 μl of DMSO to dissolve the MTT formazan crystals. Plates were shaken 20 min and absorbance was read at 540 nm using a microplate reader (Biorad-680, USA).

2.11. Immunization studies

Four to eight weeks old female Balb/c mice were selected for the immunization studies. Animals were housed in different groups ($N = 6$ in each experimental group) with free access to food and water. 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. 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), cationic transfersomes in equivalent dose of 100 μg plasmid DNA per animals was applied topically on day 1st and 14th with gentle rubbing. Mice were also immunized with intramuscular injection of naked plasmid DNA (100 μg equivalent dose in 100 μl PBS) as well as pure recombinant HBsAg (10 μg per animal) in quadriceps muscles on day 1st and 14th with same formulations for comparison purpose. Each topical formulation was dripped and subsequently spread with a pipette tip onto the skin covering an area of about 2 cm^2. Care was taken to disperse the solution over the skin without applying pressure on the skin. 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 assayed by ELISA for anti-HBsAg antibodies. Another group 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.

2.12. Analysis of humoral and cellular immune responses

HBsAg specific antibodies were analyzed by commercially available ELISA kit (AUSAB, Abott Laboratories 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 were settled down. Supernatant was centrifuged at 2000 × *g* for 20 min and the clear supernatant was used for cytokines estimation by ELISA.

2.13. 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 IgG 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

The novel cationic transfersomes based system investigated in this study was a modified version of previously reported transfersomes for topical immunization. These cationic transfersomes were prepared by using a cationic lipid, i.e., DOTMA instead of the egg PC with sodium deoxycholate (normally as used in transfersomes preparation). The prepared cationic transfersomes were optimized for lipid to sodium deoxycholate (SDC) ratio with respect to their size loading efficiency and deformability index (Table 1). Cationic transfersomes were prepared at variable DOTMA:SDC ratio (%, w/w) and various properties were determined including size (with or without plasmid DNA), plasmid loading efficiency, zeta potential and deformability index. At optimum ratio (85:15%, w/w), the cationic transfersomes were observed to be of smallest in size with highest deformability index value. High loading efficiency was also achieved at this ratio. The optimum lipid:SPC ratio was found in accordance of previous findings of Hofer et al. (2000) and Cevc et al. (1998) as they reported the optimum lipid:SPC ratio as approximately 6:1. The vesicles observed under an electron microscope revealed that these cationic vesicles appeared as unilamellar vesicles (Fig. 1).

To cross the intact mammalian skin, transfersomes should be capable of passing through pores less than 50 nm in diameter under the influence of a suitable transdermal gradient (Cevc et al., 1995). Lipid to surfactant ratio is the only key factor for determining the flexibility of the bilayers of transfersomes (Hofer et al., 2000). Increasing the concentration of membrane softening component beyond a certain level or even to the point of bilayer solubilization does not brings an advantage in terms of transcutaneous transpore efficiency.

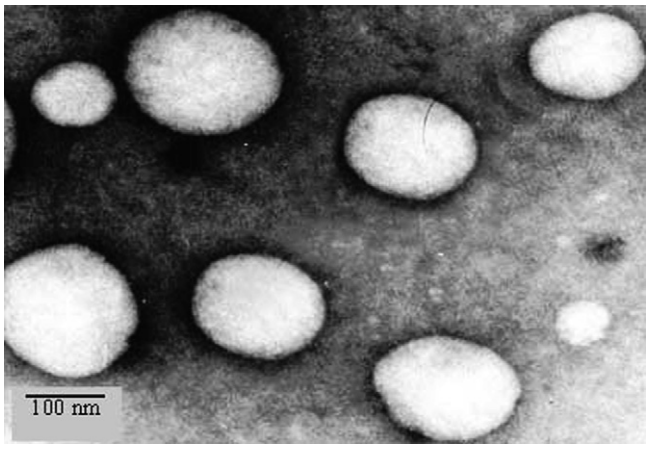

Fig. 1. Transmission electron microscopic image of DNA loaded cationic transfersomes.

Zeta-potentials of vesicular systems indirectly reflect vesicle surface net charge and can therefore be used as a determinant to evaluate the extent of interaction of the liposomal surface cationic charges with the anionic charges of DNA. A photon correlation spectrophotometric study was simultaneously used to determine the size and vesicle surface associated net charge of cationic transfersomes in the presence or absence of plasmid DNA. The particle size and zeta potential of plain cationic transfersomes and plasmid DNA loaded cationic transfersomes were plotted as shown in Table 1. Obviously as the amount of sodium deoxycholate was increased the zeta potential values decreased, however the particle sizes were measured to be approximately 80–90 nm for all the different ratios of DOTMA/sodium deoxycholate (Table 1). A similar trend was observed in size and zeta potential values for plasmid DNA loaded cationic transfersomes. Addition of plasmid DNA increased the size of the complex with relatively low zeta potentials values. The negative charge of plasmid DNA is largely responsible for the reduction in zeta potentials (Table 1).

Hofer et al. (2000) reported the mean size of transfersomes of 100 nm and this size is fairly good size for transfection however, the particle size of the novel cationic transfersomes prepared in this study was yet smaller, i.e., approximately 85 nm. As it is already shown in Table 1, the ratio of DOTMA to sodium deoxycholate used for producing the smallest particle size was 6:1 whether they were complexed with plasmid DNA or not. The zeta potentials as expected were decreased, on increasing the amount of sodium deoxycholate.

The topical carrier system should be deformable so that it can pass easily through the minute pores of the epidermis. Prepared formulations were subjected to a deformability study by extrusion measurement. The results were expressed in terms of deformability index (Table 1). Deformability was found to increase with increasing concentration of sodium deoxycholate. It was highest (114.53 ± 7.67) at DOTMA:SDC ratio

of 85:15. Further increases in the concentration of SDC resulted in decrease of deformability. The optimum content of surfactant resulted in the maximum elasticity of the transfersomal bilayers. Although vesicles were smaller in size, the amount of extruded material through 50-nm pores increased when sodium deoxycholate content was increased from 5 to 15% (w/w). Further increase in sodium deoxycholate content however did not increase the amount of extruded material through 50-nm pores, consequently there was a slight reduction in deformability. The formulation with the smallest vesicle size and maximum deformability was CT3 and selected for further studies.

Lipid-to-surfactant ratio also affects the percent DNA loading efficiency hence it was optimized by preparing formulations with different lipid-to-surfactant ratio. The optimum formulation that demonstrated good entrapment and a good elasticity value was selected. As the concentration of SDC increases the DNA loading efficiency reduces due to reduction in the amount of cationic lipid. The percent loading efficiency of optimized formulation prepared using 85:15 lipids:SDC ratio (CT3) was recorded to be 81.39 ± 4.68 (Table 1). However, increasing the concentration of sodium deoxycholate beyond this ratio led to the reduction of cationic charge of the system, thus resulting into a decreased loading efficiency.

Storage stability of prepared cationic vesicular novel carriers was also determined. The particle diameter and percent residual vesicles number of prepared optimized formulation (CT-3) were monitored over a 1-month period at refrigerated temperature ($4 \pm 1\,^{\circ}C$) and room temperature ($28 \pm 1\,^{\circ}C$). At refrigerated condition, the mean particle size was fairly stable until day 30 whereas at room temperature ($28 \pm 1\,^{\circ}C$), the mean particle size was relatively more affected (Fig. 2). The percent residual vesicles number was higher at refrigerated condition than the room temperature storage condition (Fig. 2). These results indicate that the cationic transfersomes are comparatively more stable under refrigerated condition.

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. A single visible band for pure as well as extracted DNA was observed in the gel (Fig. 3). This reveals that the preparative conditions did not affect the stability and structural integrity of plasmid DNA.

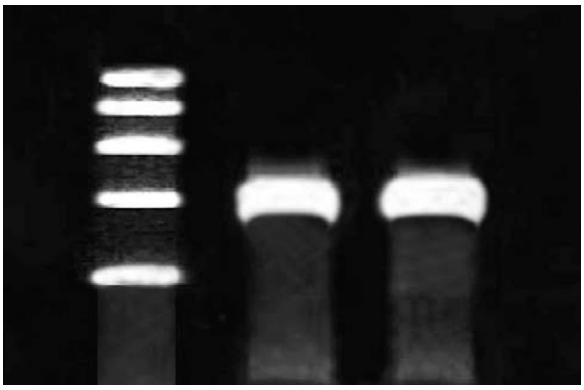

Fig. 3. Assessment of the structural integrity of DNA extracted from plasmid loaded cationic transfersomes using agarose gel electrophoresis Lane 1: marker; Lane 2: pure plasmid DNA; Lane 3: DNA extracted from plasmid loaded cationic transfersomes.

The effect of various DNA loaded cationic transfersomes on the viability of HepG2 cell line was determined using MTT assay. The viability test revealed that the non-significant difference in the cell viabilities was observed with each formulation. As shown in Fig. 4, DNA loaded cationic transfersomes were non-toxic in nature and almost >90% cells were viable when treated with each preparation under similar condition. The results further confirmed the previous findings of DNA loaded ultradeformable liposomes that these vesicles were non-toxic in nature (Lee et al., 2005).

The cellular and humoral responses obtained after administration of DNA by its de novo expression was measured using specific ELISA techniques. The specific antibody titers (anti-HBsAg) in serum obtained after topical application of cationic transfersomes, naked plasmid DNA and with the intramuscular HBsAg are shown in Fig. 5. Results showed that intramuscular injection of recombinant HBsAg (group 2) produced initial higher response as compared to DNA vaccine groups but the titer value failed to sustain for longer time period and after achieving the maximum value (after 4 weeks), it started declining. In con-

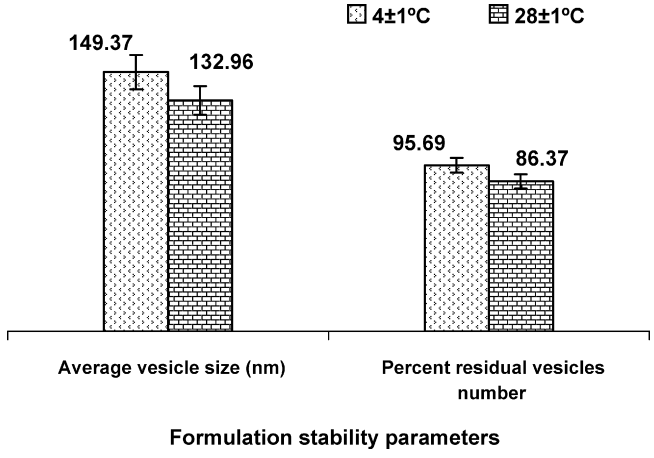

Fig. 2. Stability parameters of optimized plasmid loaded cationic transfersomes (CT-3) after 1 month of storage time. Data are shown as mean ± S.D. ($N = 3$).

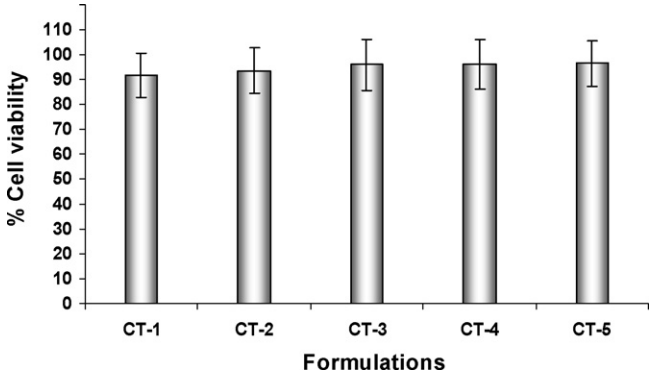

Fig. 4. Cytotoxicity of DNA loaded cationic transfersomes in HepG2 cells using MTT assay. Data are shown as mean ± S.D. of mean ($N = 3$).

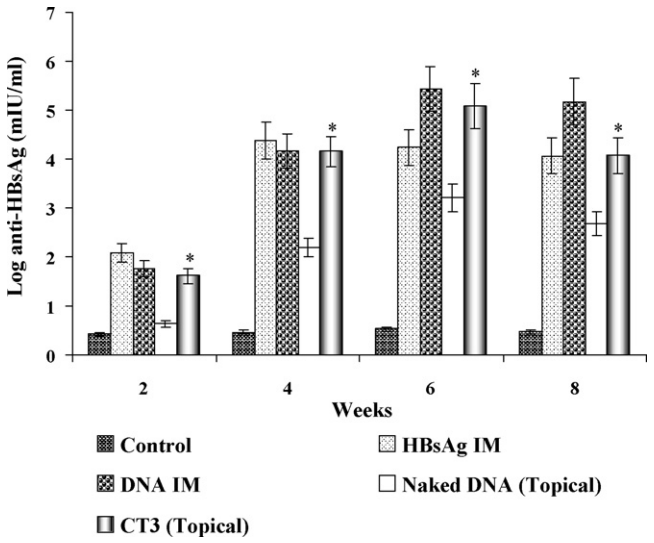

Fig. 5. Anti-HBsAg IgG antibody response in serum after immunization with different formulations. Antibody responses were analyzed after two (day 1st and day 14th) administrations in mice. Data represent titer means ± S.D. of the mean ($N = 6$). Statistical significance was calculated using Kruskal–Wallis test. *$P > 0.05$ vs. DNA (IM) and *$P < 0.05$ vs. naked DNA (topical).

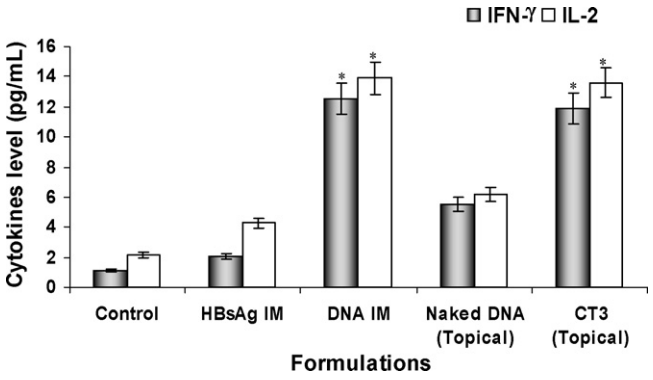

Fig. 6. Cytokine levels (IFN-γ and IL-2 in pg/ml) in spleen homogenate of mice immunized with different formulations after 4 weeks. Data represent means ± S.D. of the mean ($N = 6$). Statistical significance was calculated using Kruskal–Wallis test ($P < 0.05$). *$P < 0.05$ vs. HbsAg IM (recombinant protein).

trast, naked plasmid DNA administered intramuscularly (group 3) 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 subsequent translation before antigenic protein is expressed. This may be accounted for the delay in the initiation of humoral response. However, once DNA expression produces the antigenic protein it continues for a longer duration leading to production of better humoral response (Davis, 1996).

Results also reveal that, at all time points measured, mice immunized topically with cationic transfersomes (group 5) elicited significantly higher ($P < 0.05$) immune responses than naked DNA mice (group 4) as a result of effective permeation and subsequent transfection of transfersomes carried plasmid DNA across the intact skin. Results indicate that a clinically protective levels of antibodies (>10 mIU/ml) could detected in serum of mice immunized with topical application of cationic transfersomes after 2 weeks and the antibody 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).

Endogenous cytokine levels (IFN-γ and IL-2) were estimated in spleen homogenate after 4 weeks following administration of different formulations (Fig. 6). A significantly higher level of both IL-2 and IFN-γ ($P < 0.05$) was observed in mice immunized with DNA vaccine as against control (group 1) and antigen immunized group (group 2). Furthermore, the levels of both cytokines in the topically immunized mice with DNA vaccine formulated in cationic transfersomes were comparable to those of intramuscular naked DNA ($P > 0.05$; Kruskal–Wallis test). Further it was 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 conventional recombinant protein based vaccine though elicited higher humoral response (antibody production), however failed 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).

Antigen presenting cells (APCs) which contain both MHC-I and MHC-II molecules required in processing and presentation of antigen through both endocytic and cytosolic pathways to eventually elicit both humoral and cellular responses. The uptake of naked DNA by immunologically competent and relevant cells is reported to be minimal and involves only a minor fraction of these cells. Naked DNA is also susceptible to hydrolysis by DNAase 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 DNAase attack. The immunity induced by topical immunization appears to be long lasting, as indicated by persistence of serum antibodies. Langerhan's cells are the only antigen presenting cells in the un-inflamed epidermis (Udey, 1997); therefore they play a vital role in the induction of the systemic immune response on topical immunization. Mature Langerhan's cells express a high level of MHC (major histocompatibility complex) Class I and Class II antigens, co-stimulatory molecules and chemokine receptors. These are all important for the antigen presenting function of Langerhan's cells (Cruz and Bergstresser, 1990; Schuler and Steinman, 1998). Langerhan's cells present antigen to T cells in draining lymph nodes. They present antigen to naive T cells as well as to antigen specific T cells of CD4+ and CD8+ phenotype to stimulate both antibody and cellular immune responses. Thus, Langerhan's cells are critical determinants of immuno-consequences following topical immunization especially with cationic transfersomes an equally important vehicle for non-invasive plasmid delivery to these antigen-presenting cells. Some other cells in the vicinity of the Langerhan's cells like fibroblast cells or muscle cells may also contribute for effective processing and presentation

of endogenous antigens for improved immune responses. This study reflects that topical administration of DNA loaded cationic transfersomes 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.

4. Conclusion

Cationic transfersomes prepared in present study were capable of inducing strong humoral and cellular immune response after topical administration. Topical immunization using cationic transfersomes based DNA vaccine offers all the advantages of DNA vaccines, and in addition overcome the disadvantages of classical invasive methods of vaccination. The proposed system is simple, economical, stable, painless and potentially non-toxic.

Acknowledgments

The authors are thankful to Aldeveron USA for gift sample of plasmid DNA and M/s, Panacea Biotec Ltd., India for providing gift sample of recombinant HBsAg. Authors Mahor, Rawat and Dubey acknowledge Council of Scientific and Industrial Research (CSIR), New Delhi for the financial assistance.

References

Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature 392, 245–252.

Bergh, B.A.I., Wertz, P.W., Junginger, H.E., Bouwstra, J.A., 2001. Elasticity of vesicles assessed by electron spin resonance, electron microscopy and extrusion measurements. Int. J. Pharm. 217, 13–24.

Cevc, G., Blume, G., Schatzlein, A., Gebauer, D., 1996. The skin: a pathway for the systemic treatment with patches and lipid-based agent carriers. Adv. Drug. Deliv. Rev. 18, 349–378.

Cevc, G., Gebauer, D., Stieber, J., Schatzlein, A., Blume, G., 1998. Ultraflexible vesicles, transfersomes, have an extremely low pore penetration resistance and transport therapeutic of insulin across the intact mammalian skin. Biochim. Biophys. Acta 1368, 201–215.

Cevc, G., Schatzlein, A., Blume, G., 1995. Transdermal drug carriers: basic properties, optimization and transfer efficiency in the case of epicutaneously applied peptides. J. Control. Release 36, 3–16.

Constant, S.L., Bottomly, K., 1997. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. Annu. Rev. Immunol. 15, 297–322.

Cruz, P.D., Bergstresser, P.R., 1990. Antigen processing and presentation by epidermal langerhans cells, induction of immunity or unresponsiveness. Dermatol. Clin. 8, 633–647.

Davis, H.L., 1996. DNA-based vaccination against hepatitis B virus. Adv. Drug. Deliv. Rev. 21, 33–47.

Donnelly, J.J., Wahren, B., Liu, M.A., 2005. DNA vaccines: progress and challenges. J. Immunol. 175, 633–639.

Fry, D.W., White, J.C., Goldman, I.D., 1978. Rapid separation of low molecular weight solute from liposomes without dilution. Anal. Biochem. 90, 809–815.

Geissler, M., Tokushige, K., Chante, C.C., Zurawski, V.R., Wands, J.R., 1997. Cellular and humoral immune responses to hepatitis B virus structural proteins in mice after DNA-based immunization. Gastroenterology 112, 1307–1320.

Gupta, P.N., Mishra, V., Rawat, A., Dubey, P., Mahor, S., Jain, S., Chatterji, D.P., Vyas, S.P., 2005b. Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study. Int. J. Pharm. 293, 73–82.

Gupta, P.N., Mishra, V., Singh, P., Rawat, A., Dubey, P., Mahor, S., Vyas, S.P., 2005a. Tetanus toxoid loaded transfersomes for topical immunization. J. Pharm. Pharmacol. 57, 1–7.

Gupta, P.N., Singh, P., Mishra, V., Jain, S., Dubey, P.K., Vyas, S.P., 2004. Topical immunization: mechanistic insight and novel delivery systems. Ind. J. Biotechnol. 3, 9–21.

Hassett, D.E., Whitton, J.L., 1996. DNA immunization. Trends Biotechnol. 4, 307–312.

Hofer, C., Hartung, R., Gobel, R., Deering, P., Lehmer, A., Breul, J., 2000. New ultradeformable drug carriers for potential transdermal application of interleukin-2 and interferon-alpha: theoretic and practical aspects. World J. Surg. 24, 1187–1189.

Leclere, C., Deriaud, E., Rojas, M., Whalen, R.G., 1997. The preferential induction of a Th1 immune response by DNA-based immunization is mediated by the immunostimulatory effect of plasmid DNA. Cell. Immunol. 79, 97–106.

Lee, E.H., Kim, A., Oh, Y.K., Kook, C., 2005. Effect of edge activators on the formation and transfection efficiency of ultradeformable liposomes. Biomaterials 26, 205–210.

Mishra, V., Mahor, S., Rawat, A., Dubey, P., Gupta, P.N., Singh, P., Vyas, S.P., 2006. Development of novel fusogenic vesosomes for transcutaneous immunization. Vaccine 24, 5559–5570.

Nakane, A., Numata, A., Minagawa, T., 1992. Endogenous tumour necrosis factor, interleukin-6 and gamma interferon levels during Listeria monocytogenes infection in mice. Infect. Immun. 60, 523–528.

Perrie, Y., Gregoriadis, G., 2000. Liposome-entrapped plasmid DNA: characterisation studies. Biochim. Biophys. Acta 1475, 125–132.

Rich, J.D., Ching, C.G., Lally, M.A., Gaitanis, M.M., Schwartzapfel, B., Charuvastra, A., Beckwith, C.G., Flanigan, T.P., 2003. A review of the case for hepatitis B vaccination of high-risk adults. Am. J. Med. 114, 316–318.

Schuler, G., Steinman, R.M., 1998. Murine epidermal langerhans cells mature into potent immunostimulatory dendritic cells. Proc. Natl. Acad. Sci. U.S.A. 96, 13147–13152.

Singh, R.P., Singh, P., Mishra, V., Prabakaran, D., Vyas, S.P., 2002. Vesicular systems for non-invasive topical immunization: rational and prospects. Ind. J. Pharmacol. 34, 301–310.

Tuteja, R., 1999. DNA vaccines: a ray of hope. Crit. Rev. Biochem. Mol. Biol. 34, 1–24.

Udey, M.C., 1997. Cadherins and langerhans cells immunobiology. Clin. Exp. Immunol. 107, 6–8.

Vyas, S.P., Singh, R.P., Jain, S., Mishra, V., Mahor, S., Singh, P., Gupta, P.N., Rawat, A., Dubey, P., 2005. Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B. Int. J. Pharm. 296, 80–86.