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Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study

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Abstract

Non-invasive vaccine delivery is a top priority for public health agencies because conventional immunization practices are unsafe and associated with numerous limitations. Recently, the skin has emerged as a potential alternative route for non-invasive delivery of vaccine. Topical immunization (TI), introduction of antigen through topical application onto the intact skin, has many practical merits compared to injectable routes of administration. One of the possibilities for increasing the penetration of bioactives through the skin is the use of vesicular systems. Specially designed lipid vesicles are attracting intense attention and can be used for non-invasive antigen delivery. In the present study, elastic vesicle transfersomes, non-ionic surfactant vesicles (niosomes) and liposomes were used to study their relative potential in non-invasive delivery of tetanus toxoid (TT). Transfersomes, niosomes and liposomes were prepared and characterized for shape, size and entrapment efficiency. These vesicles were extruded through polycarbonate filter (50-nm pore size) to assess the elasticity of the vesicles. The immune stimulating activity of transfersomes, niosomes and liposomes were studied by measuring the serum anti-TT IgG titre following topical immunization. The immune response elicited by topical immunization was compared with that elicited by same dose of alum-adsorbed tetanus toxoid (AATT) given intramuscularly. The results indicate that optimal formulations of transfersomes, niosomes and liposomes could entrap 72.7 ± 3.4, 42.5 ± 2.4 and 41.3 ± 2.2% of antigen and their elasticity values were 124.4 ± 4.2, 29.3 ± 2.4 and 21.7 ± 1.9, respectively. In vivo study revealed that topically given TT containing transfersomes, after secondary immunization, could elicit immune response (anti-TT-IgG) that was equivalent to one that produced following intramuscularly alum-adsorbed TT-based immunization. In comparison to transfersomes, niosomes and liposomes elicited weaker immune response. Thus transfersomes hold promise for effective non-invasive topical delivery of antigen(s).

Keywords: Non-invasive vaccine delivery; Transfersomes; Niosomes; Liposomes; Tetanus toxoid

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1. Introduction

Immunization is one of the most efficient and cost-effective means for the prevention of various diseases. Non-invasive or needle-free delivery has become a global priority because the conventional vaccination route is associated with numerous limitations. Topical immunization (TI) is a novel vaccination strategy that utilizes topical application of vaccine antigen to intact skin to induce an immune response (Glenn et al., 2000). This non-invasive immunization strategy could decrease the risk of needle-born diseases such as hepatitis B or HIV, reduces the complications related to the physical skin penetration and improve access to vaccination by eliminating the need for trained personnel and sterile equipment (Glenn et al., 1999; Gupta et al., 2004). The invasive nature of conventional immunization practice reduces the patient compliance. If vaccine could be delivered without the use of sharps (needles and syringes) immunization practice would become safer, more acceptable and more suitable for mass use.

There is an increasing recognition of the skin as an organ of immune system. It has been recognized as a highly immune-reactive tissue containing an abundance of antigen presenting cells, especially within the epidermis. Skin is an immunologically active site and a promising vaccination route (Chen et al., 2001). Topical immunization combines the advantages of needle-free delivery while targeting the immunologically rich milieu of the skin. Vaccination through the skin may be particularly advantageous as the immunocompetent Langerhans cells (LCs) are found in abundance along the transdermal penetration pathways and these cells aligned specifically along the minute pores through which pathogens are likely to invade the body. LCs are found in close proximity to stratum corneum and represent a network of immune cells that underlie and cover 25% of the area of total surface area (Paul and Cevc, 1995). Epidermal LCs binds cutaneously encountered antigen and then process it. Carrying processed antigen they migrate from the epidermis into lymphatic vessels and finally into regional lymph nodes. Differentiation of LCs into dendritic cells takes place during this process and the dendritic cells offer the antigen to naïve $CD4^+$ T-cells that have entered the lymph nodes through the high endothelial venules (Kupper, 1990; Paul et al., 1998).

Topical immunization provides an access to the skin immune system that is dominated by densely distributed and potent professional antigen presenting cells called LCs that can be manipulated to orchestrate-specific, robust immune responses (Hammond et al., 2001). approach has potential to induce local as well as systemic immunity. Among the various approaches for topical immunization studied, vesicular carriers are gaining wide attention. Some lipid directly lowers the skin barrier potential, which resides primarily due to the stratum corneum. Hence specially designed lipid vesicles are attracting increasing attention. Various types of surfactant have been used for the preparation of non-ionic vesicles having potential for non-invasive bioactive delivery (Handjani-vila et al., 1979; Ballie et al., 1985; Kiwada et al., 1985; Hofland et al., 1991). The most recent development in vesicle design for transdermal bioactive delivery is the use of elastic vesicles, transfersomes, which differs from conventional niosomes and liposomes by their characteristic fluid membrane with high elasticity. This feature enables transfersomes to squeeze themselves through intercellular regions of the stratum corneum under the influence of transdermal water gradient (Cevc et al., 1998). Niosomes and liposomes also have potential in topical delivery of bioactives.

The aim of the work was non-invasive delivery of tetanus toxoid using different vesicular carriers, viz. transfersomes, niosomes and liposomes and to compare the respective immune responses by measuring serum IgG antibody titre. Transfersomes, niosomes and liposomes were prepared and characterized for their size, shape and entrapment efficiency. The extrusion rate of vesicles was determined to estimate the elasticity value of the bilayer membrane of vesicles.

2. Materials and methods

2.1. Materials

Soya phosphatidylcholine (SPC), cholesterol (CH), sodium deoxycholate (SDC), sephadex G-150, 6-carboxyfluorescein (6-CF) were purchased from sigma (USA). Span-85 was purchased from Himedia, India. Protein estimation kit (by BCA method) and ELISA kit were purchased from Genei, Bangalore, India. All solvents used were of analytical grade. Tetanus toxoid

(TT) was obtained as gift sample from Serum Institute of India, Pune. TT solution contained 3600 LF/ml (lime flocculation per ml) and a protein concentration of 9.0 mg/ml.

2.2. Preparation of liposomes and niosomes

Liposomes and niosomes were prepared by the reverse phase evaporation technique as reported by Sazoka and Papahadjopoulos (1978) with slight modification. Briefly, SPC and CH (7:3%, w/w) were dissolved in 5-ml diethylether to which 2 ml of aqueous phase, i.e. phosphate-buffered saline (PBS, pH 6.5) containing TT (60 LF/ml concentration in final immunization solution) was added. The mixture was sonicated (Soniweld, India) for 5 min at 4 °C. A thick emulsion was formed which was then kept over a vortex mixer in order to remove any residual ether. To this emulsion 3-ml PBS (pH 6.5) was added in order to hydrate the vesicles. Resulting large unilamellar vesicles were passed through 0.45 and 0.2 μm polycarbonate membrane (Nucleopore, The Netherlands). Similarly, TT-loaded niosomes were prepared by dissolving Span 85 and CH (1:1%, w/w) in diethyl ether and following the same procedure as described above for liposomes.

2.3. Preparation of transfersomes

Transfersomes were prepared by method as described by Paul et al. (1998) with slight modifications. In brief, ethanolic solution of SPC was mixed with SDC (85:15%, w/w) in phosphate buffer (pH 6.5) containing TT solution (60 LF/ml concentration in final immunization solution). The obtained suspension was pushed through a series of 0.45, 0.22, 0.10, 0.05 μm polycarbonate filters (Nucleopore, The Netherlands).

2.4. Vesicle morphology and size analysis

Prepared vesicular systems were characterized for their shape using transmission electron microscope (JEM-200 CX, JEOL, Tokyo, Japan). One percent phosphotungstic acid (PTA) was used as negative stain. The particle size of the prepared vesicular formulations was measured by photocorrelation spectroscopy with an Autosizer II C apparatus (Malvern Instruments, UK).

2.5. Entrapment efficiency

Prepared vesicular formulations were separated from the free (unentrapped) antigen by using a Sephadex G-150 minicolumn applying centrifugation technique as described by Fry et al. (1978). The method was repeated thrice with a fresh syringe packed with gel each time. The fraction was finally collected which was free from unentrapped antigen. The vesicular fraction was added with minimum amount of triton X-100 (0.5%, w/v) to disrupt the vesicles and liberated antigen was estimated using a BCA (bicinchoninic acid) protein assay and percent antigen entrapment was calculated.

2.6. Measurement of elasticity value

Comparative measurement of elasticity of the bilayer of transfersomes, niosomes and liposomes was carried out by extrusion measurement (Bergh et al., 2001). Briefly, the vesicles were extruded through polycarbonate filter with a pore size of 50 nm (Nucleopore, The Netherlands) at constant pressure. 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.

2.7. Immunization

The study was carried out under the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal, Ministry of Culture, Government of India). The animals were kept under standardized condition at the Pharmaceutical Departmental Animal Facility of the Dr. H.S. Gour University, Sagar, MP, India. Each group contains eight animals. The Albino rats (Wistar strain), 8–12-week-old, were used. They were carefully shaved on dorsum and rested for 24–48 h. The skin was then carefully wiped with 70% ethanol prior to application of vesicular formulation. For topical immunization 175–225 μl formulation was applied on the shaved skin over a 2 cm × 2 cm area and then left to dry. Topical immunization was done with TT-loaded transfersomes, liposomes and niosomes containing dose equivalent to 10 LF. Immune response was compared

with alum-adsorbed TT given intramuscularly. Secondary immunization was done on day 28 with formulations containing same dose of TT. Blood samples were taken on days 14, 28, 42, 56 and 90 through retro-orbital plexus vein of eye. The collected blood samples were allowed to clot and then centrifuged to separate the serum, which were stored at −20 °C until analyzed.

2.8. Preparation of rat abdominal skin membrane

The full-length shaved abdominal skin with the adjoining connective tissue was excised with care from male Wistar rats and to mimic the transdermal water gradient across the epithelial the subdermis was in contact with the phosphate-buffered saline (pH 6.5) and stratum corneum faced upward to the donor compartment. Prior to use the skin was inspected for any damage using microscopy after staining with haematoxylin and eosin.

2.9. In vitro skin permeation study

The permeation of TT-bearing transfersomes, niosomes and liposomes through the skin were determined by using Franz-diffusion cell as described previously (Fang et al., 2001). The nude rat skin was mounted on the receptor compartment with the stratum corneum side facing upward to the donor compartment. Formulation containing 40 μg of TT was applied on the skin in donor compartment. The receptor medium was 5 ml PBS buffer (pH 6.5). The receptor compartment was maintained at 37 °C with magnetic stirring at 500 rpm. At appropriate intervals 200 μl aliquots of receptor medium was withdrawn and immediately replaced with an equal volume of fresh receptor solution. The samples from the receptor medium were analyzed for antigen contents by BCA (bicinchoninic acid) method. Briefly, BCA working reagent (BWR) was prepared by mixing 50 parts reagent A (sodium carbonate, sodium bicarbonate, bicinconinic acid and sodium tartarate in 0.01 M sodium hydroxide) with one part of reagent B (copper sulphate solution).An amount of 0.2 ml of standard and unknown samples were added to the 2 ml BWR with constant agitation and then kept at 37 °C for 30 min. Absorbance of these solutions was taken at 562 nm.

2.10. Determination of IgG titre by ELISA

Specific anti-IgG antibody level in the serum was determined by ELISA as described previously (Esparza and Kissel, 1992). Hundred microliters of TT (10 μg/ml in phosphate buffer, pH 7.4) was coated to each well of Nunc-Immuno plate. The plate was incubated at 4 °C overnight. Plate was then washed three times with PBS tween-buffer. Hundred microliters of 2% BSA was added in each well and plate was incubated for 2 h at room temperature and washed three times with PBS-tween. Hundred microliters of diluted serum sample was added to each well and incubated for 2 h at room temperature. Plate was washed three times with PBS tween-buffer. Hundred microliters of diluted horseradish-peroxidase-conjugated antiglobulin-specific anti-rat IgG was added to each well and incubated for 2 h. Plate was again washed three times with PBS tween buffer and then 100 μl of substrate solution 3,3′,5,5′-tetramethyl benzidine containing hydrogen peroxide was added to each well. Plate was incubated in darkness at room temperature for 15 min. The reaction was stopped by adding 50 μl of 2 M H_2SO_4 to each well. The absorbance was measured at 450 nm using a microplate ELISA reader (Lab System Multiscan, Finland). Immune response is shown in terms of reciprocal antibody titre.

2.11. Statistical analysis

The results were expressed as mean ± standard deviation. Statistical analysis was carried out by using Student's *t*-test and statistical significance was designated as $p < 0.05$.

3. Results and discussion

3.1. Preparation of vesicular systems and their characterization

Liposomes and niosomes were prepared by reverse phase evaporation technique because the highest skin penetrating and bioactive carrying capability is frequently associated with such method (Cevc et al., 1996). Further, this method is also associated with relatively higher encapsulation efficiency of macromolecules such as antigens and peptides

(New, 1990). TT-loaded transfersomes were prepared by method as described by Paul et al. (1998). Transmission electron microscopy was used to characterize transfersomes, liposomes and niosomes. These carriers invariably appeared as unilamellar vesicles (Fig. 1 A–C).

Bioactive molecules larger than 500 Da normally do not cross the skin. This prevents non-invasive delivery of high-molecular-weight bioactive. In order to cross the intact mammalian skin, transfersomes should be capable of passing through pores of diameter less than 50 nm under influence of suitable transdermal

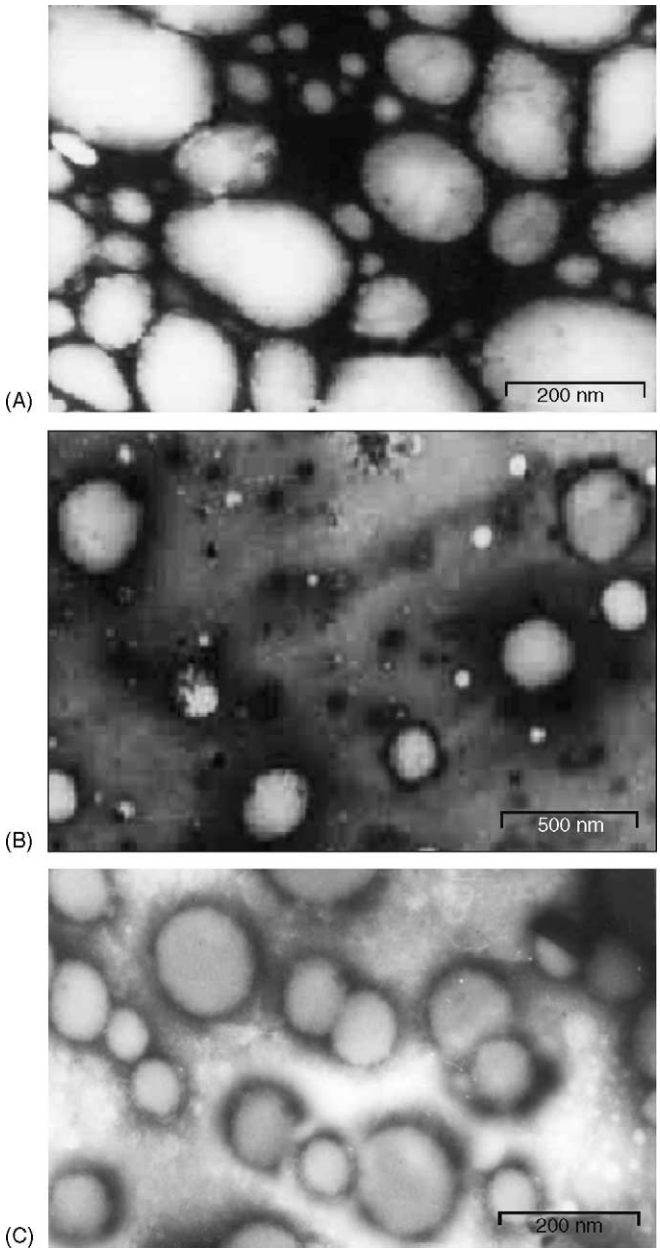

Fig. 1. Transmission electron microscope (TEM) photomicrograph of transfersomes (A), liposomes (B) and niosomes (C).

Table 1
Various formulations, their composition, entrapment efficiency, size and deformability index

Formulations	Composition	Entrapment efficiency[a]	Initial size (nm)[a]	Deformability index[a]
Transfersomes	SPC: SDC (85:15%, w/w)	72.7 ± 3.4	196 ± 10.2	124.4 ± 4.2
Liposomes	SPC: CH (7:3%, w/w)	42.5 ± 2.4	221 ± 11.5	29.3 ± 2.1
Niosomes	Span 85:CH (1:1%, w/w)	41.3 ± 2.2	214 ± 10.7	21.7 ± 1.9

[a] Mean ± S.D. ($n = 4$) (SPC: soya phosphatidylcholine; SDC: sodium deoxycholate; CH: cholesterol).

gradient (Ceve et al., 1995). Only properly optimized and moderately loaded carriers can pass through pores smaller than their own diameter. Increasing the concentration of membrane softening component beyond certain level or even to the point of bilayer solubilization brings no advantages in terms of transcutaneous transportation efficiency. Only optimum ratio of lipid and surfactant could lead to flexibility of the bilayer membrane (Hofer et al., 2000). On the basis of entrapment efficiency and deformability index optimum ratio of lipid, surfactant and cholesterol were taken for the preparation of transfersomes, niosomes and liposomes (unpublished data). The maximum entrapment efficiency 72.7 ± 3.4 was found in case of transfersomal formulation (Table 1). Transfersomes revealed significantly higher entrapment efficiency in comparison to liposomes and niosomes. The entrapment efficiency of proteins depends on interaction between the protein and lipid bilayer, lipid concentration and number of extrusion as well, if the method involves the extrusion through membrane filters. Lipid composition of the transfersomes was higher in comparison to that in liposomes and this may result in higher entrapment efficiency measured with transfersomes (Yoshioka et al., 1994). Further transfersomes contains a mixture of lipid and membrane softener, e.g. sodium deoxycholate. The lipid is a stabilizing factor and sodium deoxycholate is a destabilizing factor (Hofer et al., 2000). In the preparation method of transfersomes, the vesicles content are exchanged with the dispersion medium during breaking and resealing of phospholipid bilayer as they pass through the polycarbonate membrane filters. During the successive extrusion, the protein stayed inside the transfersomes suggesting that interaction between lipid membrane and protein did not allow free displacement of protein. Thus comparatively higher lipid concentration and successive extrusion through membrane filters may synergistically leads to higher entrapment efficiency of transfersomes in comparison to liposomes and niosomes. Higher entrapment efficiency for proteins in case of transfersomes has also been reported by Paul and Cevc (1995) (85% entrapment of gap junction protein) and Hofer et al. (2000) (84 and 80% entrapment of interferon and interleukin-2, respectively). The entrapment efficiency of liposomes and niosomes was almost equal, however, slightly less entrapment in case of niosomes estimated may be accounted to the pore formation characteristics of the Span 85 in bilayer of the niosomes.

3.2. Measurement of bilayer elasticity

The prerequisite for topical immunization is that antigen as well as its carrier/adjuvant must penetrate through the stratum corneum in order to reach epidermis where the immuno-competent cells reside. Lipid vesicle penetration through the skin is a function of carrier membrane deformability and thus the carrier system should be deformable so that it can pass easily through the minute pores present in the skin. Prepared formulations were subjected to deformability study by extrusion measurement. The results were expressed in terms of deformability index (Table 1).

Transfersomes were most deformable vesicle, which can be used to transport bioactive across the biological membrane such as skin. Deformability was found to be maximum with the transfersomal formulation (124.4 ± 4.2). These deformable vesicles cross the skin through the virtual pores between the corneocytes in the stratum corneum (Schafer-Korting, 1993). The vesicles deform so as to ooze through the pore entrance. After slipping into pore entry transfersomes move through the constriction with nearly same velocity as outside the pore. After leaving the confining section of the pore the vesicle regain its original form and penetration process start anew. Two types of transportation routes have been documented in the mammalian skin: (a) the wide cleft between clusters of 3–10 corneocytes, organized in columns; the appearants widths of such pores is quite large; 100 nm. (b)

Virtual pores (30 nm) between the individual cells in each corneocyte clusters (Schatzlein and Cevc, 1995, 1998). This intercellular track is most important for the transport of deformable vesicle through the skin. In comparison to transfersomes, niosomes and liposomes are less deformable. This may be attributed to the fact that sodium deoxycholate mainly contribute to the transfersomal deformability whereas cholesterol in niosomes and liposomes tends to form rigid vesicle. Consequently, they are less capable of passing through the barrier with pores smaller than their own diameter. Niosomes and liposomes have almost similar deformability index but still niosomes has an edge over liposomes, the reason could be membrane-softening effect of Span 85, which is a constitutive component of niosomes.

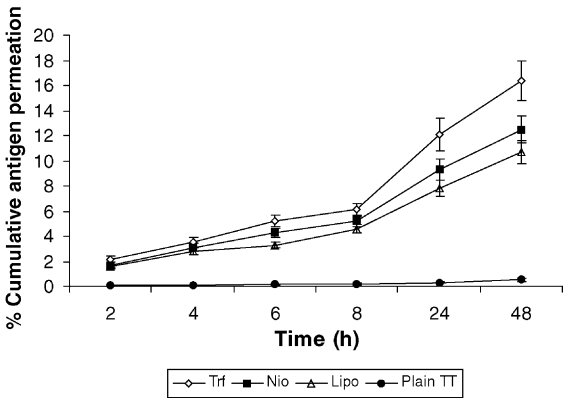

Fig. 2. Percentage cumulative antigen permeation through rat skin of TT entrapped in transfersomes, niosomes and liposomes. Plain TT applied topically as a control ($n = 4$).

3.3. In vitro skin permeation study

To increase the bioactive transport across the skin, penetration enhancers as well as other approaches have been attempted. The use of vesicular systems to increase bioactive transport across the skin is the most controversial method. In vitro skin permeation experiment was conducted using Franz-diffusion cell and permeation behavior was compared with similar size of transfersomes, liposomes and niosomes. The experiment was carried out for 48 h and withdrawn samples were analyzed using BCA method.

After 48-h cumulative permeation of TT was 16.4 ± 1.6, 12.5 ± 1.1 and 10.7 ± 0.9 in case of transfersomes, niosomes and liposomes, respectively (Fig. 2). The slow release of the entrapped antigen could be due to the large molecular size of the antigen with poor diffusivity through vesicular membrane. Further, small amount of antigen release might be attributed to the release of surface adsorbed antigen. Transfersomal formulation showed maximum permeation profile, whereas niosomes and liposomes shown relatively low permeation characteristics, because cholesterol affects profoundly their membrane properties. Incorporation of cholesterol in gel-state bilayer can induce a continuous and permanent transition to an ordered liquid crystalline state. This may attributed to slow release characteristics through niosomal and liposomal formulations. Two types of interaction between the skin and vesicles may possibly facilitate transdermal biomolecule delivery: (1) Adsorption and fusion of biomolecule-loaded vesicles onto the surface of skin lead to high thermodynamic activity gradient of the biomolecule-stratum corneum surface. (2) The effect of vesicle on stratum corneum may change the bioactive permeation kinetics due to an impaired barrier function of the stratum corneum (Touitou et al., 1994; Fang et al., 2001). The action of transfersomes as penetration enhancer may predominantly be on the intercellular lipid of stratum corneum, raising the fluidity and weakness of stratum corneum. Ultradeformable character of transfersomes supports their passage through very fine pores in the skin under suitable osmotic gradient. Phospholipids have high affinity for biological membranes. Mixing of the phospholipid of the carrier system with the skin lipid of the intercellular layers may also contribute to the permeability of the skin to lipid vesicles (Ogiso et al., 1997; Weiner et al., 1989). The presence of unsaturated fatty acid in SPC may be responsible for enhanced permeation. The packing nature of unsaturated fatty acids changed the fluidity of stratum corneum lipid structure and facilitated the permeation of bioactive (Valenta et al., 2000). Topically applied lipid vesicles affect characteristics and integrity of the skin permeability barrier. In addition, they may extract the lipid from the skin or disrupt the order within and between the corneocyte upon binding to the keratin filament. Elastic vesicle can be used to transfer bioactive rapidly into the deeper layer of the stratum corneum, after which the bioactive can permeate into the viable epidermis. This study is revealing that transfersomes have better bioac-

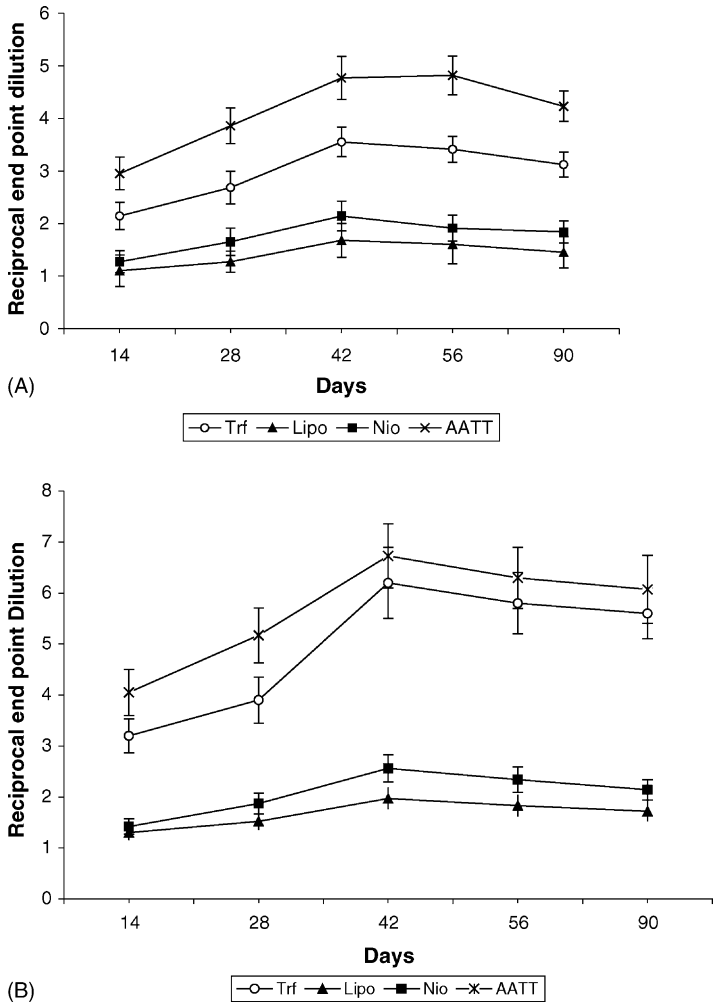

Fig. 3. A. Induction of primary serum IgG response following topical application of TT-loaded transfersomes, niosomes and liposomes. Alum-adsorbed tetanus toxoid (AATT) was given intramuscularly as a control ($n = 4$). (B) Induction of serum IgG response following topical application of TT-loaded transfersomes, niosomes and liposomes after booster dosing on day 28. Alum-adsorbed tetanus toxoid (AATT) was given intramuscularly (with booster dose on day 28) as a control ($n = 4$).

tive transportation profile compared to liposomes and niosomes.

3.4. Systemic IgG responses

We have investigated the humoral immune responses in albino rats to non-invasively (topically) and intramuscularly applied tetanus toxoid. Three different antigen carriers, transfersomes, niosomes and liposomes, were used. Following topical immunization maximum response was observed after 42 days with TT-loaded transfersomes, which was significantly less ($p < 0.05$) than that elicited by intramuscularly given AATT (Fig. 3A). Most vaccines produce their protective level of antibodies through boosting regimens. It was observed that topical immunization similarly induces memory responses that can be readily boosted. After secondary immunization on day 28, transfersomes elicited maximum immune response again on day 42. The response was significantly comparable ($p < 0.05$) to that elicited by intramuscular injection of AATT (Fig. 3B). The result is consistent with our pre-

vious studies in which we have topically administered TT-loaded transfersomes, Plain TT, physical mixture of TT and transfersomes (Gupta et al., 2005). Liposomal and niosomal formulation reflected significantly lower ($p < 0.05$) antibody titre value in comparison to transfersomes both after primary and secondary immunizations. Lower immune response elicited by niosomes and liposomes may be attributed their unequal capability to penetrate intact skin barrier. Further, for effective vaccine delivery several variables related to the nature of antigen and characteristics of the skin barrier may be other determinants. The diffusion of antigen through the stratum corneum is dependent on its physicochemical properties and its molecular interaction with the skin constituents. Transfersomes, niosomes and liposomes differ in their efficiency to breach skin barrier and deliver their payload to immunocompetent LCs. This results in the varied degree of immune response elicited by these carriers for TT. High level of antibody response after boosting indicates presence of memory B- and T-cell population evoked by primary immunization. Immune response was found to be maintained with very gradual decrease in immune responses after boosting. The results favor good immunoadjuvant action of transfersomes and reflect that it is relatively better than liposomes and niosomes for the topical delivery of bioactive (antigen). Niosomes reflected better immune response than liposomes. This may be attributed to permeation enhancement effect of non-ionic surfactant, which constitute niosomes.

Antigens in conventional delivery systems are unable to penetrate through the intact skin. Classical penetration enhancers are also inefficient to eliminate this barrier (Paul and Cevc, 1995). Thus we can infer that transfersomes-mediated antigen delivery may not be through penetration-enhancement effect. Furthermore, observed immune response may not be exclusively due to better antigen presentation at vesicle surface. A unique property associated with transfersomes is their deformability. This property in combination with sensitivity of transfersomes to water gradient across the skin makes this carrier a potential system for topical immunization. The horny region of the skin is associated with sparsely distributed pores. These pores act as permeability shunt and locally lower the skin permeability barrier. Further, these pores are potential sites for deformable bodies, which are strongly driven under the effect of trans-epidermal water gradient. Liposomes and niosomes cannot enter the intact skin spontaneously because of their low deformability. Transfersomes are unique carrier and adjuvant exploitable in transdermal immunization.

The present work is vindicating that protein antigens can be safely delivered non-invasively through the skin using elastic carrier, referred to as transfersomes. The immune response of transdermal immunization against TT using transfersomes was significantly ($p < 0.05$) comparable following secondary immunization to that achieved by intramuscular injection of same dose of AATT. For topical immunization transfersomes have an edge over niosomes and liposomes. We can surmise that this difference in immune response elicited by transfersomes, niosomes and liposomes is due to unequal capability and to the divergent tendency of these formulations especially in respect to penetration across the intact skin barrier.

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