International Immunopharmacology 11 (2011) 1516–1522

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Non-ionic surfactant vesicles mediated transcutaneous immunization against hepatitis B

Chetan Maheshwari [a,*], R.S. Pandey [a], Akash Chaurasiya [b], Ashu Kumar [c], D.T. Selvam [c], G.B.K.S. Prasad [d], V.K. Dixit [a]

[a] Department of Pharmaceutical Sciences, Dr. Hari Singh Gour University, Sagar 470003, India
[b] Faculty of Pharmacy, Jamia Hamdard, New Delhi-110062, India
[c] Defence Research and Development Establishment, Gwalior 474002, India
[d] Jiwaji Vishwavidyalaya, Gwalior, India

ARTICLE INFO

Article history:
Received 22 December 2010
Received in revised form 20 April 2011
Accepted 9 May 2011
Available online 24 May 2011

Keywords:
Niosomes
Transcutaneous immunization
HBsAg surface antigen
Cholera toxin B subunit
Confocal laser scanning microscopy

ABSTRACT

Transcutaneous immunization (TI) has many practical merits compared to parenteral routes of administration. In the present study, non ionic surfactant vesicular carrier, i.e. niosomes, was evaluated for topical delivery of vaccines using hepatitis B surface protein as an antigen and cholera toxin B as an adjuvant. Niosomes were characterized for size, shape, entrapment efficiency and in process antigen stability. In vitro permeation and skin deposition studies of antigen were performed using human cadaver skin. Skin penetration efficiency of niosomes was assessed by confocal laser scanning microscopy. The immune stimulating activity of these vesicles was studied by measuring the serum IgG titer, isotype ratio IgG2a/IgG1and mucosal immune responses following transcutaneous immunization in Balb/c mice and results were compared with the alum adsorbed HBsAg given intramuscularly and topically administered plain HBsAg solution. The result shows that optimal niosomal formulation could entrap 58.11 ± 0.71 of antigen with vesicle size range of 2.83 ± 0.29 μm. Serum IgG titers after three consecutive topical administrations were significantly better than single administration of hepatitis antigen with niosomal system, suggesting an effective stimulation of serum immune response; higher IgG1/IgG2a ratio revealed CTB mixed niosomes elicit both Th1 and Th2 responses. This study suggests that topical immunization with cholera toxin B is potential adjuvant for cutaneous immune responses when coadministered with the HBsAg encapsulated niosomes. Results also suggest that the investigated niosomes systems can be effective as topical delivery of vaccines.

1. Introduction

Transcutaneous immunization (TI) is a novel vaccination strategy based on the application of antigen together with an adjuvant onto hydrated bare skin and subsequent delivery to underlying Langerhans cells that serve as antigen-presenting cells [1]. Non-invasive vaccination onto the skin could improve vaccination programs because the procedure does not require specially trained personnel and may avoid risk associated with needle prick. A critical role in enhancing the immune response to both licensed and experimental vaccines is played by adjuvants. It appears that adjuvants are crucial to inducing sufficiently potent and functional immune responses on the skin. The uses of adjuvants have recast the fields of vaccine research for injectable, oral, and nasal immunization. Adjuvants, such as cholera toxin and heat labile enterotoxin from *Escherichia coli* and other adjuvants, and delivery techniques that can target LCs may revolutionize the future delivery of vaccines [2,3].

One of the possibilities for increasing the penetration of bioactives through the stratum corneum is the use of vesicular systems. Interactions can occur either at the skin surface or in the deeper layers of the stratum corneum. Previously it has been reported that adsorption and fusion of vesicles onto the skin surface result in the formation of lamellae and rough structures on top of the outermost corneocytes [4,5]. These delivery systems act as adjuvants to enhance the immunogenicity of antigens, which otherwise induce "weak" immune response when applied topically. Nonionic surfactant based vesicles (niosomes) that are assemblages of non-ionic amphiphiles into closed bilayer structures have also been reported to possess strong adjuvanticity [6]. Vesicular carrier systems liposomes and niosomes have been advocated for topical delivery of bioactives [7–9]. The low cost, high purity, content uniformity, greater stability and ease of storage have presented niosomes as better alternatives to liposomes. Cholera toxin (CT) and its non toxic components cholera toxin B (CTB) have been important in the development of the mucosal route as a useful and easily accessible non-invasive way to induce immunity [10–13]. Langerhans cells (LCs) are found in the epidermis, almost directly under the stratum corneum. Their superficial location

* Corresponding author. Tel.: +91 8010110041.
E-mail address: chetansemail@gmail.com (C. Maheshwari).

1567-5769/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.intimp.2011.05.007

makes these potent APCs attractive targets for vaccine delivery. LCs is thought to be phagocytes antigen in the skin and, if activated, will migrate to the draining lymph nodes where they present antigen to T cells. CT activates LCs, which leads to antigen presentation in the draining lymph node that leads to the induction of a systemic immune response [14]. Employing CT as an adjuvant for transcutaneous immunization would allow the skin to be used in a similar way.

The aim of the present study was to establish potential of niosomes as topical hepatitis B vaccine carriers. Niosomes encapsulating hepatitis B surface antigen (HBsAg) were prepared and characterized by variety of analytical methods. The specific immunological response against hepatitis B antigen elicited by niosomes was compared with that induced by administration of HBsAg vaccine through topical route and intramuscular administration.

2. Materials and methods

2.1. Materials

The HBsAg and CTB were obtained as gift samples from Shantha Biotechnics (Hyderabad, India) and Institute of Genomics and Integrative Biology, New Delhi respectively. AUSZYME monoclonal diagnostic kit was obtained from Abbott Laboratories, Chicago, IL, USA. Cholesterol, soya phosphatidylcholine (SPC), Sephadex G-100, tetramethyl benzidine (TMB), FITC-OVA, FITC-dextran (MW = 21 kDa), horseradish peroxidase (HRP) labeled goat anti-mouse IgG, IgG2a and IgG1 antibodies were purchased from Sigma (Sigma, St. Louis, USA). Span 80 (an ester of plain sorbitan with fatty acids) was purchased from Himedia (Mumbai, India). Gel electrophoresis kit was purchased from the Genei (Genei Pvt. Ltd., Bangalore, India) and used for SDS–PAGE studies. All other chemicals and reagents were of analytical grade, purchased from local suppliers and used as received. Distilled deionized water (Milli-Q^{TM} Water system, Millipore Corporation, Massachusetts, and USA) was used throughout the study.

2.2. Preparation of vesicular carrier systems

Niosomes were prepared by reverse phase evaporation method [15,16]. Briefly, Span 80 and cholesterol in a molar ratio 7:3 were dissolved in diethyl ether followed by emulsification with 2 ml aqueous solution of HBsAg solution (10–150 μg/ml) by probe sonication (Soniweld, India) for 2 min at 50 Kc/s. Thick emulsion was formed which was then kept over a vortex mixer in order to remove any residual ether. To this emulsion, 3 ml phosphate buffer (PB, pH 6.5) was added in order to hydrate the vesicles.

2.3. Entrapment efficiency

For determination of entrapment efficiency the unentrapped antigen from niosomal formulation was separated by the use of the Sephadex G-100 minicolumn centrifugation method [17]. The amount of antigen entrapped in the vesicles was then determined by disrupting the vesicles using 0.1% Triton X-100 followed by filtration using 0.22 μm membrane filter (Millipore, USA). The amount of antigen was determined by biscinchoninic acid (BCA) protein assay method for HBsAg by taking bovine serum albumin as standard.

2.4. Vesicle morphology and size analysis

Prepared vesicular systems were characterized for their shape using transmission electron microscopy (TEM) with an accelerating voltage of 100 kV. A drop of the sample was placed on to a carbon-coated copper grid to leave a thin film. Before the film dried on the grid, it was negatively stained with 1% phosphotungstic acid (PTA) and excess of the solution was drained off with a filter paper. The grid was allowed to thoroughly dry in air and samples were viewed in a transmission electron microscope (Phillips Morgagni D-268, Netherlands). For the size measurement, vesicular suspension was mixed with the appropriate medium (PB pH 6.5) and the size measurement was done by Malvern Zetasizer 3000 HS (Malvern Instruments Co., UK). Each experiment was conducted in triplicate.

2.5. In process stability

SDS–PAGE experiment was carried out to analyze the integrity of HBsAg antigen under non-denaturing condition. In presence of strong reducing agent and heat, proteins get dissociated before they were applied on the gel. SDS–PAGE experiments were performed on a 5% stacking gel and 8% resolving gel following the standard protocol. The gel was dried and the samples were applied on to the gel for electrophoresis. Protein bands were detected by Coomassie blue staining. The samples were heated to 95 °C for 5 min prior to their applications.

2.6. In vitro skin permeation and deposition studies

The in vitro skin permeation of rHBsAg-loaded niosomes and CTB, plain niosomes, plain rHBsAg solution and CTB, was studied using locally fabricated Franz diffusion cell with an effective permeation area of 2.8 cm^2. The temperature was maintained at 32 ± 1 °C. The receptor compartment contained 7 ml PBS (pH 6.5) and was constantly stirred by magnetic stirrer (Expo India Ltd., Mumbai, India) at 100 rpm. Dermatomed (~500 μm thickness) human cadaver skin from abdominal areas was obtained from District Hospital, Sagar, India, and stored at −20 °C. The skin was then carefully checked through a magnifying glass to ensure that samples were free from any surface irregularity such as tiny holes or cervices in the portion that was used for transdermal permeation studies. After assurance, the skin was mounted on a receptor compartment with the stratum corneum side facing upward into the donor compartment. Formulations containing 10 μg of HBsAg were applied on the skin in donor compartment. Samples were withdrawn through the sampling port of the diffusion cell at predetermined time intervals over 24 h and analyzed. The receptor phase was immediately replenished with equal volume of fresh diffusion buffer. After dismantling was completed, the donor compartment of the cell was rinsed carefully five times with 0.5 ml of buffer solution. The skin was removed and washed with 0.5 ml of buffer solution.

Approximately five such washings were found to be sufficient to remove >99% of the formulation when determined at time zero. All washings were collected and assayed for hepatitis B. Following the rinsing procedure, the skin patch was mounted on a board and a piece of surgical adhesive tape (1.9 cm wide and about 6 cm long) was pressed firmly to the skin surface with a spatula. The tape was of sufficient size to cover the area of skin that was in contact with the formulation. Twelve such strippings were carried out, and each strip was analyzed separately for HBsAg.

For the HBsAg analysis of the donor compartment washings, skin washings, and receiver compartment solution, enough trichloroacetic acid (10% w/v) was added to samples so that the concentration of the protein precipitant was ~5% (w/v). After overnight equilibration, the mixture was centrifuged (Remi C-24, Mumbai, India) for 15 min 3000 rpm and the supernatants and precipitates were separately assayed. For HBsAg analysis of the skin samples (tape strippings and remaining skin), a 10% (w/v) solution of trichloroacetic acid in HEPES buffer was added to the sample contained in a flask and the contents were thoroughly mixed on a Vortex mixer at 1200 rpm (Fischer Scientific, India) and further incubated for 24 h to allow intimate contact between the protein precipitant and the HBsAg residing in the skin tissue. The mixture was centrifuged for 30 min (3000*g*) and the supernatant and precipitate were assayed. The quantitative estimation of antigens was performed according to the manufacturer's instructions