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Topical immunization using a nanoemulsion containing bacterial membrane antigens

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ABSTRACT

This work describes the development of a nanoemulsion composition suitable for the topical administration of vaccines based on outer membrane vesicles. The application onto bare skin of outer membrane antigens from *Salmonella enterica* (size between 20 and 100 nm), included in a nanoemulsion, induced a clear specific antibody response. In contrast to other semisolid formulations used (i.e. simple and polyethyleneglycol ointments), the occlusive effect provided by the nanoemulsion together with the penetration enhancer effect of Labrasol® and Plurol® oleique, increased antigen uptake by epidermal and transfollicular routes. Nevertheless, when the antigenic complex was loaded into poly(anhydride) nanoparticles and then incorporated in the nanoemulsion, the specific IgG response in serum was significantly lower. These results suggest that the higher size of nanoparticles (about 230 nm) and their non-deformable nature could hamper the arrival of the antigen to the immunological inducer sites when administered on the skin. Immunohistochemistry analysis confirmed that these bacterial vesicles were able to penetrate the skin reaching the dermis only when antigens were administered in the form of nanoemulsion. Further research will determine the full potential of this formulation for topical application of this specific type of vaccines.

1. Introduction

Nowadays, the majority of vaccines are administered by inoculation with needles, with the concomitant side effects derived from accidents during manipulation including the risk of transmission of blood-borne diseases [1,2]. To date, more than twenty pathogens transmitted through such accidents have been reported, including, the human immunodeficiency virus, hepatitis B virus, or hepatitis C virus [3]. In spite of the introduction of safety programs to prevent this problem [4], percutaneous injuries remain a common incident among healthcare workers [5,6]. Apart from these risks associated to an inadequate practice, conventional immunizations are related with pain that may be also a source of anxiety and distress for patients receiving the vaccine.

In order to increase immunization compliance, reducing logistical burden and minimizing the risks of transmissible infections, different strategies have been proposed including direct vaccination through the skin. The skin contains important early-warning components of the immune system, including powerful antigen presenting cells (APCs), such as Langerhans and dendritic cells, and immunologically active keratinocytes [7,8]. Therefore, skin is now recognized as an excellent site for vaccination. It comprises a complex network of inducing and effector immune cells, and, in terms of large scale usability, the readily accessible surface of the skin provides an advantage for immunization [9]. Numerous studies have confirmed the feasibility of non-invasive, topical immunization onto the skin using protein subunit [10], DNA vaccines [11] or (even) inactivated and attenuated ones [12]. However, the very low capability of antigenic compounds (usually biomacromolecules with a high molecular weight) to cross the stratum corneum in order to reach the basal epidermis and the dermis, in which the immune cells are located, represents a major hurdle.

In order to overcome this drawback, microneedles [13], lipid vesicles (e.g. deformable liposomes) [14,15] as well as other physical and chemical acute cutaneous barrier disruptors have been proposed. These new methods include apparatus designed to penetrate the *stratum corneum* by electrical methods

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(iontophoresis) [16], particle acceleration [17], low-frequency sonophoresis [18] and microdermabrasion [19].

Another possibility for topical immunization may be the use of nanoemulsions. These dosage forms are thermodynamically stable mixtures of oil and water, stabilized by surfactant molecules, showing dispersed globules in the submicron size range [20]. For topical immunization, nanoemulsions may offer two main advantages. The former would be the possibility of including occlusive substances that may promote the transdermal absorption of lipophilic compounds [21]. The latter would be the presence of a high amount of surfactants that can act as penetration enhancers [22].

Herein, we describe the use of a nanoemulsion composition to facilitate the penetration of an antigenic composition containing outer membrane vesicles from *Salmonella enterica* serovar. Enteritidis (HE), through intact skin after topical administration in laboratory animals. For this purpose, the antigenic compound HE, either in a free form and formulated as aqueous suspension or loaded in poly(methyl vinyl ether-*co*-maleic anhydride) nanoparticles, were incorporated in different topical semisolid formulations. These nanoparticles have demonstrated a high capability to offer protection against a lethal *Salmonella* Enteritidis challenge in mice [23]. Finally, the influence of the formulation on the immunogenic response against this antigen was evaluated.

2. Materials and methods

2.1. Materials

Poly(methyl vinyl ether-*co*-maleic anhydride) or poly(-anhydride) (Gantrez® AN 119; Mw 200,000) was kindly gifted by ISP (Barcelona, Spain). 2-keto-3-deoxyoctonate (KDO), 4-chloro-1-naphthol, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), glycine, bovine serum albumin (BSA), RPMI 1640 medium and mannitol were from (Sigma-Aldrich, Madrid, Spain). BCATM Protein Assay and Microbicinchoninic acid (microBCA) protein assay were from Pierce (Rockford, USA), acrylamide slabs from Bio-Rad (Richmond, USA), trypticase-soy broth from Biomérieux (Marcy L'Etoile, France), formalin from Merck (Hellerup, Denmarck), diaminobenzidine from DakoCytomation (Cambridgeshire, UK) and molecular mass markers (Rainbow RPN 579) from Amersham Pharmacia Biotech (Freiburg, Germany). Lanolin, paraffin, cetostearyl alcohol, petrolatum, cetyl alcohol, poly(ethylene glycol) 400 (PEG 400) and poly(ethylene glycol) 4000 (PEG4000) were purchased from Roig Pharma S.A. (Barcelona, Spain) whereas Labrasol® (caprylocaproyl macrogol-8 glycerides) and Plurol® Oleique CG (polyglyceryl-6 dioleate) were from Gattefosse SA (Saint-Priest, France). Ketamine (Ketolar 50 mg/mL) was from Pfizer (Spain) and xylazine (Herfasin 20/mg/mL) from Le Vet Pharma BV (The Netherlands). Goat anti-mouse IgG horseradish peroxidase conjugated antiserum was from Nordic Immunological Laboratories (Tilburg, The Netherlands). Sulphuric acid, hydrogen peroxide, xylene, methanol, ethanol and Tween 20 were from Panreac Quimica (Castellar del Vallès, Spain). Acetone was obtained from VWR Prolabo (Fontenay-sous-Bois, France). All other chemicals and solvents used were of analytical grade.

2.2. Isolation and purification of the antigen extract (HE)

The HE antigenic complex was obtained from *S.* Enteritidis strain 3934 (wild-type, clinical isolate) grown until the stationary phase in trypticase-soy broth on a rotatory shaker (135 rpm, 37 °C, 24 h). The antigenic complex was obtained by a heat treatment of hole bacteria in saline solution in order to induce the release of small membrane vesicles, as described previously [24,25], with some modifications during the purification step. Briefly, live bacteria were homogenized in saline solution (150 mM NaCl, 10 g of packed cells per 100 mL) and heating in flowing steam for 15 min. After centrifugation at 12,000×*g* for 15 min, the supernatant (containing the antigenic extract) was recovered by tangential flow ultrafiltration (Millipore Pellicon XL PLCGC10, 10 kDa nominal molecular weight limit). The procedure was performed at a feed flow rate of 150 mL/min (Masterflex® Pump Controller), equivalent to an average transmembrane pressure of 25 psi. A volume of deionized water was added to the feed reservoir, equivalent to the amount of permeate removed. Finally, ultrafiltered material was lyophilized (Genesis 12 EL; Virtis, USA) and stored at room temperature.

2.3. Characterization of the HE antigenic complex

Total protein was determined by the BCA™ Protein Assay method with bovine serum albumin as standard. The analysis for 2-keto-3-deoxyoctonate (KDO, exclusive marker of LPS) corrected for 2 deoxyaldoses was performed by the method of Warren modified by Osborn [26].

On the other hand, the profile of proteins associated with the HE complex was assessed by electrophoresis. Thus, SDS-PAGE was performed in 12% acrylamide slabs by the method of Laemmli [27] (Bio-Rad criterion XT 12% bis-Tris). The apparent molecular masses of the proteins present in the antigenic extracts were determined by comparing their electrophoretic mobility with that of molecular mass markers.

The antigenicity of HE was evaluated by immunoblotting using sera from hyper immune rabbit. Immunoblotting were carried out as described by Towbin [28] with some modifications. Briefly, after the SDS-PAGE, the gel was transferred to PVDF (polyvinylidene fluoride papers, pore size of 0.45 μm) (Schleicher & Schuell Bioscience, Amsterdam, The Netherlands) by using a semidry electroblotter (Bio-Rad Laboratories, Richmond, USA) (200 mA, 30 min) in a transfer buffer (0.2 M glycine; 24 mM TRIS; 10% methanol [pH 8.3]). Then, the blots were placed in blocking buffer (3% skimmed milk and 0,15% Tween-20 in 10 mM phosphate-buffered saline [pH 7.4]) overnight at room temperature and, finally, incubated with serum diluted 1:100 in blocking buffer without skimmed milk for 4 h at room temperature. Peroxidase-labeled anti-rabbit antibody (Amersham Pharmacia Biotech) was used as secondary antibody (Nordic Labs, Tilburg, The Netherlands). Blots were washed and developed by incubation in a solution containing H_2O_2 and 4-chloro-1-naphthol, at room temperature in the dark.

2.4. Preparation of HE-loaded poly(anhydride) nanoparticles

Poly(methyl vinyl ether-*co*-maleic anhydride) nanoparticles were prepared by a solvent displacement method previously described [29]. Briefly, 100 mg poly(anhydride) were dissolved in 6 mL acetone. Five milligrams of HE extract were dispersed in 0.2 mL water for injection by ultrasonication for 1 min (Microson™, Misonix Inc. New York, USA). Then, this aqueous phase was added to the acetone phase containing the poly(anhydride). The mixture was incubated for 1 h under magnetic stirring at room temperature. After incubation, nanoparticles were formed by the addition of 15 mL water for injection and further incubation at room temperature for 5 min. The solvents were eliminated under reduced pressure (Buchi R-144, Switzerland) and nanoparticles were purified by centrifugation at 21,000 rpm for 20 min. The pellet was resuspended in 5.8 mL water containing 0.2 g mannitol and subsequent dilution with 6 mL acetone. Finally, the suspension was dried in a Mini Spray-dryer Büchi B191 (Büchi Labortechnik AG, Switzerland).

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