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Topical vaccination with super-stable ready to use nanovesicles

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ABSTRACT

Ultradeformable archaeosomes (UDA) are nanovesicles made of total polar archaeolipids (TPA) from the archaea *Halorubrum tebenquichense,* soybean phosphatidylcholine and sodium cholate (3:3:1 w/w). Fresh dispersions of UDA including different type of antigens are acknowledged as efficient topical vaccination agents. UDA dispersions however, if manufactured for pharmaceutical use, have to maintain colloidal stability upon liposomicidal processes such as sterilization and lyophilization ($_{SLR}$UDA), needed to extend shelf life during storage. The remaining capacity of $_{SLR}$UDA to act as adjuvants was therefore tested here for the first time. Another unexplored issue addressed here, is the outcome of replacing classical antigen inclusion into nanovesicles by their physical mixture. Our results showed that UDA behaved as super-stable nanovesicles because of its high endurance during heat sterilization and storage for 5 months at 40 °C. The archaeolipid content of UDA however, was insufficient to protect it against lyophilization, which demanded the addition of 2.5% v/v glycerol plus 0.07% w/v glucose. No significant differences were found between serum anti-ovalbumin (OVA) IgG titers induced by fresh or $_{SLR}$UDA upon topical application of 4 weekly doses at 600 μg lipids/75 μg OVA to Balb/c mice. Finally, $_{SLR}$UDA mixed with OVA elicited the same Th2 biased plus a non-specific cell mediated response than OVA encapsulated within UDA. Concluding, we showed that TPA is key component of super-stable nanovesicles that confers resistance to heat sterilization and to storage under cold-free conditions. The finding of $_{SLR}$UDA as ready-to-use topical adjuvant would lead to simpler manufacture processing and cheaper products. .

1. Introduction

Parenteral vaccination requires of needles to inject controlled amounts of antigen and adjuvant by intramuscular, subcutaneous or intradermal routes [1]. However, the skeletal muscle and subcutaneous tissue, have relatively few professional antigen presenting cells (APC) and the expression of MHC class II and co-stimulatory molecules is absent in myocytes which cannot directly prime T cells [1]. Injectable vaccines require of trained personnel and particularly in developing countries is associated to a risk of transmitting infections, due to the widespread reuse of non-sterile syringes [2]. These facts, added to the complex manufacture process of liquid vaccines, place the development of needle and pain free noninvasive immunization procedures as a top priority for public health agencies [3]. Topical vaccination is attractive since it has the potential to make vaccine delivery more equitable, safer and equally or more efficient than parenteral vaccination [4].

Topical vaccination however, is challenged by the barrier that the *stratum corneum* interposed between antigens-adjuvants and the skin-associated lymphoid tissue (SALT) lying few hundred micrometers depth from skin surface [4,5]. In conventional topical vaccination, the *stratum corneum* is disrupted by harsh physical or chemical means [6,7] and antigens are combined with high doses of powerful immunomodulatory agents such as cholera toxin, *Escherichia coli* heat-labile toxin, or Toll Like Receptors (TLR) ligands, such as imiquimod or bacterial CpG motifs [8]. The intense pro-inflammatory activity of these topical adjuvants may induce adverse skin reactions [9] and autoimmune diseases [10]. On the other hand, the damaged skin barrier causes local irritation potentiating skin infections [11]. These are the main reasons for the delayed clinical developments of topical vaccination.

In this scenario, soft matter having elasto-mechanical properties enabling the penetration of the intact *stratum corneum*, could pave the way towards safer and efficient topical vaccination. Topical ultradeformable liposomes (UDL), made of soybean phosphatidyl-

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choline (SPC) and edge activators such as sodium cholate (NaChol) have been preclinical employed from the middle 90′s, to induce systemic humoral, cellular, and secretory antigen-specific immune responses [12–17]. Moreover, we previously reported that topical immunization with ovalbumin (OVA) included within ultradeformable archaeosomes (UDA: made of total polar archaeolipids (TPA) extracted from the archaea *Halorubrum tebenquichense* plus SPC and NaChol, 3:3:1 w/w) induces long lasting OVA-specific IgG titers 2 orders higher than those induced by UDL-OVA (OVA included in UDL made of SPC and NaChol, 6:1 w/w, lacking TPA) [18]. A similar trend was found for topical immunization with *Leishmania braziliensis* antigens included in UDA [19]. Topical UDA can penetrate the *stratum corneum* to deliver OVA (MW ∼45 KDa) up to the viable epidermis [20]. Besides, UDA are much more pronouncedly captured by phagocytic cells and immature APC than TPA-lacking nanovesicles [21].

Two major drawbacks, however, are associated to topical vaccination with ultradeformable nanovesicles: their complex manufacture [22] and the poor stability during storage and use, because of the high proportion of surfactants needed to gain ultradeformability [23,24]. The manufacture process of liposomal vaccines consists of antigens suspension in aqueous inner phase [14–16] or its partition within nanovesicles bilayers [17], removal of the free from associated fraction and its quantification. Then, nanovesicles and antigen should be subjected together to vesicle size reduction, sterilization and dehydration. Such processes induce destabilizing effects both on lipid nanovesicles as well as on protein antigens [25]. The antigen inclusion is carried out assuming it is mandatory to elicit a strong antigen-specific immune response, but its avoidance may lead to a much simpler formulations manufacture. Mixing of nanovesicles and antigens, which can be done locally prior to administration, may speed and reduce the cost of industrial manufacture of a final product for topical immunization. On the other hand, nanovesicles intended for pharmaceutical use must retain colloidal, physical and chemical stability for long periods of storage, and if possible, resist to a potential loss of cold chain or, best, to be cold chain-free [25].

TPA are a mixture of *sn*2,3 glycerol ether linked fully saturated polyisoprenoid chains. TPA containing vesicles are more resistant against lipolytic enzymes, hydrolytic or oxidative attacks [26], and against some physical stress like nebulization [27], than TPA-lacking ones. We speculate that TPA may increase the structural stability of ultradeformable bilayers undergoing sterilization, lyophilization and storage. Then, stored as lyophilized, UDA could be rehydrated and mixed with antigen prior to immunization. To test this hypothesis, in this work we submitted UDA to thermal stress of heat sterilization and to freeze and drying stress of lyophilization. Aqueous dispersion of heat sterilized UDA were lyophilized ($_{SL}$UDA) and then stored at 4 and 40 °C. Finally, we screened for immunogenicity of OVA associated to fresh UDA or $_{SLR}$UDA in different ways: OVA included in the aqueous space of UDA, OVA mixed with UDA, OVA and UDA sequentially administered. We showed that TPA is key to confer UDA resistance to heat stress of sterilization and to storage under cold-free conditions. We also confirmed that $_{SLR}$UDA can be used as a ready-to-use topical adjuvant, since no antigen inclusion- only a physical mixture- was sufficient to elicit an antigen-specific systemic immune response.

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (SPC, purity > 90%) was a gift from Lipoid, Ludwigshafen, Germany. Sodium cholate (NaChol), Sephadex G-25, Sephacryl S-200, ovalbumin grade V (OVA), Aluminiumnitratenonahydrat ($Al(NO_3)_3 \times 9H_2O$) and Triton X-100were from Sigma-Aldrich, St. Louis, Missouri. Roswell Park Memorial Institute (RPMI) 1640 medium was from Gibco, Life Technologies (New York, USA). 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) was from Molecular Probes (Eugene, Oregon). DPX (*p*-Xylene-Bis-Pyridinium Bromide) was from Thermo Fisher Scientific, Waltham, USA. Ficoll was from GE Healthcare, Munich, Germany. Hypaque was from Winthrop Products, Buenos Aires, Argentina. Glycerol was from ICN Biomedicals Inc. Aurora, Ohio. Glucose, mannose and trehalose were from Anedra, Argentina. Fetal calf serum (FCS), antibiotic/antimycotic solution (penicillin 10,000 IU/mL, streptomycin sulphate 10 mg/ml, amphotericin B 25 μg/ml), glutamine, and trypsin/ethylenediaminetetraacetic acid were from PAA Laboratories GmbH (Pasching, Austria).

2.2. Archaebacteria growth, extraction, and characterization of total polar archaeolipids

Halorubrum tebenquichense archaea, isolated from soil samples of Salina Chica, Península de Valdés, Chubut, Argentina were grown in basal medium supplemented with yeast extract and glucose at 37 °C. Biomass was grown in 15 L media in a 25 L home-made stainless steel bioreactor and harvested after 96 h growth. Total polar archaeolipids (TPA) were extracted from biomass using the Bligh and Dyer method modified for extreme halophiles [28]. Between 400 mg and 700 mg TPAs were isolated from each culture batch. The reproducibility of each TPA-extract composition was routinely screened by phosphate content [29], and electrospray-ionization mass spectrometry, as described by Higa et al. [18].

2.2.1. Preparation and characterization of nanovesicles

2.2.1.1. Preparation. Archaeosomes (ARC) made of TPA, ultradeformable liposomes (UDL) made of SPC:NaChol 6:1 (w/w), ultradeformable archaeosomes (UDA) made of SPC:TPA:NaChol 3:3:1 (w/w) and liposomes (L) made of SPC were prepared by the film hydration method. Briefly, mixtures of lipids (60 mg of TPA for ARC; 120 mg of SPC and 20 mg NaChol for UDL; 60 mg of TPA, 60 mg of SPC and 20 mg of NaChol for UDA and 60 mg of SPC for L) were dissolved in 3 ml of chloroform:methanol 1:1 v/v. Then solvents were rotary evaporated at 40 °C until elimination. The lipid films were flushed with N_2 and hydrated with 3 ml of aqueous phase (10 mM Tris-HCl buffer pH 7.4 with 0.9% w/w NaCl –Tris-HCl buffer) up to a final concentration of 40 mg/ml total lipids for UDA and UDL, and 20 mg/ml for ARC and L. The resultant suspensions were sonicated (1 h with a bath-type sonicator 80 W, 80 KHz) and extruded 10 times through a sandwich of 0.2 μm and 0.1 μm pore size polycarbonate filters using a Thermobarrel extruder (Northern Lipids, Vancouver, Canada).

To prepare ovalbumin containing nanovesicles (UDA-OVA), lipid films were hydrated with 3 ml of 6 mg/ml of OVA in Tris-HCl buffer. Free OVA was removed from OVA-UDA by gel filtration on Sephacryl S-200 using the minicolumn centrifugation technique [30]. Briefly, aliquots of 300 μl of UDA-OVA were seed on a 3-ml syringe filled with Sephacryl-S200 and centrifugated during 3 min at 700 x g. Fractions of 240–300 μl were collected upon elution with 300 μl of Tris HCl buffer pH 7.4. The elution profile was determined by quantifying proteins and phospholipids in each fraction.

To prepare HPTS/DPX containing UDA and UDL (HPTS/DPX-nanovesicles), lipid films were hydrated with solution of 35 mM HPTS and 50 mM DPX in Tris-HCl buffer pH 8.7. Free HPTS and DPX were removed from HPTS/DPX-nanovesicles by gel filtration on Sephadex G-25 using the minicolumn centrifugation technique, as described before.

2.2.1.2. Size and Z potential. Size and Zeta potential were determined by dynamic light scattering (DLS) and phase analysis light