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Research paper

Liposomal constructs for antitumoral vaccination by the nasal route

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ABSTRACT

Mucosal surfaces are promising routes for vaccination. Among mucosa, airway mucosa provides the opportunity to develop non-invasive approaches for vaccine delivery. In the current study, nasal route was used to investigate the potency of highly versatile di-epitopic liposomal constructs of different size, structure and composition to exhibit antitumor efficiency after prophylactic vaccination in mice. Well-characterized small unilamellar (SUV), multilamellar (MLV), reverse-phase evaporation (REV) and ultraflexible small unilamellar vesicles (Uf-SUV), containing the ErbB2 T-cytotoxic epitope, the influenza-derived HA T-helper epitope and the lipopeptide adjuvant Pam_2CAG, were formulated. These vaccines were administered into the nasal cavity of BALB/c mice, followed by i.v. or s.c. implantation of ErbB2-surexpressing cancer cells. Nasal vaccination with the SUV vaccine resulted in an efficient antitumor activity against lung tumors and a non-significant protection against s.c. tumors. Size, structure and flexibility of liposomes did not impact vaccine immunity and antitumoral efficiency against lung tumors, in contrast to total dose of vaccine or dose of adjuvant. These results showed an undeniable interest of liposomes as lipid-based carriers for antitumor vaccine delivery by the nasal route, opening new perspectives for cancer treatment.

1. Introduction

In the past decade, mucosal surfaces equipped with mucosa-associated lymphoid tissues (MALT), which contain all the necessary players for the induction of an immune response have emerged as promising routes for vaccine delivery [1–3]. Among mucosa, nasal and lung mucosa that contain nasal- (NALT) and bronchial- (BALT) associated lymphoid tissue respectively, are very attractive for vaccination [4,5]. Due to its high accessibility the respiratory tract is suitable for mass vaccination. Furthermore, administration through the airways does not require invasive devices. It is thus painless and ensures patient compliance. There is substantial evidence in the literature that immunization through the respiratory tract triggers a local but also a systemic immune response, as well as a protective response at distant mucosa, such as the vaginal mucosa [6–8]. Indeed, mucosal surfaces comprise an integrated immune network called the common mucosal immune system, establishing communications between mucosal tissues [1–3]. Thus, vaccination through the airway mucosa could be effective in fighting local and distant infections, as well as diseases such as cancer.

Traditional vaccines are made of live-attenuated organisms, killed whole organisms or inactivated toxins. However, these vaccines, which have a high immunogenicity, may occasionally trigger side effects. As well, with live-attenuated pathogens a reversion to virulence is to be feared particularly in immune deficient patients. Therefore, recent efforts in the field of vaccinology focused on

Abbreviations: BALT, bronchial-associated lymphoid tissue; CD, cluster of differentiation; Chol, cholesterol; CV, coefficient of variation; DPG-Mal, dipalmitoyl glycerol-maleimide; ELISA, enzyme-linked immunosorbent assay; EPC, L-α-phosphatidylcholine from chicken egg; Eq., equivalent; HA, influenza hemagglutinin-derived peptide; IL, interleukin; i.p., intraperitoneal; i.v., intravenous; KC, keratinocyte chemoattractant; LPS, lipopolysaccharide; MALP, macrophage-activating lipopeptide; MALT, mucosa-associated lymphoid tissue; MLV, multilamellar vesicle; NAL, nasal lavage; NALT, nasal-associated lymphoid tissue; Pam_2CAG, *S*-[2,3-bispalmitoyloxy-(2R)-propyl]-R-cysteinyl-alanyl-glycine; PBS, phosphate buffered saline; PC, L-α-phosphatidylcholine; PDI, polydispersity index; PG, L-α-phosphatidylglycerol; s.c., subcutaneous; SDC, sodium deoxycholate; SEM, standard error of the mean; SPC, soybean L-α-phosphatidylcholine; REV, reverse-phase evaporation vesicle; SUV, small unilamellar vesicle; Th, T helper; TLR, toll-like receptor; Uf-SUV, ultraflexible small unilamellar vesicle.

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developing synthetic vaccines composed of protein, peptide or polysaccharide antigens or of nucleic acids. These vaccines require the use of particulate drug delivery systems to transport antigens or nucleic acids, to protect them from the environment, but also to increase antigen immunogenicity [9–11].

Among particulate systems that could be used for vaccine delivery, liposomes are very attractive. These phospholipid vesicles, which can deliver a wide range of molecules, considerably enhance the immunogenicity of weak protein antigens or synthetic peptides in the presence of adjuvant. As well, liposomes are highly versatile. Numerous physicochemical parameters including size, charge, structure or composition can be modified to optimize their use in vaccine delivery [12]. Over the last years, different liposomal vaccines were reported to be effective in triggering mucosal or systemic humoral or cytotoxic immune responses, in conferring protection against bacterial or viral infections and/or in inhibiting tumor development after nasal administration in mice [13–20]. However, mucosal sites in the airways that are critical for vaccine efficacy after nasal administration remain unclear. Indeed, nasal administration is an effective and non-invasive technique employed for the delivery of allergens, drugs, gene therapy or pathogens into the respiratory tract of mice. However, the protocol used to perform this administration, namely volume of administration, mouse position during and post delivery or type of anaesthesia dramatically influence the relative distribution of the administered substance between the upper and the lower respiratory tract [21,22]. In studies evaluating liposomal vaccine efficacy, this protocol varied greatly. Thus, the volume of administered vaccine exceeded generally 15 μL, reaching up to 50 μL in some works. This may result in vaccine distribution in the lower respiratory tract and raises the question of the targeted mucosa [13,15–20]. Taking into account the convenience of nasal administration compared to administration into the lower respiratory tract, it seems necessary to clarify whether nasal delivery *per se* is sufficient to get liposomal vaccine efficacy. Besides, physicochemical characteristics may influence activity of a liposomal vaccine. A wide range of physicochemical factors has been explored in the design of liposomal constructs dedicated to nasal vaccination. However, due to the lack of comprehensive studies, critical factors necessary to get an optimal response remain unknown [23].

In this context, we investigated here the activity of a liposomal vaccine administered in the nasal cavity (designated nasal administration) or in the whole respiratory tract (designated airway administration) in mice bearing lung or s.c. tumors overexpressing the human tumor protein antigen ErbB2. This vaccine was constructed with the various elements required for an effective antitumor activity, namely a $TCD8^+$ epitope derived from the ErbB2 protein, a $TCD4^+$ epitope derived from influenza hemagglutinin (HA) and the Pam_2CAG lipopeptide toll-like receptor 2/6 ligand as adjuvant. In addition to the mode of administration, several factors that may influence efficacy of this vaccine after nasal delivery were assessed, namely vaccine dose, as well as liposome composition, structure, size and flexibility. To do so, we prepared small unilamellar vesicle (SUV) vaccines containing different amounts of adjuvant, and/or peptides, vaccines made of multilamellar (MLV) or reverse-phase evaporation (REV) vesicles, as well as ultraflexible liposomes (Uf-SUV), also known as transfersomes.

2. Material and methods

2.1. Vaccine preparation

2.1.1. Reagents

Chicken egg yolk L-α-phosphatidylcholine (EPC), soybean L-α-phosphatidylcholine (SPC), cholesterol (Chol) and sodium deoxycholate (SDC) were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Chicken egg L-α-phosphatidylglycerol (PG) was purchased from Avanti Polar Lipids (Alabama, USA). The thiol-functionalized peptide anchor dipalmitoyl glycerol-maleimide (DPG-Mal) and the lipopeptidic adjuvant Pam_2CAG were synthesized by our team as previously described [13,24]. The ErbB2 peptide (CG-^{p63}TYLPTNASL^{p71}, MW = 1139 g/mol, 82% of purity) and the influenza virus hemagglutinin-derived peptide HA (^{p307}PKYVKQNTLKLAT^{p319}-C, MW = 1606 g/mol, 92% of purity) were obtained from Genecust (Dudelange, Luxembourg). The cysteinyl-glycine or cysteine residues added at the N- or C-terminus of the peptides allowed their coupling to the maleimide function of the peptide anchor. All other chemicals and reagents were of analytical grade and commercially available.

2.1.2. Vaccine composition

Five different vaccines were prepared: a SUV vaccine, a low-adjuvanted SUV vaccine, a REV vaccine, a MLV vaccine, and an Uf-SUV vaccine. SUV, MLV and REV liposomes were prepared from EPC, PG and Chol [14]. Transfersomes were prepared form SPC and SDC [25]. To graft the peptides onto the liposomes, the amphiphilic anchor DPG-Mal was inserted in the liposome bilayer at a fixed peptide molar ratio. This addition requires an equivalent decrease in PC ratio in order to keep constant the overall charge of the liposomes. Then, the two peptides were coupled onto the surface of preformed vesicles at equimolar quantities. SUV, MLV and REV vaccines, except the low-adjuvanted SUV vaccine, had the same composition, namely EPC/PG/Chol/DPG-Mal/Pam_2CAG with a molar ratio of 75/20/50/5/0.2. This composition was chosen according to our previous study [14]. Adjuvant ratio in the low-adjuvanted SUV vaccine was 0.04 instead of 0.2. Composition of Uf-SUV vaccine was SPC/SDC/DPG-Mal/Pam_2CAG with a molar ratio of 68/27/5/0.2.

2.1.3. Liposome preparation

MLV were prepared by the lipid film hydration method. Briefly, EPC, PG, Chol, DPG-Mal and Pam_2CAG were dissolved in chloroform/methanol (9/1 v/v) and mixed in a Pyrex round-bottom tube. The solvent was removed by rotary vacuum evaporation for 1 h. The resulting lipid film was hydrated in 10 mM Hepes buffer (pH 6.5) containing 5% w/v sorbitol at a final phospholipid concentration of 15 mM to obtain MLV suspension. SUV were obtained from the MLV suspension after sonication (1s cycle every 3s) for 50 min at room temperature under a continuous flow of argon, using a Vibra Cell 75041 ultrasonicator (750 W, 20 kHz, Fisher Bioblock Scientific, Illkirch, France) equipped with a 3 mm-diameter tip probe set at 40% amplitude. For REV formulation, a lipid film was prepared as described for MLV formulation, and resuspended in freshly prepared milli-Q water-saturated diethyl ether. One-third of the required volume of hydratation buffer (10 mM HEPES, 5% sorbitol, pH 6.5) was then added and the resulting two-phase system was sonicated (2–3 min) with the sonicator probe until a one-phase dispersion that didn't separate for at least 15 min after sonication was obtained. The diethyl ether was removed by rotary vacuum evaporation under reduced pressure (≈300 bar) resulting in REV formation. The rest of hydratation buffer was then added and the liposomes were placed under vacuum to remove traces of residual ether. All preparations were centrifuged at 10 000 *g* for 2 (REV) or 10 min (SUV) to remove any titanium dust originating from the sonicator probe.

2.1.4. Transfersome preparation

Uf-SUV were prepared as described before by Cevc et al. [25]. Briefly, a chloroform/methanol solution containing SPC, DPG-Mal and Pam_2CAG was evaporated under vacuum in a Pyrex tube for

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