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Synthetic long peptide-based vaccine formulations for induction of cell mediated immunity: A comparative study of cationic liposomes and PLGA nanoparticles

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ABSTRACT

Nanoparticulate formulations for synthetic long peptide (SLP)-cancer vaccines as alternative to clinically used Montanide ISA 51- and squalene-based emulsions are investigated in this study. SLPs were loaded into TLR ligand-adjuvanted cationic liposomes and PLGA nanoparticles (NPs) to potentially induce cell-mediated immune responses. The liposomal and PLGA NP formulations were successfully loaded with up to four different compounds and were able to enhance antigen uptake by dendritic cells (DCs) and subsequent activation of T cells *in vitro*. Subcutaneous vaccination of mice with the different formulations showed that the SLP-loaded cationic liposomes were the most efficient for the induction of functional antigen-T cells *in vivo*, followed by PLGA NPs which were as potent as or even more than the Montanide and squalene emulsions. Moreover, after transfer of antigen-specific target cells in immunized mice, liposomes induced the highest *in vivo* killing capacity. These findings, considering also the inadequate safety profile of the currently clinically used adjuvant Montanide ISA-51, make these two particulate, biodegradable delivery systems promising candidates as delivery platforms for SLP-based immunotherapy of cancer.

1. Introduction

Peptide-based vaccine formulations offer several advantages over protein-based vaccines, as peptides can be easily synthesized and characterized, and are generally more stable [1] and better processed [2] than whole proteins. Synthetic peptides derived from tumour- associated antigens (TAAs) have attracted considerable interest as a basis for cancer vaccines, and vaccination with synthetic long peptides (SLPs), containing all the CTL and T_H epitopes of a TAA, has been applied in mouse models with superior efficacy to protein antigen [2] or minimal MHC class I restricted epitopes [3,4]. In contrast to short peptides, SLPs cannot bind directly to MHC molecules, but have to be taken up and processed by DCs like regular pathogens, inducing a stronger immune response, owing to the activation of both $CD4^+$ and $CD8^+$ T cells [4–6]. However, peptides alone are poorly immunogenic and need to be combined with adjuvants such as immune modulators and/or delivery systems in order to properly activate the innate and adaptive arms of the immune system [1].

Over the past few years, delivery systems that elicit strong immune responses, such as nano-emulsions and particulate delivery systems, have been extensively studied. These include MF59 (Novartis) and AS03™ (GlaxoSmithKline), squalene-based oil-in-water emulsions, which have been approved in Europe for use in the Fluad® and Pandemrix™ influenza vaccines, respectively [7]. Despite the efficacy of these emulsions as influenza vaccine adjuvants, and though some degree of T_H1 responses have been observed, still they lack the ability to stimulate strong T cell responses [8]. Montanide (ISA-51, Seppic) water-in-oil (w/o) emulsions have shown to elicit CTL responses in clinical studies, and have been applied to formulate SLPs in several clinical therapeutic cancer vaccination trials [9–15]. However, the use of Montanide has some important limitations, such as non-biodegradability, limited long-term stability, poorly defined release properties, suboptimal efficacy, and in some cases induction of local adverse side effects [16,17]. Therefore, alternative delivery systems for SLP-based vaccines are highly needed.

Studies have shown that peptide-based vaccines may benefit from particulate delivery systems that mimic the size and structure of a pathogen, facilitating uptake by DCs and increasing cross-presentation of the

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peptide [18–20]. Importantly, they can harbour multiple vaccine components and be actively or passively targeted to DCs, also shaping the induced immune response *via* specific receptors, such as toll-like receptors (TLRs), by incorporation of TLR ligands (TLRLs). Among several particulate delivery systems, both liposomes and polymeric particles have been widely studied.

We have previously studied the application of poly-(lactic-co-glycolic-acid) (PLGA) NPs [21] and cationic liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [22] for the encapsulation of a 24-amino acid-long SLP (referred to as OVA24) harbouring the CTL epitope SIINFEKL of ovalbumin (OVA). Encapsulation of this SLP in PLGA NPs led to a significant enhancement of MHC class I antigen presentation and $CD8^+$ T cell activation compared to free SLP *in vitro* [21]. The SLP-specific $CD8^+$ T cell frequency induced *in vivo* by a liposomal SLP formulation containing poly(I:C) showed a 25 fold increase compared to poly(I:C)-adjuvanted free SLP. Furthermore, intradermal immunisation of mice with SLP-liposomes combined with poly(I:C) led to a strong cytotoxic activity, in contrast to vaccination with a mixture of free SLP and poly(I:C) [22].

In this study, considering the different physicochemical properties that cationic liposomes and PLGA NPs have, we further investigated the potential of both systems in a direct comparative study. For that purpose, we studied the co-delivery of two SLPs containing the CTL (OVA24) and the T helper (T_H, OVA17) epitopes of OVA together with poly(I:C) and $Pam3CSK_4$, a TLR3 and TLR2/1 ligand, respectively, in comparison to the clinically used adjuvants Montanide ISA-51 and SWE, a squalene oil-in-water emulsion. OVA24/OVA17-loaded PLGA NPs and liposomes with or without the TLR-ligands were characterized for particle size, zeta-potential, and for peptide and TLR loading efficiencies. The obtained formulations were assessed *in vitro* and *in vivo* for their potency to induce $CD8^+$ and $CD4^+$ T cell immune responses. The observed T cell immune responses induced by our particulate formulations were superior to the ones observed with the emulsions (Montanide ISA-51 or SWE), with the liposomal formulation outperforming PLGA NPs. These findings reinforce that particulate systems are promising delivery vehicles for clinical application in cancer immunotherapy.

2. Materials and methods

2.1. Materials

The ovalbumin-derived SLP OVA24 [DEVSGLEQLESIINFEKLAAAAAK], including the CTL epitope SIINFEKL, and the short peptide OVA8 [SIINFEKL] were produced and purified at the GMP facility of the Clinical Pharmacy and Toxicology Department at the Leiden University Medical Center [5]. The ovalbumin-derived SLP OVA17 [ISQAVHAAHAEINEAGR], including the helper T_H-epitope AAHAEINEA, was produced in the Immunohematology and Blood Transfusion Department of the Leiden University Medical Centre. The lipids DOPC and DOTAP were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and the TLR ligands (poly(I:C) and $Pam3CSK_4$) with their labelled analogues (rhodamine and fluorescein) were obtained from InvivoGen (Toulouse, France). Resomer® RG 502H was purchased from Boehringer Ingelheim (Ingelheim, Germany). 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), dichloromethane (DCM), dimethyl sulfoxide (DMSO), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (ACN) was obtained from Biosolve BV (Valkenswaard, the Netherlands), and PVA 4-88 (31 kDa) was purchased from Fluka (Steinheim, Germany). Sodium hydroxide was purchased from Boom (Meppel, Netherlands). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Eugene, Oregon, USA). Acetonitrile (ACN), chloroform, and methanol were obtained from Biosolve BV (Valkenswaard, the Netherlands) and Vivaspin 2 centrifuge membrane concentrators were purchased from Sartorius Stedim Biotech GmbH (Goettingen, Germany). Iscove's modified Dulbecco's medium (IMDM; Lonza Verniers, Belgium) was supplemented with 8% (*v*/*v*) foetal calf serum (Greiner Bioscience, Alphen a/d Rijn, the Netherlands), 50 μM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, Netherlands), 100 IU/mL penicillin and 2 mM glutamine (Life Technologies, Bleiswijk, the Netherlands). Deionized water with a resistivity of 18 MΩ·cm was produced by a Millipore water purification system (MQ water). Montanide ISA-51 was purchased from Seppic SA (Paris, France). Squalene oil-in-water emulsion (SWE) contained 3.9% (*w*/*v*) squalene, 0.5% (*w*/*v*) Tween 80 and 0.5% (*w*/*v*) Span 85 in 10 mM citrate buffer pH 6.5 and it was manufactured by the Vaccine Formulation Laboratory of the University of Lausanne. Phosphate buffer was composed of 7.7 mM $Na_2HPO_4 \cdot 2H_2O$ and 2.3 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.4 (10 mM PB, pH 7.4). MQ water and 10 mM PB, pH 7.4, were filtered through a 0.22-μm Millex GP PES-filter (Millipore, Ireland) before use. Phosphate-buffered saline, which was used for all the *in vitro* and *in vivo* assays was purchased from B.Braun (Meslungen, Germany). All other chemicals were of analytical grade and all aqueous solutions were prepared with milli Q water.

2.2. Mice

Female C57BL/6 ($H\text{-}2^b$) mice were purchased from Charles River (L'Arbresle, France) and congenic CD45.1 (Ly5.1) mice were bred at the Leiden University Medical Centre animal facility and used at 8–14 weeks of age according to the Dutch Experiments on Animal Act, which serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe.

2.3. Liposome preparation

Cationic liposomes loaded with SLPs were prepared by using the thin film dehydration–rehydration method, as described previously [22]. Briefly, DOTAP and DOPC (1:1 molar ratio) in chloroform were mixed in a round-bottomed flask to reach a concentration of 10 mg total lipid per mL of final liposome dispersion. The formed dry film was rehydrated with 2 mL of 1 mg/mL OVA24 and/or OVA17 in ACN/H_2O 1:1 (*v*/*v*); for the liposomes loaded with both OVA24 and OVA17, the aqueous solution of the SLPs was first adjusted to pH 8.5. For poly(I:C)-loaded liposomes, the ligand (including 0.5% fluorescently-labelled equivalent) in a total concentration of 200 μg/mL was added dropwisely to the dispersion, while for the $Pam3CSK_4$-loaded liposomes, the TLR ligand was dissolved in chloroform together with the lipids, before the dry film formation. After the lipid film hydration, the liposome dispersion was snap-frozen in liquid nitrogen, followed by overnight freeze-drying. Dehydrated–rehydrated liposomes were generated by gradually adding 10 mM PB, pH 7.4, to the freeze-dried lipid cake. Liposomes were down-sized by high-pressure extrusion at room temperature using a Lipex extruder (Northen Lipids Inc., Canada) and concentration of peptide-loaded liposomes was performed by using a VivaSpin 2 centrifugation concentrator (PES membrane, molecular weight cut-off (MWCO) 300 kDa) as described previously [22].

2.4. PLGA NPs preparation

Nanoparticles loaded with OVA24 and/or OVA17 and/or TLRLs were prepared by using a double emulsion with solvent evaporation method [21]. In brief, 50 mg of PLGA dissolved in 1 ml of dichloromethane, with or without 0.25 mg $Pam3CSK_4$ (and 0.1% $Pam3CSK_4$ Rhodamine-labelled), was emulsified under sonication (30 s, 20 W) with 1.4 mg OVA24, 1 mg OVA17, 1 mg poly(I:C) (and 0.1% poly(I:C) fluorescein-labelled, dissolved in 50% ACN in 0.25 mM NaOH + 400 μL Hepes pH 8.0). To this first emulsion (w1/o), 2 ml of 1% PVA solution was added immediately, and the mixture was emulsified again by sonication (30 s, 20 W), creating a double emulsion (w1/o/w2). The emulsion was then added dropwise to 10 ml of extraction medium (0.3% *w*/*v* PVA) previously heated to 40 °C under agitation, to allow quick solvent