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Induction of humoral and cellular immune responses by antigen-expressing immunostimulatory liposomes

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ABSTRACT

Recently we have shown that liposomes can be used as artificial microbes for the production and delivery of DNA-encoded antigens. These so-called antigen-expressing immunostimulatory liposomes (AnExILs) were superior in inducing antigen-specific antibodies compared to conventional liposomal protein or DNA vaccines when tested in mice after i.m. immunization. In this study, we investigated the capacity of AnExILs to induce T-cell responses. By using a plasmid vector encoding a model antigen under control of both the prokaryotic T7 and the eukaryotic CMV promoter we hypothesized that antigen production could lead to CTL activation via two distinct routes: *i.* production of antigens inside the AnExILs with subsequent cross-presentation after processing by APCs and *ii.* endogenous production of antigens after AnExIL-mediated transfection of the pDNA. Although we were not able to demonstrate transfection-mediated expression of luc-NP in mice, i.m. injection of AnExILs producing luc-NP resulted in T-cell responses against the encoded NP epitope, as determined by tetramer staining. T-cell responses were comparable to the responses obtained after i.m. injection of naked pDNA. In order to find out whether CTL activation was caused by cross-presentation of the exogenous antigens produced inside AnExILs or by endogenous antigen production from transfection with the same pDNA source a second study was initiated in which the contribution of each of these effects could be separately determined. These results demonstrate that the observed T-cell responses were not exclusively caused by cross-presentation of the AnExIL-produced antigens alone, but were rather a combination of dose-dependent antigen cross-presentation and low levels of endogenous antigen production.

1. Introduction

Immunotherapy of cancer requires the conversion of weak tumor antigens into strong immunogens in order to overcome the often weakened or tolerized immune system of the cancer patients. Moreover, to prevent occurrence of tumor resistance to the immunotherapy, mixtures of antigens rather than single tumor associated antigens are preferred [1]. DNA vaccines have been extensively explored for cancer immunotherapy (reviewed by [2–4]). The popularity of DNA can be ascribed to its ease of manipulation, production, and formulation in addition to the possibility to incorporate multiple antigenic epitopes in one vector. Moreover, since DNA vaccination leads to antigen production within the cytosol, it often induces strong CTL responses needed for eradication of tumor cells [5]. However, in spite of high expectations based on their efficacy in preclinical models, immunogenicity of first generation DNA vaccines in clinical trials was shown to be poor, and despite recent improvements in delivery methods for DNA vaccines, no DNA vaccines have yet been licensed for human use [3].

Recently, we have developed an entirely new concept of vaccination that entails the production of protein antigens inside liposomes using cell-free protein synthesis systems. These so-called antigen-expressing immunostimulatory liposomes (AnExILs) combine the production and delivery of antigens in one system [6,7]. It has two major advantages over conventional DNA vaccines. First, AnExILs do not only rely on the often inefficient transfection of antigen-encoding DNA into the vaccinee's cells in order to be effective. In addition to expression of endogenous antigens in the vaccinee's cells, the antigens will be exogenously produced inside the AnExILs prior to delivery. Second, since AnExILs make use of bacterial proteins (extracts or a mix of recombinant proteins) for cell-free protein synthesis, they have a strong adjuvant effect which is expected to strengthen the immune

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response against the produced antigens. Previous work indeed demonstrated that AnExILs, when compared to conventional DNA, protein or mixed DNA/protein liposomal vaccines, were superior in inducing antigen-specific antibody responses, which could be directly ascribed to the adjuvant-effect of AnExILs [6].

The objective of this study was to find out whether AnExILs are, besides generation of antibodies, also capable of inducing T-cell responses against the produced antigens, which is a prerequisite for further development of AnExILs as cancer vaccine. For this, a model CTL epitope was genetically fused to the C-terminus of reporter enzymes (luciferase or β-galactosidase) to assess antigen production, quality and degree of antibody production and epitope specific T-cell responses. The results show *i.* the capacity of AnExILs to induce NP-specific T-cell responses and *ii.* interesting clues to the mechanism of the T-cell induction with AnExILs.

2. Experimental section

2.1. Materials

Egg-derived L-α-phosphatidylcholine (EPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-polyethylene glycol (PEG) 5000 (DSPE-PEG 5000) and 1,2-dioleoyl-*sn*-glycero-3-([N(5-amino-1-carboxypentyl) iminodi-acetic acid] succinyl) (DOGS-NTA) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). Luria Broth, 2-mercaptoethanol, adenosine-5′-triphosphate (ATP), phosphoenol-pyruvate (PEP), cytidine-5'-triphosphate (CTP), guanosine-5′-triphosphate (GTP), 3′-5′-cyclic adenosine monophosphate (cAMP), folinic acid, cholesterol (CHOL) and β-galactosidase enzyme (400 IU/mg) and each of the 20 encoded amino acids were purchased from Sigma (Saint Louis, MO, USA). The Fluorescein di-β-D-galactopyranoside (FDG) was supplied from Marker Gene Technologies (Eugene, OR, USA). *E-coli* tRNA, creatine kinase and creatine phosphate were obtained from Roche (Basel, Switzerland). Uridine 5′-triphosphate (UTP) and T7 polymerase were supplied from Fermentas (Burlington, Ontario, Canada). Dithiothreitol (DTT), deMan-Rogosa-Sharpe (MRS) Broth and pyruvate kinase (PK) were from Flucka (Seelze, Germany). Rabbit polyclonal anti-β-galactosidase IgG and Cy-5 conjugated goat IgG anti-rabbit immunoglobulin was from Abcam (Cambridge, UK). Horseradish Peroxidase (HRP)-labeled goat anti-mouse total IgG and HRP-labeled rabbit anti-mouse IgG1 were purchased from Invitrogen (Breda, The Netherlands). HRP-labeled Rat monoclonal anti-mouse IgG2a was obtained from Abcam (Cambridge, The United Kingdom). PEG 8000 was from Promega (Madison, WI, USA). All other materials used were of analytical or pharmaceutical grade.

2.2. Plasmids

Plasmid pIVEX2.2EM-LacZ-NP encoding *E. coli* β-galactosidase fused to H3N2 influenza nucleoprotein epitope ($NP_{366–374}$, ASNENMDAM), under control of the prokaryotic T7 promoter, was used for *in vitro* production of β-galactosidase-NP, as a model antigen for vaccination studies. Plasmid pVAX-LacZ-NP, under control of eukaryotic CMV promoter was used in DNA vaccines for immunization studies. Furthermore, pDUAL.GC-Luc-NP, encoding firefly luciferase fused to NP epitope, with both prokaryotic T7 and eukaryotic CMV promoters, which allows *in vitro* and *in vivo* expression of Luc-NP protein using single plasmid DNA, was used for *in vivo* antigen expression and immunization against influenza NP epitope.

2.3. Preparation of cell-free protein synthesis system and AnExILs

The *E. coli* Rosetta-gami™ strain, which contains pRARE encoding rare tRNA codons and is devoid of endogenous β-galactosidase enzyme (Novagen, The Netherlands), was used to make S30 bacterial extract as described previously [7]. A coupled *in vitro* transcription/translation reaction mixture (further referred to as IVTT mix), consisted of 30% (v/v) S30 extract, 175 μg/ml tRNA, 250 μg/ml creatine kinase, 5.8 mM magnesium acetate, 260U T7 polymerase, and 50% (v/v) low-molecular-weight mix (LM mix) containing: 110 mM HEPES, 3.4 mM DTT, 2.4 mM ATP, 1.6 mM CTP, 1.6 mM GTP, 1.6 mM UTP, 0.8 M creatine phosphate (CP), 0.65 mM cAMP, 0.05 mM folinic acid, 0.21 M potassium acetate, 27 mM ammonium acetate, 2 mM each of the 20 amino acids, and 8% (v/v) PEG8000, was used for protein synthesis.

Neutral PEG-liposomes consisting of EPC, CHOL and DSPE-PEG 5000 with a molar ratio of EPC:CHOL:PEG 5000 = 1.6:0.9:0.025 were prepared as described previously [7]. For preparation of AnExIL formulations with antigen expressed inside/outside and inside liposomes (further referred to as AnExIL and AnExIL-In), 100 μl of IVTT mixture and pDNA, was used to rehydrate a batch of 6 μM of PEG-lipid cakes in order to form liposomes encapsulating IVTT mix and pDNA. The liposomes were incubated on ice for 10 min to complete the rehydration process. For AnExIL-In protein expression outside liposomes was inactivated by adding RNase with a final concentration of 10 μg/ml, to the liposomal suspension. Both AnExIL and AnExIL-In were incubated at 30 °C for 3 h to allow protein synthesis to complete.

2.4. Mice for immunization and in vivo imaging of antigen expression

Female C57BL/6 (B6) mice, 6-8 weeks old (Charles River, Netherlands), were housed in groups of 5 and maintained in the animal facility of Utrecht University with a 12 h day and night schedule, while food and water were *ad libitum*. The experiments were approved by the Ethical Committee for Animal Experimentation of Utrecht University.

2.5. Immunization with AnExIL-pDUAL vaccine and imaging of in vivo antigen expression

AnExIL-pDUAL vaccine containing pDUAL-Luc-NP, which can be expressed both in bacterial cell-free expression system and *in vivo* in mammalian cells, was prepared as described earlier in the section of preparation of AnExILs. Briefly, 100 μl of IVTT mix with either 50 μg (100 nM) or 100 μg (200 nM) of pDUAL-LUC-NP was used to rehydrate a batch of freeze-dried liposomes and incubated for 3 h at 30 °C to produce Luc-NP antigen *in vitro*. For imaging of *in vivo* antigen expression and immunization, mice (5 per group) were vaccinated i.m. twice with three-week intervals. The animals received 100 μl of different formulations (Table 1), divided over two injections of 50 μl, which were injected in each of the hind leg muscles. Seven days after the last immunization, mice were sacrificed, spleens were dissected and single cell suspensions of splenocytes were analyzed for cellular immune responses against NP epitope.

For imaging of *in vivo* antigen expression in vaccinated mice, at the days 0, 1 and 4 post immunization, mice were anesthetized with isoflurane (Abbott Laboratories) and injected intraperitoneally with 100 μl solution of the substrate luciferin (25 mg/ml, Promega) supplemented with 1.25 mM ATP and 10 min later the luminescence produced by active luciferase was acquired during 5 min with a photon imager (Biospace Lab). The signal from the areas of interest (ROIs) was analyzed using the Biospace Lab M^3 vision software.

Table 1
Immunization study design.

AnExIL-pDUALvaccines ($n = 5$, 2 immunizations i.m.)	Injection	
	Amount (μl)	DNA dose (μg)
AnExIL-Luc-NP (pDUAL-Luc-NP)	(2 × 50)	(50)
AnExIL- Luc-NP (pDUAL-Luc-NP)	(2 × 50)	(100)
Control naked DNA (pDUAL-Luc-NP)	(2 × 50)	(100)