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Elucidating the mechanisms of protein antigen adsorption to the CAF/NAF liposomal vaccine adjuvant systems: Effect of charge, fluidity and antigen-to-lipid ratio

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ABSTRACT

The reverse vaccinology approach has recently resulted in the identification of promising protein antigens, which in combination with appropriate adjuvants can stimulate customized, protective immune responses. Although antigen adsorption to adjuvants influences vaccine efficacy and safety, little is generally known about how antigens and adjuvants interact at the molecular level. The aim of this study was to elucidate the mechanisms of interactions between the equally sized, but oppositely charged model protein antigens α-lactalbumin and lysozyme, and i) the clinically tested cationic liposomal adjuvant CAF01 composed of cationic dimethyldioctadecylammonium (DDA) bromide and trehalose-6,6′-dibehenate (TDB) or ii) the neutral adjuvant formulation NAF01, where DDA was replaced with zwitterionic distearoylphosphatidylcholine (DSPC). The effect of liposome charge, bilayer rigidity, isoelectric point and antigen-to-lipid ratio was investigated using dynamic light scattering, transmission electron microscopy, differential scanning calorimetry, intrinsic fluorescence and Langmuir monolayers. The net anionic α-lactalbumin adsorbed onto the cationic liposomes, while there was no measureable attractive interaction with the zwitterionic liposomes. In contrast, the net cationic lysozyme showed very little interaction with either types of liposome. Adsorption of α-lactalbumin altered its tertiary structure, affected lipid membrane packing below and above the phase transition temperature, and neutralized the liposomal surface charge, resulting in reduced colloidal stability and liposome aggregation. Langmuir studies revealed that α-lactalbumin was not squeezed out of DDA monolayers upon compression, which suggests additional hydrophobic interactions.

Such interactions are thus likely to affect the way vaccine antigens are presented to antigen-presenting cells, and may play an important role for the efficacy of the vaccine-induced immune response. These studies thus exemplify the importance of characterizing the molecular interactions between the vaccine antigen and adjuvant along with immunogenicity and efficacy studies.

1. Introduction

In recent years, the reverse vaccinology approach towards subunit vaccine design has resulted in the identification of a number of promising pathogen-derived recombinant protein antigens with significantly improved safety profiles, as compared to the live attenuated and whole inactivated pathogens that traditionally have been used as vaccines [1]. However, since highly purified protein-based antigens in general are weakly immunogenic by themselves, co-administration of appropriate adjuvants is required to potentiate the immune response [2]. Aluminum-based adjuvants have routinely been used in marketed human vaccines to stimulate long-lived protective immune responses, but their applicability is limited by the fact that they only induce antibody-mediated immune responses [3]. Stimulation of cell-mediated and/or mucosal immunity is though a prerequisite for preventing more difficult infectious diseases like tuberculosis, malaria and AIDS with vaccines [4]. A number of novel adjuvant systems that can induce such immune responses are therefore in clinical development [5].

A promising example is the cationic liposomal adjuvant CAF01 (Statens Serum Institut, Denmark), which is based on a binary mixture of the cationic surfactant dimethyldioctadecylammonium (DDA)

Abbreviations: Cryo-TEM, Cryo transmission electron microscopy; DDA, dimethyldioctadecylammonium bromide; DLS, dynamic light scattering; DSC, differential scanning calorimetry; DSPC, distearoylphosphatidylcholine; SOI, site of injection; TDB, trehalose-6,6′-dibehenate

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bromide and the immunopotentiator α,α′-trehalose 6,6′-dibehenate (TDB) [6]. This adjuvant has been tested in phase 1 clinical trials in combination with the tuberculosis fusion antigen Ag85B-ESAT6 (H1) (NCT ID: NCT00922363) and an HIV-1 peptide mix (NCT ID: NCT01141205; NCT01009762), respectively. These gel state DDA/TDB liposomes potentiate a strong $CD4^+$ T-cell response characterized by a mixed Th1/Th17 profile [7–9]. Their adjuvant properties are highly dependent on their physicochemical properties like surface charge, size and membrane rigidity, which are decisive for the adjuvant mechanism(s) and thus the vaccine efficacy and safety [10–12]. Studies have thus shown that co-administration of antigens with such cationic liposomes leads to the induction of stronger and qualitatively different antigen-specific immune responses than co-administration with neutral or anionic liposomes of similar thermotropic phase behavior [13,14]. In addition, the gel state of the liposomes is apparently a prerequisite for their adjuvant activity, because replacement of the DDA component with unsaturated or shorter-chain analog abolishes their adjuvant activity [10]. Compared to neutral/anionic liposomes with similar thermotropic phase behavior, the CAF01 adjuvant has distinct advantages since it mediates the avid binding and retention of the antigen(s) at the site of injection (SOI) and enhances immunogenicity, eventually resulting in strong cell-mediated immune responses [7,11]. However, a prerequisite for the strong adjuvant effect of CAF01 is that the antigen is co-localized with the immunopotentiator, either through encapsulation or by strong surface adsorption: Kamath et al. showed that administration of free antigen (TB vaccine candidate H1) one day before or simultaneous to vaccination with the vaccine containing H1 complexed to CAF01 reduced the ability of the vaccine to induce a CMI response. This was related to the presence of high amounts of dendritic cells exposed only to the antigen, which due to lack of co-stimulation by the adjuvant would create temporary anergy to activation of antigen-specific T-cells [8]. It is thus pivotal to understand interactions at the molecular level between vaccine candidates and adjuvants in order to ensure optimal vaccine efficacy.

Electrostatic interactions are generally considered to be the main driving forces for surface adsorption of protein antigens onto cationic liposomes [11,15,16]. However, little is generally known about how antigens and adjuvants interact at the molecular level. This is primarily because the application of most analytical methods for characterization of antigen-adjuvant mixtures is limited by i) the structural complexity of protein antigens and adjuvants, ii) the particulate nature of most adjuvants and iii) the relatively low protein antigen doses required for efficacy [17]. Interactions between liposomes and proteins can be measured by using a range of analytical methods; indirect measurements include for example an assessment of the colloidal stability, the permeability of the liposomal membrane and the structural changes of the proteins upon adsorption, or direct measurements including e.g. single molecule techniques, isothermal titration calorimetry (ITC) and monolayer techniques.

In the present study, we performed systematic adsorption studies by investigating the two equally sized, but oppositely charged highly pure and well-characterized model proteins α-lactalbumin and lysozyme (Table 1) and the cationic liposomal adjuvant CAF01 or the neutral adjuvant formulation NAF01, in which DDA has been replaced with zwitterionic distearoylphosphatidylcholine (DSPC). The aim was to elucidate their mechanisms of interaction via biophysical investigations by applying a number of different analytical methods. These included dynamic light scattering (DLS) and cryo-transmission electron microscopy (cryo-TEM). Subsequently, the effect of the interactions on the membrane thermotropic phase behavior as well as the protein structure was investigated by applying differential scanning calorimetry (DSC) and intrinsic fluorescence, respectively. Finally, the molecular interactions were studied further by using the Langmuir monolayer technique.

2. Materials and methods

2.1. Materials

DDA and DSPC were obtained from Avanti Polar Lipids (Alabaster, AL, USA). TDB was synthesized by Clausen-Kaas A/S (Farum, Denmark). The model proteins were obtained from Sigma-Aldrich (St. Louis, MO, USA); α-lactalbumin (L5385, ≥85%), α-lactalbumin Ca^{2+} depleted (L6010, ≥85%), and lysozyme (L6876, >90%). MeOH and $CHCl_3$ (extra pure) were purchased from VWR (Leuven, Belgium) and Merck (Darmstadt, Germany), respectively. Purified Milli-Q water was used for all buffers. The protein stock solutions were prepared in 10 mM Tris buffer (pH 7.4), and their concentrations were determined by UV spectroscopy at 280 nm by using a NanoDrop spectrophotometer (Thermo scientific, Wilmington, DE, USA) applying published extinction coefficients.

2.2. Preparation of liposomes by the thin film method

The liposomes were prepared by the thin film method essentially as described previously [6], but with a few modifications. Briefly, weighed amounts of DDA, TDB and DSPC were dissolved in $CHCl_3$–MeOH (9:1, v/v) and aliquots from these stocks were mixed in a 50 mL round-bottomed flask resulting in lipid mixtures of different molar ratios (Table 2). The total lipid content per batch was 22.34 μmol. The organic solvent was evaporated under vacuum resulting in the formation of thin lipid films. The films were stripped twice with EtOH and dried overnight to remove trace amounts of the organic solvents. The lipid films were rehydrated with Tris buffer (10 mM, pH 7.4) and sonicated for 5 min using a Branson 2510 Ultrasonic Cleaner (Danburry, CT, USA), followed by heating at 80 °C in a water bath. Every 10 min, the dispersions were whirl-mixed vigorously by using a VELP Scientifica wizard vortex mixer (Usmate, Milano, Italy) combined with tip-sonication for 20–120 s by applying a 150 W Branson tip-sonicator (85-% of the duty cycle) to reduce the size and to avoid size reduction by e.g. extrusion that causes an unnecessary loss of lipid during processing.

The average liposome size distribution and polydispersity index (PDI) were analyzed by DLS by using the photon correlation spectroscopy technique. The surface charge of the particles was estimated by analysis of the zeta potential (laser-Doppler electrophoresis). For the size measurements, the samples were diluted 10 times, whereas for the zeta potential measurements, the samples were diluted 300 times in 10 mM Tris buffer to a lipid concentration of approximately 14.9 μM. The measurements were performed at 25 °C by using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. For viscosity and refractive index, the values of pure water were used. Malvern DTS v.6.20 software was used for data acquisition and analysis. A Nanosphere™ Size

Table 1
Physicochemical characteristics of α-lactalbumin and lysozyme [18–21].

Protein	Dimensions (nm)	M_w (Da)	pI	Net charge at pH 7.0	Hydrophobicity (Cal/residue)[a]	Surface hydrophobicity[b]
α-Lactalbumin	2.3 × 2.6 × 4.0	14,175	5.5[c]	−5.3[c]	1150	1.66
Lysozyme	4.5 × 3.0 × 3.0	14,300	11.1[c]	8.4[c]	970	7.49

[a] Calculated from the amino acid sequence.
[b] Determined as the retention coefficients from hydrophobic column chromatography.
[c] Calculated from PROPKA 3.1 from PDB file (2LYZ.pdb/1F6S.pdb).