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Squalene containing solid lipid nanoparticles, a promising adjuvant system for yeast vaccines

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ABSTRACT

Potent adjuvant systems are required for subunit and single antigen based vaccines to provide sufficient immunogenicity. Furthermore, adjuvants can reduce the required number of immunisations and the antigen amount. Squalene nanoemulsions, like MF59® and AddaVax™, are potent, safe and well characterised adjuvant systems and approved for use in humans. Here, we developed squalene containing solid lipid nanoparticles, which can be sterilised by steam sterilisation and stored as freeze-dried power together with a yeast-based vaccine. Detailed size measurements using dynamic and static light scattering were applied, as the immune stimulating effect of squalene emulsions is mainly dependent on the particle size. The size range of AddaVax™ (120–170 nm) was favoured for the developed squalene containing solid lipid nanoparticles. Differential scanning calorimetry (DSC) and H NMR studies were performed to characterise the interactions of the incorporated liquid squalene with the solid hard fat matrix. A homogeneous distribution as liquid domains in the solid glyceride structure was suggested for the liquid squalene. The developed adjuvant was compared with Freund's adjuvant and a commercially available squalene nanoemulsion in a vaccine trial in the mouse model with a yeast-based vaccine directed against the infectious bursal disease virus. All squalene-based adjuvants showed excellent biocompatibility and provided immune stimulating properties comparable to Freund's adjuvant.

1. Introduction

Pathogens are recognised by the immune system via multiple mechanisms, like size, cell wall composition, surface charge, hydrophobicity, pathogenic DNA and specific characteristics [1–4]. Especially subunit and single antigen vaccines are limited by a low immunogenicity. Thus, several adjuvant systems have been developed to compensate this vaccine downside and to minimise the number of immunisations and antigen amount [5]. Jules Freund developed the Freund's adjuvant of emulsified antigen and *Mycobacteria tuberculosis* in paraffin oil in 1937, which is still regarded as the gold standard in immune stimulation, but it shows massive side effects [6,7]. Squalene nanoemulsions like AddaVax™ or MF59® have been widely used and are well-characterised and biocompatible adjuvants [8,9]. Thus, the most prominent squalene nanoemulsion MF59® has been applied in more than 150 million influenza vaccines since its approval in 1997 [10]. Different from the classical water in oil adjuvants like Freund's adjuvant, which generate an antigen depot at the injection side, squalene nanoparticles induce a local inflamed and immune competent region at the injection side [11]. As the injection of the single ingredients of MF59® or squalene in water emulsions in the size range of 1 μm did not affect an immune reaction, the size distribution in the nanometre range around 160 nm plays an important role [12–14].

Here we developed a squalene containing solid lipid nanoparticle formulation, which can be stored together with a yeast-based vaccine as dry powder. *Kluyveromyces lactis* based vaccines expressing the virus capsid forming protein VP2 of the infectious bursal disease virus (IBDV) already showed its great potential as anti-IBDV yeast-based vaccines [15]. The study focuses on the detailed size measurement of the developed nanoparticle formulations and the characterisation of interaction between the hard fat matrix and the liquid squalene. Finally, all adjuvants were investigated in a vaccine trial in a mouse model.

2. Materials and methods

2.1. Adjuvant preparation

The developed adjuvant JS-3o was prepared by the hot melt emulsion technology. The lipid phase of squalene (Sigma-Aldrich,

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Munich, Germany) and Softisan 154 (Cremer Oleo, Hamburg, Germany) as solid lipid matrix was mixed in 1:1 ratio at 65 °C. The 10% lipid (50% squalene and 50% Softisan 154), stabilised with 5% Poloxamer 188 (Kolliphor P188, BASF, Ludwigshafen, Germany) as an emulsifier, was pre-emulsified in 10 mM sodium citrate solution pH 6.5 using an Ultra-Turrax® T18 (IKA, Staufen, Germany) at 20000 rpm for 5 min. The pre-emulsions were further processed at 65 °C with a high pressure homogeniser (EmulsiFlex C5, Avestin, Ottawa, Canada) to achieve a comparable size distribution like AddaVax™ (120–170 nm). The compositions of the commercial AddaVax™ formulation and the new formulation JS-3o are compared in Table 1. The squalene concentration was 43 mg per ml in AddaVax™ and 50 mg per ml in JS-3o.

Lyophilisation of the adjuvants was performed with 10% sucrose (Fluka, Buchs, Switzerland) and 2% polyvinyl alcohol (Gohsenol EG-05PW, Nippon Gohsei, Düsseldorf, Germany) as cryoprotectants. The samples were frozen rapidly with liquid nitrogen at −196 °C and lyophilised on a Christ Alpha 2–4 freeze-drier (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) in combination with a Vacuubrand RC 6 vacuum pump (Vacuubrand GmbH, Wertheim, Germany) for 24 h at 0.05 mbar. The lyophilisate was reconstituted by adding water for injection to the original volume and gentle agitation.

2.2. Photon Correlation Spectroscopy (PCS)

PCS measurements were carried out on a Zetasizer Nano ZS (Malvern Instruments, Malvern, United Kingdom) and the Zetasizer software 6.30. All samples were diluted with 0.22 μm filtered bidistilled water to a lipid concentration of 0.025% and measured in quintuplicate in the automatic mode, suggesting 12–14 sub runs each. After 180 s equilibration time, measurements were performed at 25 °C in the backscattering mode at 173° to prevent multiple scattering events.

2.3. Static light scattering

Static light scattering measurements were made on a Mastersizer 2000 (Malvern Instruments, Malvern, United Kingdom) combined with a Hydro 2000 S wet dispersion unit. The samples were measured at a laser obscuration of 5% corresponding to 11–15% obscuration of the blue laser in purified water. Series of 5 runs were evaluated by the Mastersizer 2000 software version 5.60 using the Mie theory, assuming spherical particles with a refractive index of 1.494, absorption of 0.001 and a refractive index of 1.33 for the dispersant as optical properties.

2.4. Differential Scanning Calorimetry (DSC)

Thermal analyses were performed on a Differential Scanning Calorimeter 200 (Netzsch, Selb, Germany) in closed aluminium pans. The measurement cycle consisted of a heating segment from 5 °C to 85 °C, followed by a cooling step to 5 °C and a second heating cycle to 85 °C. Between the steps a dwell time of 3 min at the last temperature was applied. DSC scans were performed with heating and cooling rates of 5 K per minute and under continuous nitrogen flow of 10 L per minute. The maxima of the DSC curve were selected as melting points and the minima of the DSC curves were selected as solidification points. Prior to the DSC measurements, the particles were concentrated at 100,000 g for 15 min on an Optima MAX XP ultracentrifuge (Beckmann Coulter, Brea, USA). This did not change the melting behaviour or the size distribution of the particles. The particles were easily re-dispersible and provided a better signal to noise ratio.

Table 1
Composition of AddaVax™ and JS-3o.

AddaVax™		JS-3o	
0.5% (w/w)	Tween 80	5% (w/w)	Poloxamer 188
0.5% (w/w)	Span 85	5% (w/w)	Softisan 154
4.3% (w/w)	Squalene	5% (w/w)	Squalene
	Citrate buffer pH 6.5		Citrate buffer pH 6.5

2.5. ^{1}H Nuclear Magnetic Resonance (^{1}H NMR)

^{1}H NMR experiments were carried out on a 500 MHz spectrometer (Unity Inova 500, Varian, Palo Alto, USA). The formulations were measured at 25 °C using 20% D_2O containing 0.75% trimethylsilylpropionate as internal standard. To improve signal to noise ratio 32 spectra were accumulated.

2.6. Preparation of blood serum

Mouse blood was allowed to clot for 1 h at room temperature and then placed at 4 °C for 16 h. Serum was obtained after the blood was centrifuged at 10,000*g* at 4 °C for 90 s. The serum was centrifuged again and complement inactivated for 30 min at 56 °C. Serum samples were preserved with 0.02% sodium azide and stored at 4 °C.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

The antibody titre against IBDV in mice was determined with an IDEXX (IDEXX Laboratories, Westbrook (Maine), USA) IBD enzyme immune assay for chicken. The tests were carried out as described in the manual with the following modifications for the use in mice. Mice sera were diluted 1:50 and an anti-mouse-HRP antibody (Sigma-Aldrich, Munich, Germany) was used as the secondary antibody instead of the anti-chicken HRP antibody. The 2nd antibody was diluted 1:5000 in 50 mM tris HCl pH 8.0, 140 mM sodium chloride and 1% fetal calf serum (sample buffer). As positive control mab63 [16], a monoclonal antibody directed against IBDV VP2 was diluted 1:100, and the sample buffer and the secondary antibody were measured as negative controls to verify the performance of the test. The absorption at 650 nm was used for quantification of the antibody amount.

2.8. Animal handling

All animal trials were approved by the local authorities of Saxony-Anhalt and performed in accordance with the animal welfare act. Mice were housed under controlled conditions (22 °C and 12 h dark/light cycle) in groups of 5 animals and provided with water and nutrition ad libitum. Female BALB/c mice (Charles River Laboratories, Wilmington, USA) at an age of 6 weeks were used for the adjuvant studies. For a better handling and to prevent injuries to the animals during vaccination, these were sedated by treatment with 2% isoflurane for 30 s. For blood sampling the mice were anaesthetised with isoflurane/oxygen mixture with 4% isoflurane initially for 1 min and 2% isoflurane as maintenance dose. To prevent the body from cooling, mice were placed on a warming plate (35 °C). Finally, the mice were sacrificed by cervical dislocation.

2.9. Evaluation of adjuvants

Evaluation of adjuvants in mice were performed in groups of 5 animals. All samples were prepared freshly using the yeast, the adjuvant, water for injection and 10× PBS pH 7.4 to ensure isotonicity and euhydrie. Each mouse received three doses of each 0.1 mg heat inactivated yeast at day 0; 14 and 28 subcutaneously. Blood was collected to estimate the antibody titre 7 days after the

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