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Research Paper

Design of a Liposomal Candidate Vaccine Against *Pseudomonas aeruginosa* and its Evaluation in Triggering Systemic and Lung Mucosal Immunity

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Purpose. To design and evaluate liposomal constructs capable of inducing a potent systemic and airway humoral response to *Pseudomonas aeruginosa*
Methods. Liposomes contained a peptide derived from *P. aeruginosa* pilin protein as B epitope, a peptide derived from *Influenza* hemagglutinin protein as Th epitope, the TLR agonist Pam_3CAG or Pam_2CAG as adjuvant, and a mannosylated lipid as dendritic cell targeting agent. These constructions were administered to mice intraperitoneally (i.p.) or intranasally (i.n.). Their immunogenicity was evaluated by measuring B epitope-specific immunoglobulins in the serum and the airways by ELISA.
Results. The B epitope, in its native form or after substitution of a cysteine by a serine, induced high systemic IgG titers when formulated in the presence of Pam_3CAG or Pam_2CAG and administered i.p.. No IgA response was observed in the airways upon injection of candidate vaccines by i.p. route, whatever the B epitope or the adjuvant. However, i.n. vaccination resulted in a significant local production of IgA. Finally, the production of IgG was more rapid when mannose was incorporated.
Conclusions. All liposomal candidate vaccines tested induced the production of IgG and/or IgA directed against an immunogenic peptide from *P. aeruginosa*. Liposomal constructs could be attractive in the vaccination against *P. aeruginosa*.

KEY WORDS: airway mucosal immunity; liposomes; *Pseudomonas aeruginosa*; vaccination.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative pathogen that causes respiratory tract infections. Pulmonary infection with mucoid strains of *P. aeruginosa* is a main cause of nosocomial infections (1) and a major factor of morbidity and mortality in patients with cystic fibrosis (2). Although antibiotics are available to treat *P. aeruginosa* infections, these treatments are hampered due to the remarkable ability of the bacteria to develop multi-resistance, which is particularly dramatic for cystic fibrosis patients (3,4). During the past decade, search for alternative approaches to fight *P. aeruginosa* has led to an intensive research on vaccine strategies that will confer protection against infection (5). A variety of active vaccination approaches have been thus evaluated, including conjugate vaccines incorporating proteins or/and carbohydrates (6,7), DNA vaccine composed of plasmids encoding different antigens (8), as well as heterologous delivery systems expressing immunogenic *P. aeruginosa* antigens (9).

In search for the design of new vaccines, liposomes are of great interest. These phospholipid vesicles act as protein, epitope and/or adjuvant carriers (10) and have been extensively used to enhance immunogenicity of proteins. They are characterized by a marked absence of toxicity and a low intrinsic immunogenicity. These vesicles also offer a wide range of options for the design of vaccine constructs that include size and (phospho)lipid composition of the vesicles and mode of association of antigens, adjuvants such as lipopeptidic Toll-like receptor (TLR) ligands and cytokines (IL-2…) (11–13). During the past years, we have developed liposome-based vaccines that were very effective in eliciting strong and long-lasting immune responses against small peptides (14). Optimal constructions included a B epitope

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ABBREVIATIONS: BCA, Bicinchoninic acid Protein Assay Reagent; Chol, Cholesterol; DPGMal, DiPalmitoylGlycerol-Maleimide anchor; HA, T helper peptide derived from influenza hemagglutinin protein (HA307–319); i.p., intraperitoneal; i.n., intranasal; Mal, Maleimide; PAKCys, native *P. aeruginosa* pilin protein strain PAK epitope (KCTSDWDEQFIPKGCSK); PAKSer, modified *P. aeruginosa* pilin protein strain PAK epitope (KCTSDWDEQFIPKG*S*SK); Pam_2CAG, *S*-[2,3-bis(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteinyl-alanyl-glycine; Pam_3CAG, *S*-[2,3-bis (palmitoyloxy)-(2*R*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-alanyl-glycine; PC, L-α-egg phosphatidylcholine; PG, L-α-egg phosphatidyl-DL-glycerol; SEM, Standard Error of Measurement; SUV, Small Unilamellar Vesicles; Th, T helper; TLR, Toll Like Receptor.

covalently conjugated to the surface of liposomes of diameter less than 100 nm, together with a Th "universal" epitope and an adjuvant that improved immune response. Conjugation of the epitopes was achieved by incorporating in the liposomes a reactive anchor with adjuvant properties (15). This approach was successfully applied to the development of a vaccine against *Streptococcus mutans* by the design of liposomes containing a B epitope originating from a *S. mutans* surface adhesin and a "universal" Th epitope from tetanus toxin linked to the triacetylated lipopeptide and Toll-like receptor agonist, Pam_3Cysteinyl-Alanyl-Glycine (Pam_3CAG) as adjuvant (14,15).

Liposomal vaccines have been reported to be effective in inducing mucosal immunity (16). In animal models, i.n. administration of liposome-encapsulated ricin toxin or plasmid DNA encoding influenza virus hemagglutinin was shown to elicit a potent local production of specific-IgA, which are the dominant Ig isotype in the upper respiratory tract and play a critical role in preventing pathogen colonization (17,18). Thus, the aim of this work was to design and evaluate, in the mouse, liposomal constructions able to provide the highest systemic and lung antibody response against *P. aeruginosa.* As B epitope, we selected a 17 amino-acid peptide derived from the C-terminal receptor-binding region (residues 128–144) of *P. aeruginosa* pilin protein strain PAK (19). Taking into account the role of pili in the adherence of *P. aeruginosa* to the host cell, this peptide may lead to the production of specific antibodies capable of counteracting infection by blocking bacterial attachment. In our constructions, this B epitope was used as native (PAKCys) or after substitution of one of its cysteine residue by a serine (PAKSer). Indeed, the region 128–144 of pilin protein strain PAK contains two cysteine residues that form a disulfide loop in the native protein. To evaluate the influence of this peptide configuration on the immune response, the cysteine residue 142 of the native epitope was replaced by a serine leading to a peptide that will obviously adopt a linear configuration in the construction. Each B epitope was conjugated to liposomes together with a 13 amino-acid peptide derived from influenza hemagglutinin protein (HA307–319) as Th "universal" epitope and the triacetylated Pam_3Cysteinyl-Alanyl-Glycine (Pam_3CAG) or diacetylated Pam_2Cysteinyl-Alanyl-Glycine (Pam_2CAG) TLR agonist as adjuvant. To design these diepitope liposomal constructions, we synthesized an adjuvant free thiol-reactive DiPalmitoyl-Glycerol-Maleimide anchor (DPGMal) able to incorporate to the liposome bilayer due to its amphiphilic properties and allowing conjugation of Th or B epitopes containing cysteine residue through its maleimide function. This approach permits to control the epitope/adjuvant ratio. To optimize the constructions and considering our recent *in vitro* results, we also evaluate the influence of incorporation to the formulation of a mannosylated molecule acting as dendritic cells targeting agent. The different constructions were administered to mice by systemic (intraperitoneal, i.p.) or local (intranasal, i.n.) route.

MATERIALS AND METHODS

Animals

Nine-week-old male BALB/c mice were purchased from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France). Animals were maintained under controlled environmental conditions with a 12 h/12 h light/dark cycle. Food (UAR-alimentation, Villemoisson, France) and tap water were available ad libitum. Animal experimentation was conducted according to the "Principles of Laboratory Animal Care" and with the approval of the government body that regulates animal research in France.

Synthetic Peptides

The three peptides, PAKCys (KCTSDWDEQFIPKGCSK), PAKSer (KCTSDWDEQFIPKG*S*SK), and influenza virus haemagglutinin-derived HA307–319 (PKYVKQNTLKLAT-C) were obtained from NeoMPS (Strasbourg, France). The purity of the peptides, as assessed by HPLC, was at least 85%.

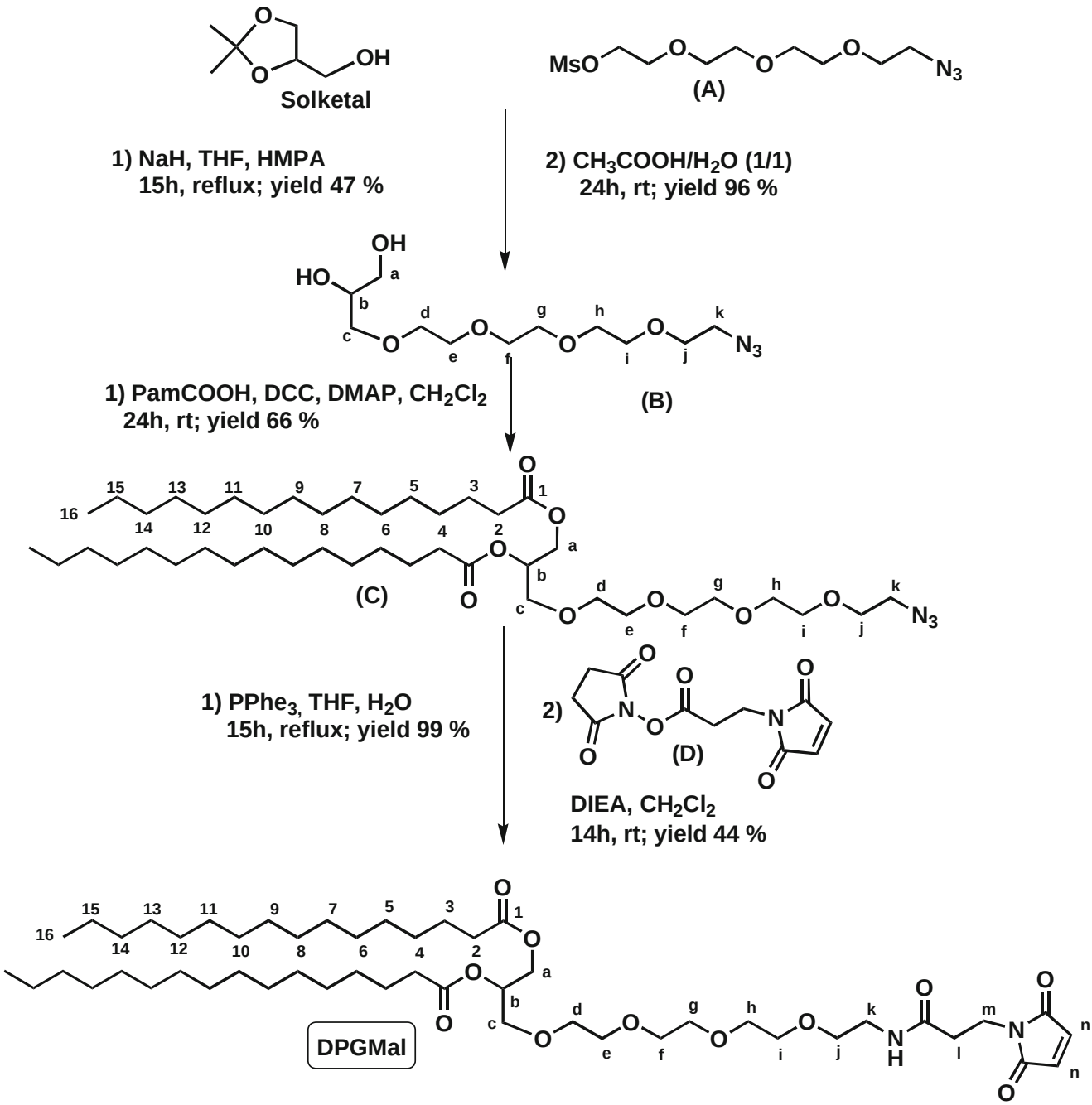
Synthesis and Characterization of Lipopeptide Adjuvants, DPGMal Anchor and Mannosylated Lipid

All chemicals were of analytical grade and commercially available. All NMR spectra were recorded at 200 and 300 MHz (^{1}H), as well as 50 and 75 MHz (^{13}C) on a Bruker Advance spectrometer. NMR chemical shifts were expressed in ppm relative to internal solvent peaks, and coupling constants were measured in Hertz. ESITOF (electrospray ionization time-of-flight) spectra were recorded on a Perseptive Biosystem Mariner 5155 spectrometer. The m/z range 200–2100 was scanned using an ion-spray voltage of 4500 V. The nozzle ranged between 30 and 60 V. TLCs were performed on Merck silica gel 60 Kieselgel F254. Column chromatographic separations were carried out by flash chromatography using silica gel (Merck) with a particle size of 0.040–0.063 mm.

Lipopeptidic adjuvants were synthesized according to Roth A. *et al.* 2004 (20). Mannosylated amphiphilic derivative was synthesized according to the process described in Espuelas S. *et al.* (submitted). It was composed of a dimannosyl head group connected to a lipid moiety *via* a polyethylene glycol spacer ($DOGP_4$Mannose).

The DPGMal anchor was synthesized following three steps (Scheme 1).

Synthesis of 1-azido-3,6,9,12-tetraoxapentadecane-14,15-diol (B). Compound A was synthesized according to Höltke, (2005) (21). For synthesis of compound B, a solution of racemic solketal (1.3 g; 9.5 mmol) and HMPA (17 ml) in anhydrous THF (50 ml) was added to a suspension of NaH 60% (0.94 g; 23.6 mmol) in anhydrous THF (18 ml) under argon. The mixture was heated at reflux for 15 min, then cooled at room temperature. A solution of the compound A (3.5 g; 11.8 mmol) in anhydrous THF (15 ml) was slowly added to the reaction medium. The mixture was heated at reflux for 15 h, then quenched with water (30 ml) and extracted three times with ethyl acetate. The combined organic layers were washed twice with a 1 N HCl solution, twice with a saturated $NaHCO_3$ solution and with saturated NaCl solution, then dried over $MgSO_4$ and evaporated to dryness. The crude was purified by silica gel column chromatography with a cyclohexane/ethyl acetate (5/5) mixture. The acetal compound was obtained as an



Scheme 1. Synthesis of the DPGMal anchor.

oil (1.4 g; yield=47%) and was dissolved in a mixture acetic acid/water (1/1 *v*/*v*, 10 ml). The mixture was stirred at room temperature for 24 h, then toluene was added (100 ml) and the reaction medium was evaporated to dryness. The crude product was dissolved in CH_2Cl_2, dried over $MgSO_4$ and evaporated to give the compound B as a yellow oil (1.2 g; yield=96%).

^{1}H NMR (300 MHz; $CDCl_3$) δ ppm: 2.87 (s; 2OH); 3.39 (t; *J*=5 Hz; 2Hk); 3.5–3.65 (m; 18Ha, c–j); 3,85 (m; Hb).

^{13}C NMR (75 MHz; $CDCl_3$) δ ppm: 51,3 (Ck); 64.48 (Ca); 70.6 (Cc); 71.1; 71.13; 71.17; 71.23; 71.3 (7Cd–j); 73.6 (Cb).

ES-MS *m*/*z*: 316.18 ([M+Na]+), Exact mass: 293.16 g mol^{-1}.

Synthesis of 1-azido-3,6,9,12-tetraoxapentadecane-14,15-diyl dipalmitate (C). Palmitic acid (PamCOOH) (3.6 g; 14 mmol), DCC (2.9 g; 14 mmol) and DMAP (200 mg; 1.63 mmol) were added to a solution of the compound B (1.2 g; 4.1 mmol) in CH_2Cl_2 (45 ml). The mixture was stirred under argon at room temperature for 24 h and then filtered. The mixture was washed twice with a saturated $NaHCO_3$ solution. The organic phase was dried over $MgSO_4$, filtered and evaporated to dryness. The crude was purified by silica gel column chromatography with a cyclohexane/ethyl acetate (8/2) mixture, to give the solid C (2.1 g; yield=66%). TLC: Rf=0.35 (cyclohexane/EtOAc 8/2).

TLC Rf=0.35 (cyclohexane/EtOAc 9/1)

^{1}H NMR (300 MHz; $CDCl_3$) δ ppm: 0.87 (t; *J*=7 Hz; 6H16); 1.24 (m; 48H4–15); 1.59 (m; 4H3); 2.3 (m; 4H2); 3.38 (t; *J*=5 Hz; 2Hk); 3.64 (m; 16Hc–j); 4.14 (dd; *J*=6.5 Hz; *J*= 12 Hz; 1Ha); 4.32 (dd; J=3.7 Hz; *J*=12 Hz; 1Ha); 5.19 (m; Hb).

^{13}C NMR (75 MHz; $CDCl_3$) δ ppm: 14.8 (2C16); 23.3 (2C15); 25.6 (2C3); 29.8; 29.98; 30.1; 30.15; 30.33 (20C4–13); 32.6 (2C14); 34.8; 35.0 (2C2); 51.3 (Ck); 63.3 (Ca); 70.1; 70.25; 70.58; 70.89; 71.16; 71.32; 71.58 (9Cb–j); 173.8; 174.1 (2C1).

ES-MS *m/z*: 792,6727 ([M+Na]+), Exact mass: 769,62 g mol^{-1}.

Synthesis of 19-(maleimido)-17-oxo-4,7,10,13-tetraoxa-16-azanonadecane-1,2-diyl dipalmitate (DPGMal). In order to deprotect the compound C (2 g; 2.6 mmol), the residue was added to a solution of triphenylphosphine (2.04 g; 7.8 mmol) and water (400 µL) in THF (25 ml). The mixture was heated at reflux for 15 h and then concentrated. The product was purified by silica gel column chromatography with a CH_2Cl_2/MeOH (9/1) mixture with 2% triethylamine. The amine compound was obtained as a white solid (1.9 g; yield=99%) immediately used in the next step. Compound D (2,5-dioxopyrrolidin-1-yl 3-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl) propanoate), synthesized according to Thibaudeau (2005) (22) (0.175 g; 0.66 mmol), was added to a solution of previous described amine (0.31 g; 0.4 mmol) in CH_2Cl_2 (10 ml) with DIEA (56 µL; 0.32 mmol). The mixture was stirred 14 h at room temperature under argon, then the solvent was evaporated to dryness. The crude product was purified by silica gel column chromatography with a CH_2Cl_2/MeOH mixture (gradient 40/1 to 20/1). The compound of interest (DPGMal) was obtained as a white solid (160 mg; yield=44%).

TLC Rf=0.25 (CH_2Cl_2/MeOH 9/1)

^{1}H NMR (300 MHz; $CDCl_3$) δ ppm: 0.87 (t; *J*=6.7 Hz; 6H16); 1.24 (m; 48H4–15); 1.59 (m; 4H3); 2.27 (m; 4H2); 2.50 (t; *J*=7.2 Hz; 2Hl); 3.41 (m; 2Hc); 3.51 (t; *J*=5.3 Hz; 2Hk); 3.64 (m; 14Hd–j); 3.83 (t; *J*=7.1 Hz; 2Hm); 4.13 (dd; *J*=6.4 Hz; *J*=12 Hz; 1Ha); 4.32 (dd; *J*=3.4 Hz; *J*=12 Hz; 1Ha); 5.19 (m; Hb); 6.34 (t; NH); 6.68 (s; 2Hn).

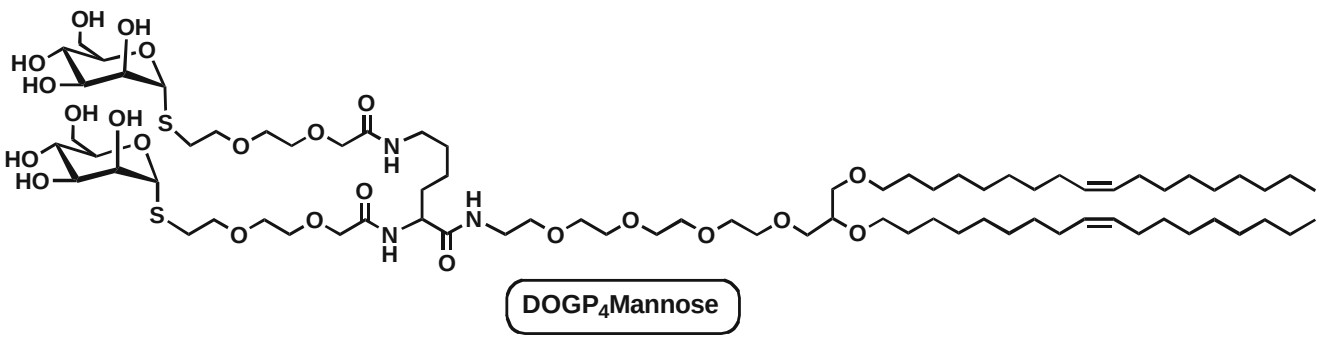
^{13}C NMR (75 MHz; $CDCl_3$) δ ppm: 14.8 (2C16); 23.3 (2C15); 25.6 (2C3); 29.72; 29.93; 29.97; 30.12; 30.3 (20C4–13); 32.6 (2C14); 34.7; 34.9; 35.2 (2C2; Cl; Cm); 39.9 (Ck); 63.3 (Ca); 70.15; 70.32; 70.55; 70.81; 71.13; 71.27; 71.54 (9Cb–j); 134.8 (2Cn); 170.3 (1Cqamide); 171.1 (2Cqmal); 173.7; 174.1 (2C1).

ES-MS *m/z*: 917.7 ([M+Na]+), Exact mass: 894.65 g mol^{-1}.

Preparation of Candidate Vaccines

Preparation of liposomes. Liposomes were prepared by mixing in chloroform: egg yolk L-α-phosphatidylcholine (PC, Sigma-Aldrich), L-α-phosphatidyl-DL-glycerol (PG, Sigma-Aldrich) and cholesterol (Chol, Sigma-Aldrich) in a 73/20/50 molar ratio, the DPGMal anchor and the lipopeptide adjuvant (Pam_3CAG or Pam_2CAG) at a final concentration of 2% and for formulation of targeted liposomes, $DOGP_4$Mannose (6 mol% mannose, Scheme 2). After solvent evaporation under high vacuum, the dried lipid film was hydrated in 10 mM Hepes buffer (pH 6.5) containing 5% (*w/v*) sorbitol by vortex mixing (1 ml for 10 µmol lipids). The suspension was sonicated for 1 h (5 s cycles interrupted for 1.25 s) using a 3 mm diameter probe sonicator (Vibra Cell, Sonics and Material Inc., Danbury, CT, USA) at 300 W. The small unilamellar vesicle preparation was finally centrifuged at 10 000 *g* for 10 min to remove the titanium dust originating from the probe. The size of the liposomes was determined by dynamic light scattering using a Zeta-master 3000 (Malvern Instruments, Paris, France). Their PC content was quantified using an enzymatic assay with a commercial test kit (Phospholipids B, Oxoid, Dardilly, France). In brief, 0–10 µl of liposomes were applied in triplicates in a 96 well microplate before addition of 200 µl phospholipid B reagent solution, prepared according to the manufacturer's instructions. The plates were then incubated at 37°C for 15 min. In presence of choline, a red colour appeared due to formation of a phenol-4-aminoantipyridine complex. Choline chloride was used for calibration curve. Absorbance was measured at λ=490 nm using a microplate reader (Bio-Rad, model 550, Marnes-La-Coquette, France).

Peptide conjugation. Reduction of the disulphide bonds of each peptide with tris(2-carboxyethyl)phosphine (Sigma-Aldrich) (0.7 eq *vs* peptide) was first performed for 15 min under argon. Peptides were conjugated to freshly prepared liposomes by incubating the preparation in a first step with the B epitope (PAKCys or PAKSer) at 0.5 molar eq. of peptide *vs* surface accessible thiol-reactive maleimide function of DPGMal for 2 h (final molar ratio of 2.5%). In a second step, the PAK-coupled liposomes were incubated with the Th epitope HA307–319 for 2 h. Couplings were performed at room temperature, under argon, in 10 mM Hepes buffer (pH 6.5) containing 5% (*w/v*) sorbitol. A ten-fold excess of 2-mercaptoethanol *vs* DPGMal was added to the preparation to derivate all unreacted maleimide groups. Then, the liposomal preparation was dialysed



Scheme 2. Structure of the dimannosylated lipid derivative ($DOGP_4$Mannose).

extensively against 10 mM Hepes buffer (pH 7.4) containing 5% (*w*/*v*) sorbitol, to eliminate unconjugated peptides and excess of reagents. No significant changes in vesicle size (less than 5%) were measured after conjugation of the peptides.

Quantification of the Peptides Associated to Liposomes

Quantification of the peptides associated to vesicles was achieved using the BCA Protein Assay Reagent (Pierce, Brebière, France) in 96 well microplates. The contribution of the liposomes alone was evaluated using the same conditions. The yields of conjugated peptides were calculated *versus* the quantity of peptide able to be coupled to the maleimide functions exposed on the external surface of the vesicles.

Animal Immunization

Mice (4 to 8 animals per group of treatment) were immunized on days 1, 7 and 21 by i.p. or i.n. route and received one booster injection on day 71. For immunization by i.p. route, animals were injected with 100 µl/injection of liposome preparation containing 15 µg of PAK peptide. Animals that were immunized by i.n. route received 25 µl/injection of liposome preparation containing 3.75 µg of PAK peptide. Intranasal administrations of the constructions (12.5 µl per nostril) were carried out under anaesthesia (50 mg/kg ketamine and 3.33 mg/kg xylazine given i.p.).

Collection of Biological Fluids for Ig Determination

Blood samples were harvested on the day prior to the first immunization (preimmune serum) and on days 20, 70 and 78 of the protocol of vaccination. Sera were collected by centrifugation (3,000 g for 15 min at 4°C) and stored at −20°C until use. Bronchoalveolar lavage (BAL) fluids were harvested on day 78 after animal euthanasia and blood collection. Briefly, the trachea was canulated and the airways were washed with 2 instillations of 0.5 ml ice-cold saline. Fluids recovered from these lavages were centrifuged (160 g for 5 min at 4°C) and the resulting supernatant was stored at −20°C until use.

Determination of IgG and IgA

Levels of PAK-specific IgG and IgA were determined in serum and BAL fluids by enzyme-linked immunosorbent assay using PAKCys peptide conjugated to bovine serum albumin (PAKCys-BSA, NeoMPS, Strasbourg, France) as coating agent. For ligation to BSA, a tyrosine residue was grafted at the N-terminal end of PAKCys. Ligation was carried out in the presence of bisdiazobenzidine (BDB) leading to PAKCysTyr-BDB-BSA. In presence of oxygen, an intramolecular dimerization of the two cysteines of PAKCys occurred mimicking the natural loop of the peptide. For a vaccine application, an antibody response against the natural epitope of *P. aeruginosa* is necessary. This is the reason why, whatever the injected epitope, all the ELISA experiments were conducted using PAKCys-BDB-BSA. Microtiter plates were coated overnight at 4°C with 2 µg of peptide/well (in 100 µl 0.1 M sodium bicarbonate buffer, pH 8.3) and blocked for 2 h at 37°C with 1% bovine serum albumin in phosphate buffered saline. Serial dilutions of serum samples or BAL fluids were then incubated overnight at 4°C. After three washings with phosphate-buffered saline containing 0.05% Tween-20, a biotinylated anti-mouse IgG (Tebu-Bio) or IgA (BD Pharmingen) antibody was added to the wells and incubated for 1 h at room temperature. The plates were then washed and incubated with an extravidin–horseradish peroxidase conjugate (Sigma-Aldrich) for 30 min at room temperature. After final washings, the horseradish peroxidase substrate, tetramethylbenzidine (BD Pharmingen) was added and coloration was allowed to develop for 15–20 min. The reaction was stopped by addition of 0.5 M H_2SO_4 and absorbance intensity was read at 450 nm. Serum levels of PAK-specific IgG were expressed as antibody titers. Antibody titers were determined as the reciprocal of the highest immune serum dilution that gave an absorbance twice the one of the pre-immune serum sample. BAL fluid levels of PAK-specific IgA were expressed as absorbance as a function of the reciprocal of bronchoalveolar lavage dilution.

Statistical Analysis of the Data

Data are presented as means±SEM. Groups were compared with respect to Ig titers using Student's *t*-test for normally distributed data or Mann–Whitney test when data were not normally distributed (SigmaStat, version 2.0, Jandel GmbH, Erkrath, Germany). Data were considered significantly different when $p<0.05$.

RESULTS

Synthesis of the DGPMal Anchor

Synthesis of DGPMal anchor is depicted on Scheme 1. Product A was prepared according to the method of Höltke et al. (2005) (21). Racemic solketal was treated by NaH to give alcoholate function in position 3. This salt reacted by nucleophilic substitution on the mesylate function of compound A. Then, the acetal group was removed by aqueous acid conditions to give B. The diol B was esterified with 2 palmitoyl acid chains by using dicyclohexyl carbodiimide (DCC) as activator for acid functions and dimethyl aminopyridine (DMAP) as catalyst. The azido function was reduced by Staudinger's method using triphenyl phosphine and water in THF to give the amine function. This last function reacted with product D (22) to give the final product DPGMal. Structure of DPGMal anchor and all intermediates was identified by mass spectroscopy, ^{1}H and ^{13}C NMR.

Composition and Characterization of the Liposomal Candidate Vaccines

The classical thin lipid film method was used to formulate four liposome constructions incorporating the DPGMal anchor, PAKSer or PAKCys as B epitope, the Th epitope HA307–319 and Pam_3CAG or Pam_2CAG as adju-

Table I. Composition and Characterization of the Liposomal Constructions Used in the Present Study

Constructions	1	2	3	4
Anchor	DPGMal	DPGMal	DPGMal	DPGMal
Th peptide	HA	HA	HA	HA
B peptide	PAKSer	PAKCys	PAKCys	PAKCys
Adjuvant	Pam_3CAG	Pam_3CAG	Pam_2CAG	Pam_2CAG
$DOGP_4$Mannose	No	No	No	Yes
Ligation of B epitope (%)	97	100	80	80
Size (nm)	109±45	112±34	100±11	114±40

vant. In one of these formulations, a mannosylated lipid (Scheme 2) able to target dendritic cells *in vitro* was also incorporated (Espuelas *et al*, submitted). Composition of the different constructions is presented in Table I. The B and Th epitopes were successively added to the preformed vesicles to allow their ligation to the maleimide anchor. The proportion of anchor in constructions 2, 3 and 4, that contained PAKCys with two cysteines, was increased to 7.5% (as compared to 5% in construction 1) to allow the B epitope to be attached at the same final proportion than in construction 1 that contained PAKSer. The increased DPGMal proportion in these liposomal formulations implied to perform a sonication at 65°C probably due to the increasing bilayer rigidity. In the fourth liposomal formulations, small (≤110 nm) and homogeneous unilamellar vesicles were prepared (Table I). Furthermore, high ligation yields of the epitopes (≥80%) were obtained (Table I).

Immunogenicity of PAKCys and PAKSer Epitopes

Immunogenicity of the two B epitopes was compared by injecting mice i.p. with construction 1 or 2 containing PAKSer and PAKCys, respectively. The two formulations contained as other entities the Th epitope HA307–319 and the adjuvant Pam_3CAG (Table I). Fig. 1 depicts the kinetics of IgG titers measured in the serum of both groups of animals. As shown on this figure, the two candidate vaccines elicited a fast production of IgG in sera that resulted in significant IgG titers at day 20. Titers decreased at day 70, but boost injection at day 71 led to a further, although statistically non-significant increase in IgG production for PAKSer at day 78. Vaccination induced no IgA response in the airways, whatever the B epitope (not shown).

Influence of the Nature of the Adjuvant

Influence of the nature of the adjuvant was assessed by injecting mice i.p. with construction 2 or 3, containing Pam_3CAG and Pam_2CAG, respectively. The other entities of the formulations were the B epitope PAKCys and the Th epitope HA307–319 for both constructions (Table I). As shown on Fig. 2, the two constructions elicited a significant IgG production in the serum of mice, with the same kinetic profile. However, after the boost, this production was more pronounced ($p<0.05$) with the formulation that contained Pam_2CAG. No IgA response was observed in the airways, whatever the formulation (not shown).

Comparison of Route of Vaccination

In the absence of IgA response in the airways after vaccination by i.p. route with liposomal construction 1, 2 or 3, intranasal (i.n.) vaccination was carried out with construction 3 and the data obtained were compared to the response elicited by this formulation after i.p. administration. As shown on Fig. 3 (panel A), intranasal administration of construction 3 triggered no systemic production of IgG until the boost and some response at day 78, when compared to the one elicited by i.p. injection. However, it lead to a potent production of IgA in the airways (Fig. 3, panel B). Similar results were obtained with construction 1 which differs from construction 3 in terms of B epitope (PAKSer) and adjuvant (Pam_3CAG; not shown), suggesting that route of administration rather than composition is critical in eliciting a mucosal response.

Influence of the Incorporation of Mannose as Targeting Agent of Dendritic Cells

A mannosylated lipid was incorporated to a formulation containing the peptide PAKCys, the Th epitope HA307–319

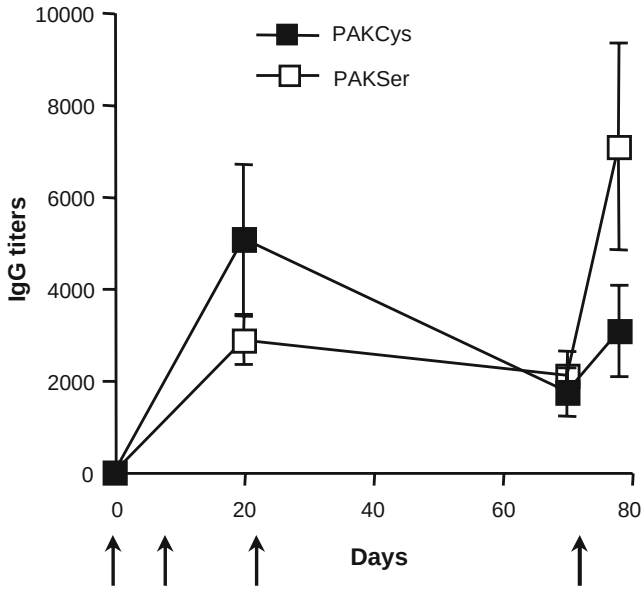


Fig. 1. Titers of anti-PAK IgG in the serum from mice immunized intraperitoneally with constructions containing the B epitope PAKCys (*closed squares*) or PAKSer (*open squares*). The other entities of the constructs were the Th epitope HA307–319 and the adjuvant Pam_3CAG. Data are means±SEM of n = four to seven animals. *Arrows* indicate days of injections.

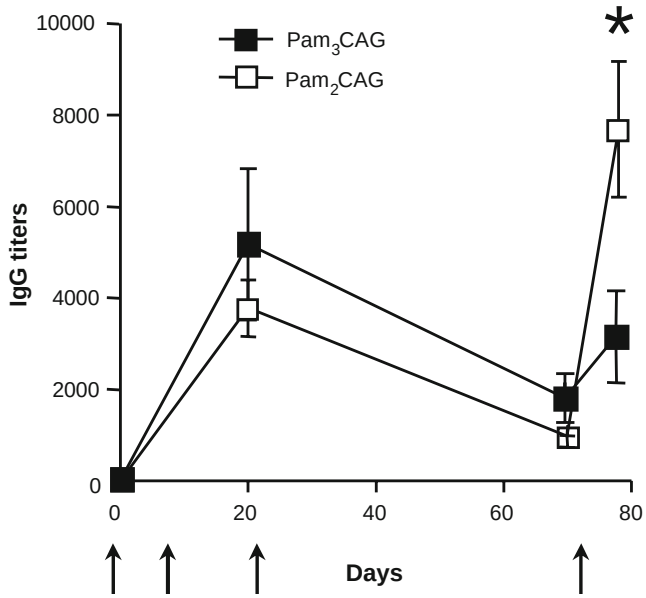


Fig. 2. Titers of anti-PAK IgG in the serum from mice immunized intraperitoneally with constructions containing the adjuvant Pam_3CAG (*closed squares*) or Pam_2CAG (*open squares*). The other entities of the constructs were the B epitope PAKCys and the Th epitope HA307–319. Data are means±SEM of n = four to five animals. Statistically significant differences at $*p<0.05$ when compared to Pam_3CAG. *Arrows* indicate days of injections.

and Pam_2CAG and the resulting construction (construction 4, Table I) was evaluated and compared to construction 3 after i.n. vaccination. As shown on Fig. 4, whereas i.n. administration of construction 3 triggered no systemic production of IgG until day 78, administration of construction 4 lead to a significant production at day 20 that was further increased at days 70 and 78. Therefore, incorporation of mannose in the formulation permitted an earlier systemic immune response upon mucosal vaccination (statistically significant differences between the two constructions at days 20 and 70). However, it didn't change intensity of the response, since at day 78 mice injected with constructions 3 and 4 exhibited similar IgG titers. Intensity of IgA response in the airways was not modified either by the presence of mannose (not shown).

DISCUSSION

The aim of the present study was to design a vaccine able to induce a potent systemic and lung humoral response to the bacteria *P. aeruginosa*. Liposome formulations were used for this purpose, since they act as multivalent vectors of protein, epitope and/or adjuvant and are potent inducers of mucosal immunity. To elaborate this vaccine, we used the strategy previously developed in our group for liposome-based vaccines against the bacteria *Streptococcus mutans.* Accordingly, constructions were formulated with the critical entities necessary to induce a potent immune response, namely a B epitope, a Th "universal" epitope (14) and an adjuvant (23). However, several modifications were introduced in the design of these constructions. At first, whereas in our previous constructions conjugation of the epitopes to the liposomes implied the use of a reactive anchor with adjuvant properties, we here synthesized an adjuvant free thiol-reactive DiPalmitoylGlycerol-Maleimide anchor able to incorporate into the liposome bilayer due to its amphiphilic properties and allowing conjugation of B or Th epitopes containing cysteine residues through its maleimide function (24). Furthermore, this approach aimed at controlling the epitope/adjuvant ratio. DPGMal was built with a PEG spacer arm of defined length (four ethyleneglycol units) in order to provide an optimal accessibility of the liposomal peptide ligands to their receptors. Our data showed that this new ligation strategy allowed easy coupling of the different peptides with a yield of conjugation greater than 80%. Another particularity in the design of the present liposomal constructions was the peptide used as B epitope. This peptide in its native form (PAKCys) contains two cysteine residues that form a disulfide bridge. Taking into account that peptide conjugation to DPGMal anchor occurs at cysteine residues, this particularity led us to investigate immunogenicity of two forms of the peptide: the native form (PAKCys) and a modified form in which one of

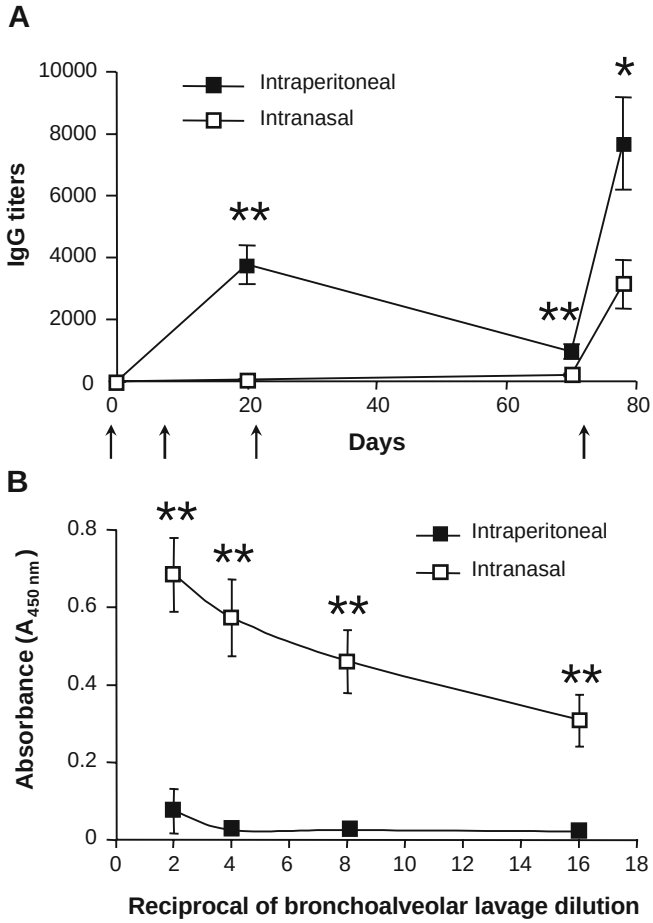


Fig. 3. Anti-PAK IgG in the serum (**A**) and IgA in the airways (**B**) from mice immunized by i.p. (*closed symbols*) or i.n. (*open symbols*) route. The construction contained the B epitope PAKCys, the adjuvant Pam_2CAG and the Th epitope HA307–319. Data are means±SEM of n = five to eight animals. Statistically significant differences at $*p<0.05$ or $**p<0.01$ when compared to intranasal route (**A**) and to intraperitoneal route (**B**). *Arrows* indicate days of injections.

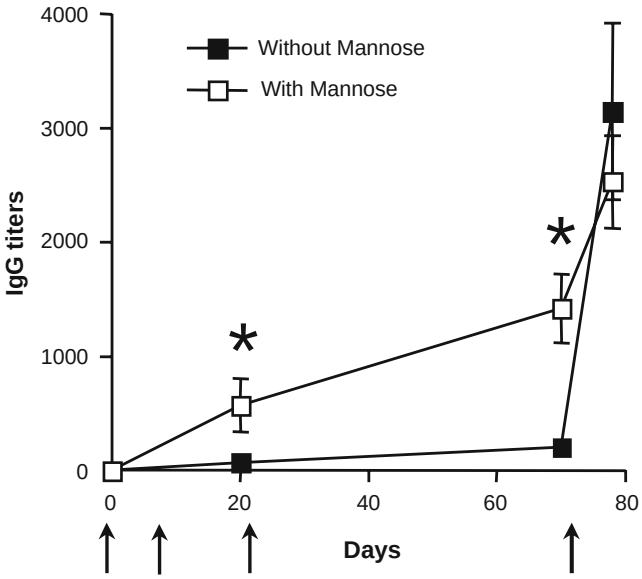


Fig. 4. Titers of anti-PAK IgG in the serum from mice immunized intranasally with a construction containing (*open symbols*) or not (*closed symbols*) a dimannosylated lipid as dendritic cell targeting agent. The other entities of the constructs were the B epitope PAKCys, the adjuvant Pam_2CAG and the Th epitope HA307–319. Data are means±SEM of n = six to eight animals. Statistically significant differences at $*p<0.05$ when compared to construction without mannose. *Arrows* indicate days of injections.

the cysteine residues was changed to a serine (PAKSer). For formulation, this particularity led us also to increase the proportion of the anchor in constructions containing the native peptide as compared to constructions containing the modified peptide to allow PAKCys to be incorporated at the same final proportion than PAKSer. The increased DPGMal proportion in the formulations implied to perform a sonication at 65°C probably due to the increasing bilayer rigidity. Nonetheless, ligation yield of the two B peptides was similar and characteristic of constructions containing the PAKCys epitope did not differ significantly from constructions containing the PAKSer peptide. A last modification in our liposomal formulation was the incorporation of a mannosylated molecule aimed at acting as dendritic cells targeting agent. This molecule was composed of a dimannosyl head group connected to a lipid moiety *via* a polyethylene glycol spacer (Scheme 2). As amphiphilic molecule, this lipid incorporated easily into the bilayer of liposomes (12). Our data showed that its incorporation had no impact on the characteristics of our formulations.

When evaluated in the mouse upon i.p. vaccination, constructions formulated with PAKCys or PAKSer as B epitope, the Th "universal" epitope HA307–319 and Pam_2CAG or Pam_3CAG as adjuvant, triggered a specific IgG response in the serum, whatever the nature of the B epitope or the adjuvant. For all constructions, this response followed the same kind of kinetic. It was already present at day 20, declined during the following weeks, and eventually increased again after the boost. Comparison of intensity of the serum IgG response triggered by the different formulations suggested however that PAKSer is more potent that the native peptide PAKCys as B epitope when formulated in the presence of the adjuvant Pam_3CAG, since vaccination with PAKSer resulted in a more important response at day 78 than vaccination with PAKCys. However, this hypothesis should be enounced with caution, since no statistically significant difference was observed at this time point between PAKSer and PAKCys. Likewise, the difference in IgG levels observed at day 78, namely 7 days after the boost, could also be explained by a difference in the kinetic of the response elicited by the boost. In the construction, conjugation of B epitope occurs through SH function(s) of cysteine residue(s). Therefore, PAKSer with one cysteine in its sequence keeps a linear conformation, whereas PAKCys, with two cysteines reacts with two maleimide functions and acquires a cyclic configuration capable of mimicking closely the conformation of the native B epitope. Our data therefore show that linearization of the B epitope does not affect significantly its immunogenicity. This result is interesting in terms of formulation, since peptide conjugation through one reaction site allows a simple and univocal ligation.

Comparison of intensity of the serum IgG titers triggered by the different formulations formulated with PAKCys or PAKSer as B epitope, the Th "universal" epitope HA307–319 and Pam_2CAG or Pam_3CAG as adjuvant evidenced also that the adjuvant Pam_2CAG led to a better response than Pam_3CAG, when formulated in the presence of PAKCys. This observation is in keeping with the *in vitro* results of Mühlradt *et al*. (2002) on primary resident peritoneal exudates macrophages showing that substitution of the free N-terminus of mycoplasmal Pam_2 derivative lipopeptides with a palmitoyl group, leading to Pam_3 derivatives, decreased their specific activity (25). Interestingly, PAKCys formulated in the presence of the potent Pam_2CAG adjuvant led to similar IgG response than PAKSer formulated in the presence of Pam_3CAG, demonstrating that vaccination with PAKCys may trigger high serum IgG titers 7 days after the boost provided that a potent adjuvant is used.

Induction of a mucosal response in the airways, and particularly of protective IgA, which are critical to confer defence mechanisms against pathogens, remains a challenge for vaccine development. Liposomal vaccines have been reported to be effective in inducing mucosal immunity (16). We therefore evaluated IgA production in the bronchoalveolar lavage fluids of mice vaccinated intraperitoneally with our different formulations. No lung mucosal response was then observed whatever the formulation. One of the most effective methods to induce mucosal immunity in the upper respiratory tract is i.n. immunization. Therefore, this mode of administration was assessed for two of our formulations: construction 1 (PAKSer-Pam_3CAG) and construction 3 (PAKCys-Pam_2CAG). Upon i.n. vaccination, both constructions led to a potent production of IgA in the airways, which is in agreement with previous studies using liposome-based vaccines (18). Although they appeared late in the course of the vaccination protocol, namely at day 78, significant titers in specific-IgG were also observed in the serum of mice vaccinated locally, which is particularly interesting since i.n. vaccination usually lead to no or very little systemic IgG response. Taken altogether, our constructs were effective in triggering a lung humoral response provided that they were given locally. This local response was associated with a systemic response when a boost was introduced. Interestingly as well, doses of B and

Th epitopes used for local vaccination were 4 times lower than the ones used for systemic vaccination.

Dendritic cells are involved in the process and presentation of antigens to naïve T cells and play therefore a key role in immune responses against pathogens (26). As the principal mucosal-resident antigen-presenting cells, they are particularly involved in mucosal immunity (26). Dendritic cells express at their surface the mannose receptor (MR) that has been implicated in the recognition of mannose residues exposed at the surface of selected pathogens and in the internalization of mannosylated antigens, resulting in enhanced antigen presentation to T cells (27). Therefore, the use of mannosylated peptides or carriers has been envisaged as an approach to target dendritic cells and enhance activity of synthetic vaccines. In this regard, we recently demonstrated that liposomes bearing a dimannosylated lipid efficiently targeted human dendritic cells *in vitro* (Espuelas *et al*, submitted). In the present study, we consequently assessed the effect of incorporation of this lipid in our formulations based on PAKCys peptide and Pam_2CAG. The presence of this targeting molecule did not have any effect on intensity of the IgG and IgA responses measured after the boost. However, the kinetic of IgG production in serum was influenced. Indeed, IgG appeared earlier at a higher titer when the mannosylated construction was used, suggesting that addition of a dendritic targeting molecule may help to get a more rapid systemic immune response upon i.n. vaccination.

CONCLUSION

A variety of vaccination approaches have been investigated as strategies to prevent *P. aeruginosa* infection. These approaches used conjugate vaccines incorporating proteins or/and carbohydrates, DNA vaccine composed of plasmids encoding different antigens, as well as heterologous delivery systems expressing immunogenic *P. aeruginosa* antigens. In the present study, we have evaluated totally synthetic and controlled liposomal constructions that incorporated different entities necessary to induce a potent humoral response, as a new approach. Although several parameters influenced activity of the constructions, all the candidate vaccines tested induced a systemic and/or local production of specific antibodies directed against an immunogenic peptide from *P. aeruginosa* in the mouse, suggesting for the first time that epitope formulation based on liposomes could be attractive in the vaccination against the bacteria. According to our results, a construction that would contain PAKSer as B epitope, Pam_2CAG as adjuvant and a mannose derivative as DC targeting agent seems the most promising candidate vaccine. In future studies, we propose to further characterize the immunological response triggered by this construction (IgG subtypes, cellular immunity, kinetic of IgA production) and to evaluate its efficacy in an animal model of infection.

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