brief articles

molecular pharmaceutics

Effect of Glycoamphiphiles on the Solubilization and Dendritic Cell Uptake of a Lipopeptide: A Preliminary Study

Dominique Bonnet,*,†,‡ Gerhild Angyalosi,† Alexandra Demory,† Valérie Santraine,† Arnaud Boulet,† Corentin Spriet,§ Laurent Héliot,§ Hélène Gras-Masse,†,|| and Cyrille Grandjean*,†

SEDAC-Therapeutics, Parc Eurasanté, Le Galénis, 85, rue Nelson Mandela, 59120 Loos, France, and FRC3-CNRS and UMR CNRS 8525, Institut de Biologie de Lille, 1, rue du Professeur Calmette, 59000 Lille, France

Received May 23, 2005

Abstract: The selective delivery of antigens to professional antigen-presenting cells represents a promising approach to improve vaccine efficacy. Addition of a glycoamphiphile to a lipopeptide, whose interest for vaccination is now well-established, greatly favors its solubilization in aqueous solutions through the formation of mixed vesicles. Flow cytometry experiments indicate that this formulation does not diminish the uptake of the lipopeptide by the dendritic cells (DCs). These preliminary results suggest a possible straightforward, noncovalent targeting of cocktail-lipopeptide vaccines to the DCs via carbohydrate receptor-mediated endocytosis.

Keywords: Lipopeptide; glycoamphiphile; dendritic cell; vesicle; targeting

Introduction

Lipopeptide vaccines have proven their efficacy in eliciting neutralizing antibodies, T-helper, and even cytotoxic T-lymphocyte responses against various pathogens or cancers.[1−10] A cocktail of five lipopeptides is currently under investigation in a phase II clinical trial as a therapeutic vaccine against HIV.[11] Despite the great interest of such compounds in the medical field, their further development suffers from their

* To whom correpondence should be adressed. D.B.: Université Louis Pasteur, UMR 7081 CNRS, Faculté de Pharmacie, 74, route du Rhin, 67401 Illkirch Cedex, France; tel, +33 (0)390244236; fax +33 (0)390244310; e-mail, dominique.bonnet@pharma.u-strasbg.fr. C.G.: SEDAC-Therapeutics, Parc Eurasanté, Le Galénis, 85, rue Nelson Mandela, 59120 Loos, France; tel, +33 (0)146655041; fax, +33 (0)390244310; e-mail, grandjean.cyr@wanadoo.fr.

† SEDAC-Therapeutics.

‡ Present address: Université Louis Pasteur, UMR 7081 CNRS, Faculté de Pharmacie, 74, route du Rhin, 67401 Illkirch Cedex, France.

§ FRC3-CNRS.

|| UMR CNRS 8525.

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10.1021/mp050039g CCC: $30.25
Published on Web 08/23/2005

lack of solubility. Indeed, due to the presence of the lipophilic moiety, such constructs are generally sparsely soluble in water and prone to aggregation, making vaccine dose formulation difficult.[12,13] In this context, we report a novel strategy aiming to improve the solubility of lipopeptides in aqueous media by adding carbohydrate amphiphiles to favor the formation of mixed vesicles. In addition, a careful choice of the carbohydrates and design of the amphiphiles might deeply influence the delivery of the lipopeptides to the immunocompetent cells, in particular to the DCs. Indeed, the specific targeting of one among the numerous C-lectin receptors expressed by DCs is of great significance since each of them not only serves as antigen receptor but also regulates the migration of the DCs and their interaction with lymphocytes.[14] Interest in targeting receptors like the mannose receptor was first demonstrated by Tan et al. in vitro.[15] It has now been confirmed by using peptides embedded in liposomes, generally decorated with mannosylated or anti-receptor antibody addresses.[16−22] The targeting of lipopeptides has been envisaged only recently following a covalent strategy, i.e., by using lipid polylysine core peptide bearing sugar residues.[7,23] Here we report that palmitoylated-lysinyl trees decorated with mannose, galactose, or quinic acid residues efficiently solubilize a rhodaminated lipopeptide through noncovalent association. Toxicity and internalization of the vesicles thus formed were assessed by preliminary flow cytometry experiments performed on DCs.

Experimental Section

General Procedures. Analytical RP-HPLC separations were performed upon a Beckman Gold System using a C4 (4.6 × 250 mm) column at a flow rate of 1 mL min^{-1} (monitoring and analysis), with detection at 215 nm, at 50

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°C. Gradient A/B: 100/0% to 0/100% over 30 min. Semi-preparative RP-HPLC separations were performed on a Waters prep 4000 RP-HPLC preparative system, using a Hypersil hyperprep C-18 (300 Å, 10 × 260 mm) column at a flow rate of 5 mL min^{-1}, with detection at 215 nm at 50 °C. Gradient A/C: 100/0% to 70/30% over 5 min, then to 40/60% over 30 min. Solvent system A: 0.05% TFA in water. Solvent system B: 0.05% TFA in 40% CH_3CN−60% water. Solvent system C: 0.05% TFA in 40% *i*-PrOH−60% water. MALDI-TOF-MS spectra were recorded on a Voyager-DE-STR spectrometer. UV light optical microscopy was performed on Axioplan2 Zeiss using a Zeiss No. 15 filter, with 20 × 1.25 zooming.

Preparation of the Carbohydrate and Glycomimetic Clusters. Relevant data concerning both syntheses and characterization of the monosaccharide precursors and the clusters have been previously reported.[24]

Synthesis of Rhodaminated Lipopeptide 10. Solid-Phase Synthesis. The synthesis was performed at a 0.20 mmol scale on a Fmoc-Lys(Mtt)-PEG-PS resin (0.16 mmol/g) using standard Fmoc/*t*-Bu chemistry on Perseptive Pioneer peptide synthesizers. The amino acids (7 equiv) were activated using TBTU/HOBt/DIEA (7/7/14 equiv) in DMF, double coupling. Each coupling step was followed by a capping treatment with Ac_2O/DIEA/DMF (3/0.3/96.7). Side chain protections were as follows: Asn(Trt), Gln(Trt), Glu(O-*t*-Bu), Lys(Boc) or Lys(Mtt), Ser(*t*-Bu), Thr(*t*-Bu), Tyr(*t*-Bu). Fmoc amino acids were purchased from Senn Chemicals, 5-(and 6)-carboxytetramethylrhodamine from Molecular Probes, and [*N*,*N*′-tri(*tert*-butyloxycarbonyl)hydrazino]acetic acid from Novabiochem.

The Mtt protecting group of the lysine side chain was removed by treatment with a solution of 1% TFA in CH_2Cl_2 (7 times). Monitoring of the deprotection was realized as described in the literature.[25] [*N*,*N*′-Tri(*tert*-butyloxycarbonyl)hydrazino]acetic acid (156 mg, 0.40 mmol) was then introduced onto the peptidyl resin swollen in DMF using TBTU (128 mg, 0.40 mmol)/HOBt (53 mg, 0.40 mmol)/DIEA (203 μL, 1.20 mmol). Following peptide elongation and N-terminal acylation, the Mtt group on the ϵ-amino group of Lys^5 was deprotected as described above. The 5-(and 6)-carboxytetramethylrhodamine (112 mg, 0.24 mmol) was then manually coupled (twice) onto the ϵ-amino group with TBTU (77 mg, 0.24 mmol)/HOBt (32 mg, 0.24 mmol)/DIEA (122 μL, 0.72 mmol) activation in DMF. The completion of the reaction was monitored using a Kaiser test.[26] Finally the peptidyl-resin was washed with DMF, CH_2Cl_2, and Et_2O and dried under reduced pressure. Cleavage and deprotection steps were performed using a TFA (12.5 mL)/thioanisole (766 μL)/phenol (765 mg)/EDT (384 μL)/TIS (153 μL) mixture for 2 h at room temperature and in the dark. Following precipitation in a cold heptane/diethyl ether (1/1) mixture (200 mL), the residue was dissolved in water, freeze-dried, and purified by RP-HPLC. The collected fractions were freeze-dried to afford **9** as a pink powder (34 mg, 6% overall yield). MALDI-TOF: $[M + H]^+$ calcd 2634.4; found 2635.7. RP-HPLC purity (215 nm): 90%.

Lipidation by Chemical Ligation. Compound **1** (4.1 mg, 8.5 μmol) was dissolved in a *t*-BuOH/water (70:30) mixture (6.79 mL) using an ultrasonic bath. A 3.5 mL (4.4 μmol) portion of this solution was added dropwise to hydrazinopeptide **9** (11.5 mg, 3.7 μmol). The resulting solution was stirred for 4 h at 30 °C. Following completion of the reaction, monitored by RP-HPLC, the reaction mixture was diluted with AcOH/water (5/95) and freeze-dried to furnish lipopeptide **8**. MALDI-TOF: $[M + H]^+$ calcd 2986.7; found 2986.1. RP-HPLC purity (215 nm): 89%.

Preparation of the Lipopeptide/Glycoamphiphile Mixture. Typical procedure for Rho-TT-Pam/$(Qui)_4$-Pam 1/16 ratio: Rhodaminated lipopeptide **8** (1 mg, 294 nmol) was dissolved in 70 μL of an 80% acetic acid/water mixture using an ultrasonic bath and further diluted with water to furnish a 294 μM solution of **8** in 5% acetic acid/water. A 582 μM solution of $(Qui)_4$-Pam in 5% acetic acid/water was prepared following the same protocol. Then 50 μL of the former solution (15 nmol) was added to 410 μL of the latter one. The resulting solution was rapidly freeze-dried to give a white powder. Water (150 μL) was added dropwise on the powder, and the resulting solution was kept for 30 s in an ultrasonic bath and for a further 10 min at 37 °C to give a clear 100 μM solution of Rho-TT-Pam ready for solubilization assays. For biological assays, the 100 μM stock solution was diluted 5 times with either PBS (pH 7.2−7.4) or 0.9% aqueous NaCl.

Culture of Human Dendritic Cells from Peripheral Blood. DCs were generated from blood samples provided by the EFS (Etablissement Français du Sang Nord-Pas-de Calais, Lille, France) after CD14 positive selection (Miltenyi Biotech) as previously described.[27]

Lipopeptide and Mixed Micelle Uptake. After 5 days of differentiation, immature dendritic cells or CD14 negative cells were harvested and washed in PBS. They were first preincubated for 10 min on ice or at 37 °C, depending on the labeling conditions, then pulsed for 20 min at the same temperature either with the lipopeptide alone or with the glycoamphiphile/lipopeptidic mixture, washed three times with cold PBS, and then fixed for 20 min in paraformaldehyde solution (1%). The cell-associated fluorescence was further analyzed with the flow cytometer Epics XL-MCL

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system (Coulter Corporation). Results are expressed in arbitrary units, called mean fluorescence intensity (MFI).

Cell Toxicity Assessment. To assess cell viability Trypan blue staining was used. Briefly, after the usual incubation, in the same conditions as for flow-cytometry analysis, the samples were stained with a freshly prepared Trypan blue solution, and then dead cells (stained in blue) were counted using an optical microscope. Viability for each sample is expressed as the percentage of living cells. Toxicity of the compounds was calculated as follows:

$$\text{toxicity} = (\text{baseline viability} - \text{sample viability}) \times 100/\text{baseline viability}$$

Experiments were performed three times independently. Baseline viability varied from 82% to 92% (at 4 °C) and from 65% to 95% (at 37 °C), respectively.

Results and Discussion

Synthesis of Glycoamphiphiles and Rhodaminated Lipopeptide. The preparation of glycoamphiphiles was envisaged following a hydrazone chemoselective ligation[28] between polar heads and lipophilic tails. This strategy allows the separate handling of the hydrophobic and hydrophilic moieties until their assembly, performed at the very last step of the synthesis in high yield and purity: thus, tetravalent quinoylated, mannosylated, and galactosylated hydrazino-L-lysinyl trees **2**−**4**, respectively, have been synthesized and further coupled to a slight excess of palmitoylated α-oxo-aldehyde **1**[29] in 80/20 2-methyl-2-propan-2-ol/water for 5 h at 30 °C to almost quantitatively give the corresponding amphiphiles **5**−**7** referred to as $(Qui)_4$-Pam, $(Man)_4$-Pam, and $(Gal)_4$-Pam, respectively (Figure 1).[24]

Both mannosylated and quinoylated L-lysinyl trees have been shown to be effective ligands of the DC MR, quinic acid acting as a mannose bioisostere,[27,30] whereas a galactosylated cluster, though not optimized, might target a different receptor, like the DC-asialoglycoprotein receptor, which has been evidenced recently.[31]

Considering the length of the palmitoyl tail, we elected to use tetravalent carbohydrate clusters to ensure that the amphiphiles would self-assemble in micelles in aqueous solutions. This property indeed depends on both the surface area of the polar headgroup and the length of the lipidic tail.[32,33] For instance, monosaccharide surfactants like *N*-alkylglucosylacrylamides, displaying a small polar head, are not soluble in water when the alkyl chain length is longer than 14 carbons.[34]

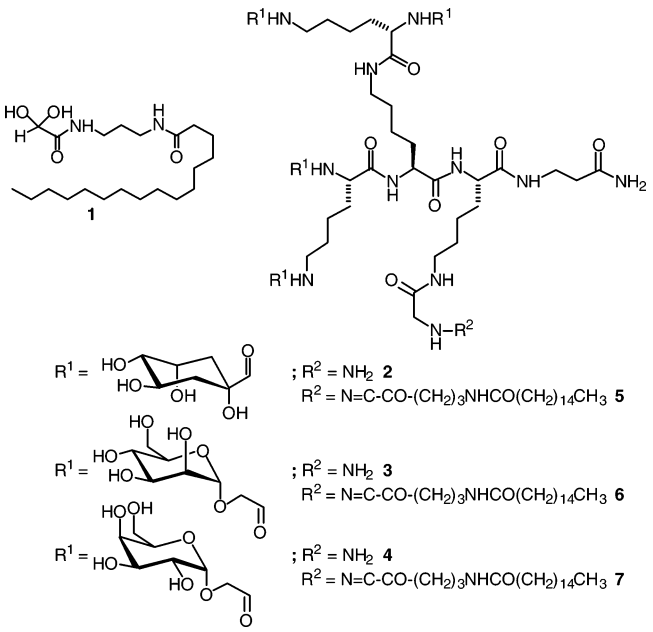

Figure 1. Palmitoylated α-oxo-aldehyde **1**, tetravalent clusters **2**−**4**, and their corresponding ligated amphiphiles **5**−**7**.

Critical micelle concentrations of amphiphiles **5**−**7** are very low, in the 5×10^{-5} to 10^{-5} M range, as determined by surface tension measurements and in good agreement with values obtained for nonionic amphiphiles bearing a hexadecyl hydrocarbon chain.[24]

To evaluate the effect of these palmitoylated glycoclusters on the lipopeptide solubilization, we planned to prepare a model lipopeptide: the promiscuous T-helper tetanus toxoid sequence, $TT^{830-846}$, was selected owing to its propensity to aggregate, even in the absence of a lipidic modification,[35] and its well-established use to study immune responses. Again, the lipidation step was envisaged following a hydrazone chemoselective ligation performed on the peptide

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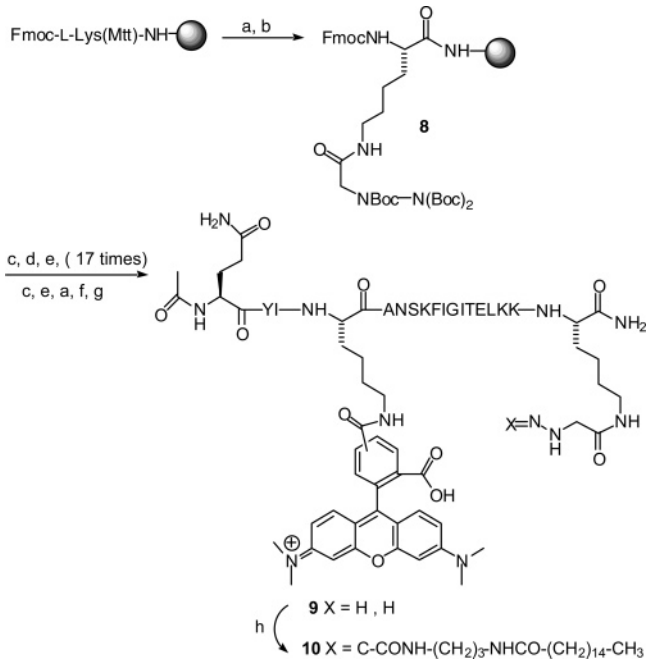
Scheme 1. Synthesis of the Lipopeptide **10**[a]



[a] Reagents and conditions: (a) TFA/CH_2Cl_2 (1/99); (b) [*N*,*N*′-tri(*tert*-butyloxycarbonyl)hydrazino]acetic acid/TBTU/HOBt/DIEA (2/2/2/6 equiv), DMF; (c) piperidine/DMF (20/80); (d) AA/TBTU/HOBt/DIEA (7/7/7/14 equiv), DMF; (e) Ac_2O/DIEA/DMF (3/0.3/96.7); (f) 5-(and 6)-carboxytetramethylrhodamine TBTU/HOBt/DIEA (1.2/1.2/1.2/3.6 equiv), DMF, (twice); (g) TFA/thioanisole/phenol/EDT/TIS, room temperature, 2 h; (h) **1** (1.2 equiv), *t*-BuOH/H_2O (70/30), room temperature, 5 h.

labeled with rhodamine. This fluorescent probe would be useful to assess its internalization by the DCs in the presence or absence of glycoamphiphiles.

Thus, peptide **9** was synthesized on a PEG-PS resin preloaded with a Fmoc-L-Lys(Mtt) peptidyl residue using the Fmoc/*tert*-butyl chemistry (Scheme 1).[36]

A hydrazino function was next introduced using [*N*,*N*′-tri(*tert*-butyloxycarbonyl)hydrazino]acetic acid,[37] after selective TFA removal of the 4-methyltrityl protective group[25] to give peptidyl resin **8**. Following peptide elongation and N-terminal acylation, the 4-methyltrityl protective group on the ϵ-amino group of Lys[5] was removed as described above and the peptidyl resin further labeled with 5-(and 6)-carboxytetramethylrhodamine. Rhodaminated-peptide **9** was obtained in 6% overall yield following cleavage from the resin, RP-HPLC purification, and freeze-drying. Finally, this intermediate was cleanly converted to its palmitoylated analogue **10** referred to as Rho-TT-Pam, upon coupling with oxo-aldehyde **1**.

Solubility Assays. The particle size of each formulation was determined by optical microscopy using Metamorph software. The area of each particle on the glass slide was compared to the total area of the particles. The measurement was performed from a 100 μM concentration of Rho-TT-Pam in water with an increasing amount of amphiphiles, the concentration of the latter remaining below their cmc. Rho-TT-Pam was found barely soluble in water at a 100 μM concentration forming large aggregates (Figure 2a). The size of more than 85% of the particles was found to be superior to 25 μm in diameter (Figure 2b). As expected, average particle sizes diminished in the presence of an increasing amount of amphiphiles, e.g., $(Qui)_4$-Pam increasing from 1/1 up to 1/16 molar ratios (Figure 2c–j). At 1/1 or 1/4 Rho-TT-Pam/$(Qui)_4$-Pam molar ratios, particles appeared as heterogeneous populations and some large aggregates were still observed by miscroscopy (Figure 2c,e). As seen in Figure 2f, every size range was present, none of them being

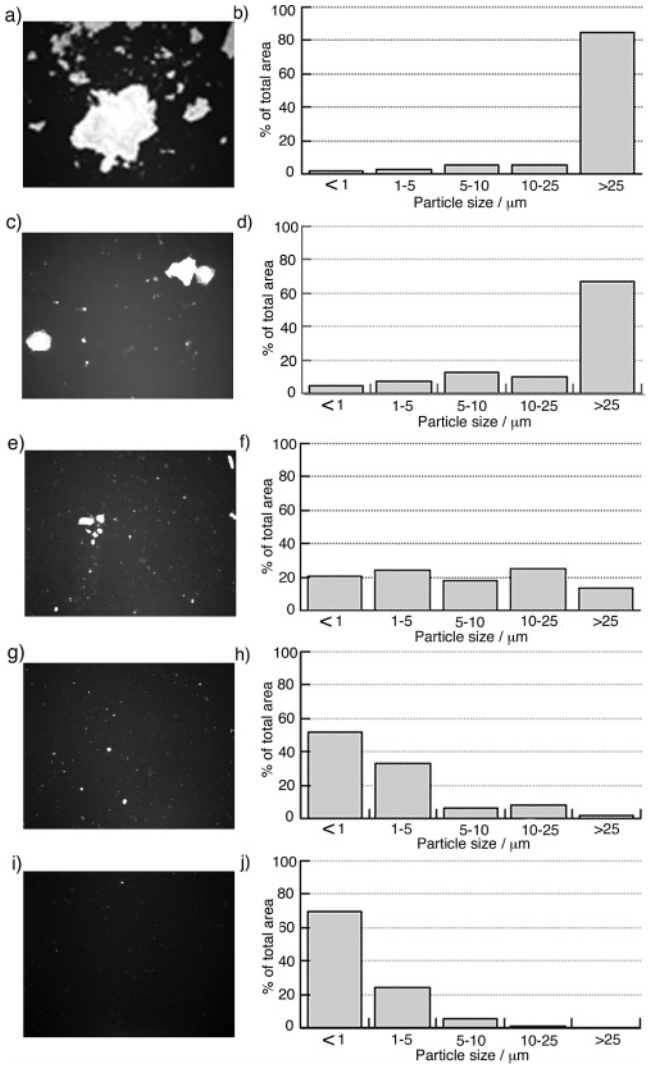


Figure 2. UV light optical micrographs (a, c, e, g, i) and particle size distribution histograms (b, d, f, h, j) at 100 μM concentration in water: (a, b) Rho-TT-Pam alone; (c, d) Rho-TT-Pam/$(Qui)_4$-Pam, 1/1 molar ratio; (e, f) Rho-TT-Pam/$(Qui)_4$-Pam, 1/4 molar ratio; (g, h) Rho-TT-Pam/$(Qui)_4$-Pam, 1/8 molar ratio; (i, j) Rho-TT-Pam/$(Qui)_4$-Pam, 1/16 molar ratio.

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predominant. Apparent solubility was achieved at a 1/8 Rho-TT-Pam/$(Qui)_4$-Pam molar ratio but was slightly improved at a 1/16 molar ratio. At these molar ratios, particles having a size superior to 25 μm represented only 2% of the total surface or were totally absent, respectively. In a parallel manner, particles having a size inferior to 1 μm contributed to 52% and 70% of the total surface, respectively.

Taken together, these results clearly indicated the positive effect of the amphiphiles on the solubilization of Rho-TT-Pam. In addition, the size of the particles obtained with a 1/16 Rho-TT-Pam/$(Qui)_4$-Pam molar ratio was found in accordance with the size of particles taken up by DCs. Indeed, recent studies have shown that the DCs could internalize particles within vesicles formed by cell surface ruffles that are up to 5 μm in diameter.[38,39]

DC Uptake Assays. A 20 μM concentration for Rho-TT-Pam, used alone or mixed with a glycoamphiphile, was selected to assess its uptake by DCs. In the latter case, glycoamphiphile was added to obtain the 16/1 glycoamphiphile/lipopeptide molar ratio which was found optimal. Internalization of substances or microorganisms by DCs generally proceeds via endocytosis or receptor-mediated endo- and macropinocytosis.[40] Uptake of lipopeptides has been shown to take place via both mechanisms.[41,42] For the latter case, the receptor, which was involved in the uptake, binds to the lipid moiety of the lipopeptide.

However, passive uptake via membrane insertion, via flip-flop internalization, or through membrane breach might not be ruled out and has been experienced using nonendocytic cells such as Kupfer's cells.[43]

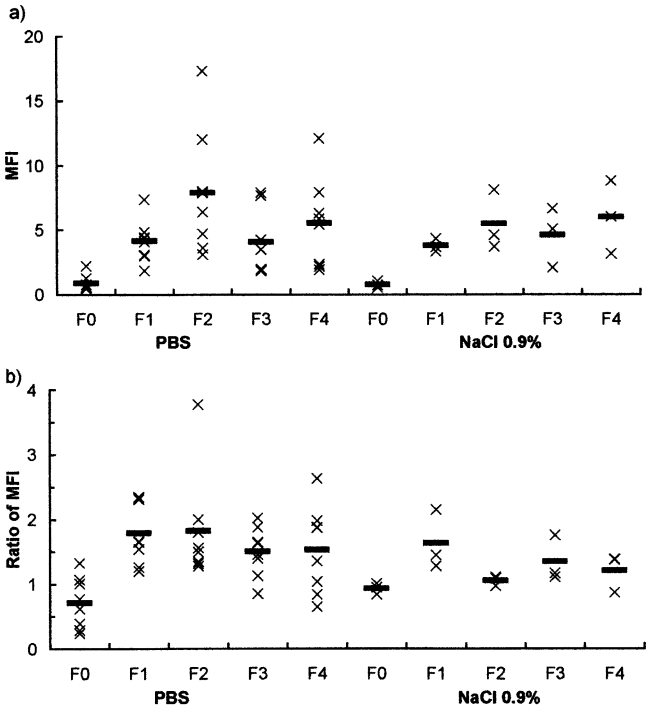

Figure 3. Rho-TT-Pam uptake by immature DCs. Cells were incubated with the different formulations for 20 min at 37 or 4 °C in PBS or NaCl 0.9%. (a) Cell-associated fluorescence, due to the uptake of Rho-TT-Pam at 37 °C, was quantified by MFI (arbitrary units of fluorescence). (b) Index of endocytosis represented as the 37 °C/4 °C ratios of the MFIs measured for each independent experiment. F0: background (NMK). F1: Rho-TT-Pam. F2: Rho-TT-Pam/$(Gal)_4$-Pam. F3: Rho-TT-Pam/$(Man)_4$-Pam. F4: Rho-TT-Pam/$(Qui)_4$-Pam.

In a first series of eight independent experiments, the uptake of Rho-TT-Pam in the presence or absence of glycoamphiphile was examined by flow cytometry. As shown in Figure 3a, the uptake of Rho-TT-Pam is slightly enhanced when formulated with $(Gal)_4$-Pam or $(Qui)_4$-Pam and equivalent when using $(Man)_4$-Pam in PBS (pH 7.2−7.4) at 37 °C. To assess the influence of the buffer composition upon the solubility, three other experiments were carried out in saline NaCl 0.9%. A better solubility of Rho-TT-Pam in the presence of a 16/1 molar ratio of amphiphiles was observed by microscopy in NaCl than in PBS. However, no significant difference in the uptake was observed (Figure 3a).

Each experiment was also performed at 4 °C to further characterize the nature of the uptake. As represented in Figure 3b, the index of endocytosis, estimated by the uptake ratio at 37 °C compared to that at 4 °C, is superior to 1 for all formulations, showing a significant endocytosis. This index is not enhanced when mixed vesicles are used instead of the lipopeptide alone. However, one should consider that endocytosis of lipopeptides was previously shown to proceed efficiently and rapidly.[41]

This high degree of endocytosis was strongly supported by the comparison of Rho-TT-Pam uptake in immature versus mature DCs. It is well-known that, in immature DCs (after 5 days of culture), receptor-dependent pinocytosis but

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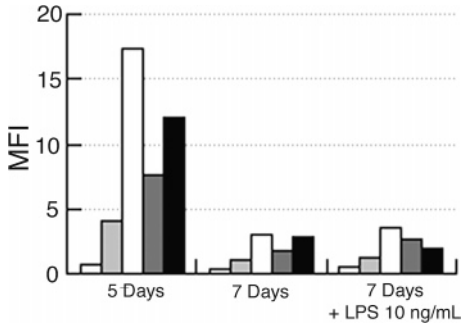

Figure 4. Comparison of the Rho-TT-Pam uptake by immature or mature DCs. The different formulations were incubated for 20 min at 37 °C in PBS with DCs after 5 days of culture or 7 days of culture or with mature DCs. Cell-associated fluorescence, due to the uptake of Rho-TT-Pam at 37 °C, was quantified by mean fluorescence intensity (MFI) (arbitrary units of fluorescence). Left to right within each group of five bars: background (light gray), Rho-TT-Pam (medium gray), Rho-TT-Pam/$(Gal)_4$-Pam (white), Rho-TT-Pam/$(Man)_4$-Pam (dark gray), Rho-TT-Pam/$(Qui)_4$-Pam (black).

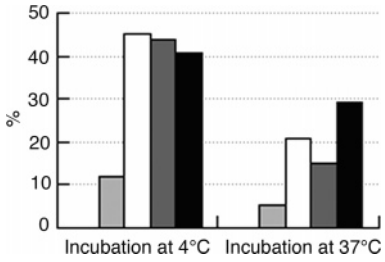

Figure 5. Toxicity of the mixed vesicles on DCs. Cells were incubated with the different formulations for 20 min at 37 °C or 4 °C in PBS, and dead cells were counted on an optical microscope after Trypan blue staining. Percent of toxicity is calculated as (baseline viability − sample viability) × 100/baseline viability. The results shown are representative of at least three independent experiments. Baseline viability varied from 82% to 92% (at 4 °C) and from 65% to 95% (at 37 °C). Left to right within each group of four bars: Rho-TT-Pam (medium gray), Rho-TT-Pam/$(Gal)_4$-Pam (white), Rho-TT-Pam/$(Man)_4$-Pam (dark gray), Rho-TT-Pam/$(Qui)_4$-Pam (black).

also nonspecific endocytosis are maximal and start to decline during maturation.[40] Therefore, we tested uptake of DCs after 5 days of culture (immature DCs) and after 7 days of culture and DCs that were treated for 48 h with 10 ng/mL of LPS, to induce maturation. As expected, upon maturation (in the presence or absence of LPS), endocytosis of mixed vesicles was strongly diminished (Figure 4).

At this stage, we were unable to confirm by flow cytofluorimetry whether or not the MR partially mediates the uptake of $(Qui)_4$-Pam and $(Man)_4$-Pam. In particular, competitive inhibition assays[24] using mannan, a bacterial polysaccharide that binds to the MR, were not conclusive (data not shown). Presumably, confocal microscopy experiments would be helpful to qualitatively characterize the different mechanisms simultaneously involved in the uptake.[44]

Finally, DC viability was affected when DCs were incubated with the different formulations of the lipopeptide. In each case, viability diminished but the toxic effect was less pronounced when Rho-TT-Pam alone was used rather than mixed vesicles (Figure 5).

The difference might be ascribed to the amount of product used but also to the surface active properties of the amphiphiles. According to that, toxicity was more important at 4 °C than at 37 °C: endocytosis being blocked at low temperature, passive insertion of the free amphiphiles into the cell membrane, which is deleterious, may be maximized. However, one can expect to modulate the toxicity by varying the nature of the lipophilic moiety.[45] Besides, residual toxicity can be desirable as it may contribute to the appearance of the immune response by facilitating cross-presentation[46] or by simply providing inflammatory signals.

Conclusion

In this preliminary study, we demonstrated that glycoamphiphiles might be useful to solubilize a lipopeptide prone to aggregation and that the resulting mixed micelles were taken up mainly via endocytosis by DCs in vitro. Further experiments are required to fully characterize the exact nature of the vesicles formed and to qualitatively define the endocytic pathways involved in the internalization.

However, most importantly the next step will consist of determining the immune response obtained and of studying a possible adjuvant effect when glycoamphiphile-lipopeptide mixed vesicles are being used compared to the same lipopeptide cocktail used alone or formulated with a non-DC membrane-receptor ligand polyol amphiphile.

Abbreviations Used

DC, dendritic cell; MR, mannose receptor; RP-HPLC, reverse-phase high performance liquid chromatography; TFA, trifluoroacetic acid; *i*-PrOH, propan-2-ol; MALDI-TOF-MS, matrix-assisted laser desorption ionization mass spectroscopy; PEG-PS, poly(ethylene glycol)-polystyrene; Fmoc, 9-fluorenylmethoxycarbonyl; Mtt, methyltrityl; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; HOBt, 1-hydroxybenzotriazole; DIEA, diisopropylamine; Trt, trityl; *t*-Bu, *tert*-butyl; Boc, *tert*-butyloxycarbonyl; EDT, ethanedithiol; TIS, triisopropylsilane; Rho,

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5-(and 6)-carboxymethylrhodaminyl; Pam, palmitoyl; TT, 830−846 fragment of tetanus toxoid; MFI, mean fluorescence intensity; cmc, critical micelle concentration; DMF, dimethylformamide; Man, mannose; Gal, galactose; Qui, quinic acid.

Acknowledgment. We thank Nathalie Fardel and Linda Baptista for their dedication to this work.

MP050039G