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Neo-mannosylated liposomes: Synthesis and interaction with mouse Kupffer cells and resident peritoneal macrophages

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In order to target liposomes to cells expressing at their surface mannose receptors, e.g. mouse Kupffer cells and peritoneal macrophages, we have developed a new synthetic strategy which allows a chemically well defined preparation of neo-mannosylated vesicles. α-D-Thiomannopyranoside residues, substituted with a hydrophilic spacer arm and functionalized with a sulfhydryl group, were covalently coupled to preformed large unilamellar vesicles containing 4-(*p*-maleimidophenyl)butyryl phosphatidylethanolamine. Liposomes, containing 15 mol% of mannosyl residues, were specifically aggregated with concanavalin A; this aggregation could be reversed by an excess of free methyl α-D-mannopyranoside indicating that the surface ligands were freely accessible to the lectin. The neo-mannosylated liposomes presented in vitro an increased binding to cells possessing α-D-mannose specific binding sites. At 37°C a specific binding, up to 9-fold compared to control vesicles, was observed. These neo-mannosylated vesicles represent attractive tools for targeting bio-active molecules to macrophage-associated diseases.

Introduction

The natural uptake of large liposomes by macrophages can be exploited as a means for drug delivery. This passive targeting was used for example to activate the tumoricidal properties of macrophages, by liposome-associated immunomodulators [1–4], or for the treatment of diseases linked to macrophage-resident microorganisms [5] and parasites [6]. However, the incorporation into vesicles of ligands capable of interacting with macrophage surface receptors was shown to markedly promote their uptake by these cells [7–9]. In order to increase the interaction of liposomes with macrophages we have now prepared neo-mannosylated vesicles. Among the different receptors present at the surface of macrophages, the lectin specific for the binding of α-D-mannose residues is attractive for liposome targeting. It is expressed by a variety of macrophages [10], e.g., peritoneal [11,12], lung alveolar [13–16] and splenic macrophages [17], as well as by Kupffer cells [18–20]. Moreover, following its internalization, the mannose receptor is continuously recycled–back to the cell surface where it can take part to new rounds of endocytosis [21]; this property is responsible for an efficient cellular uptake of the ligands of this receptor.

Our synthesis of neo-mannosylated liposomes is based on a versatile method developed by Martin and Papahadjopoulos [22], which involves the reaction under mild conditions between preformed vesicles (REV) containing MPB-PE, a reactive lipid derivative, and a ligand possessing a thiol group. The glucidic residue was conjugated to the liposomes via a hydrophilic spacer-arm whose length can be easily extended. Two types of derivatization were chosen for the mannosyl moiety: (i) an *O*-glycosidic bond and (ii) a thioether bond; this latter linkage is known for its chemical and metabolic stability [23] and, moreover, substitution of the anomeric oxygen by sulfur is not detrimental to the affinity of the mannosyl residue for its receptor [24]. The neo-mannosylated liposomes prepared by our method could be aggregated specifically by the plant lectin concanavalin A. The results obtained in the present study on the interaction of the neo-mannosylated liposomes with murine Kupffer cells and resident peritoneal macrophages indicate that the targeted liposomes present much higher affinities for those cells than the unconjugated vesicles.

Abbreviations: Man, D-mannose; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; SATA, succinimidyl-*S*-acetylthioacetate; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; SMPB, succinimidyl-4-(*p*-maleimidophenyl)butyrate; MPB-PE, 4-(*p*-maleimidophenyl)butyryl phosphatidylethanolamine; Con A, concanavalin A; REV, reverse phase evaporation vesicles; PBS, phosphate-buffered saline; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Materials and Methods

Cholesterol (recrystallized in methanol), phosphatidylethanolamine (egg yolkd), fluorescamine, 5,5'-dithiobis(2-nitrobenzoic acid), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, concanavalin A, mannan, methyl α-D-mannopyranoside and α-D-mannose pentaacetate were purchased from Sigma Chemical Co. [^{3}H]Inulin (average M_r 5200) 4.9 Ci/mmol) was from Amersham. SMPB was from Pierce Chemical Co. and MPB-PE was prepared according to Martin and Papahadjopoulos [22]. SATA was synthesized according to Duncan et al. [25] and SPDP according to Carlsson et al. [26]. Phosphatidylcholine was extracted from egg yolk and purified according to Nielsen [27]. 5(6)-Carboxyfluorescein was obtained from Eastman Kodak Co. and purified according to Ralston et al. [28]. Dulbecco's modified Eagle's medium, fetal calf serum were obtained from Gibco BRL (France) and the culture plates from Falcon. Thin-layer chromatography (TLC) was performed on silica gel F-254 (Merck) plates and the spots were detected by a sulfuric acid-ethanol (5 : 95, v/v) spray. All other reagents were of analytical grade.

Synthesis of the functionalized p-aminophenyl-α-D-mannosyl residue

p-(*N'*-(*N*-(3-Mercaptopropionyl)-3-amino-2-propanol)thiourea)phenyl-α-D-mannose (compound **5a**) was synthesized by a procedure outlined in Fig. 1a. The known intermediary *p*-phenylisothiocyanate-α-D-mannopyranoside (**2a**) [29,30] was obtained from *p*-aminophenyl-α-D-mannose. In brief, **1a** (300 mg; 1.1 mmol) was converted into **2a** by reaction (4 h at room temperature) with *N*,*N'*-thiocarbonyldiimidazole (Merck) (300 mg; 1.68 mmol) in ethanol (50 ml) [31]. The reaction product (83% yield), recrystallized in ethanol, gave a single spot on thin-layer chromatography with R_f 0.32 (chloroform/methanol, 1 : 1); it was used to prepare *p*-(*N'*-(3-amino-2-propanol)thiourea)phenyl-α-D-mannose (compound **3a**). To the isothiocyanate **2a** (60 mg; 0.19 mmol) in ethanol (20 ml) was added 1,3-diamino-2-propanol (180 mg; 2 mmol). After 16 hours, under stirring at room temperature, the mixture was purified by chromatography on a 20 × 2 cm Dowex 50-X2 (200–400 mesh; H^+ form). After a washing with 100 ml water, **3a** was obtained by elution with 0.33 M hydrochloric acid (300 ml; flow rate: 2 ml/min), and after lyophilization, as a colorless sirup (32% yield). Thin-layer chromatography on silica gel plates (chloroform/methanol, 1 : 1) indicated a single spot (R_f 0.14). SPDP (40 mg; 0.17 mmol) dissolved in 0.4 ml ethanol, was added to a solution of **3a** (0.055 mmol) in 4 ml 50 mM sodium phosphate buffer (pH 7.0) [32]. After one hour at room temperature, under argon, the unreacted amino-mannose derivative was adsorbed on a 25 × 2 cm Dowex 1-X2 (formate form) column. Product **4a**, which was eluted with water (TLC: R_f 0.48; chloroform/methanol, 1 : 1), was thiol deprotected by addition of $NaBH_4$ (0.227 mg; 6 μmol) at pH 7.0 (adjusted by HCl 1 M) to give **5a**. The excess of borohydride was destroyed by acid (pH 4.0). Compound **5a** was used immediately without further purification (yield 11%).

Synthesis of the functionalized 1-thio-α-D-mannosyl residue

1'-Deoxy-1'-*N'*-(*N*-(mercaptoacetyl)-3-amino-2-propanol)thioacetamide-α-D-mannopyranoside (compound **8b**) was synthesized by the procedure outlined in Fig. 1b. The known intermediary 1'-deoxy-1'-thioacetic acid-α-D-mannopyranoside [33] (**4b**) was obtained from penta-*O*-acetyl-α-D-mannose (**1b**) by procedures adapted from literature [34,35]. In brief, **1b** (5 g; 12.8 mmol) was converted into 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide (**2b**) in 70% yield by reaction (3 h at room temperature) with 33% (w/v) HBr in glacial acetic acid (20 ml) containing 0.1 ml acetic anhydride. Chloroform (100 ml) was then added and the organic phase was washed successively with ice water, 0.2 M $NaHCO_3$, dried (anhydrous sodium sulfate) and evaporated. The crude material consisting of a colorless syrup, giving a single spot on TLC (R_f 0.63; toluene/ethyl acetate, 1 : 1), was used without further purification to prepare 1'-deoxy-1'-thiopseudourea-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside hydrobromide (**3b**). The bromide **2b**) (3.5 g; 8.5 mmol) in anhydrous acetone (50 ml) was heated under reflux in the presence of thiourea (0.8 g; 10.5 mmol) according to Matta et al. [36]. After 3 hours, the reaction mixture was extracted with chloroform (20 ml) and **3b**, obtained in 95% yield, gave a single spot on TLC (R_f 0.41; toluene/ethyl acetate, 1 : 1). A mixture of **3b** (3.8 g; 7.8 mmol) and iodoacetic acid (3.3 g; 18 mmol) in 20 ml acetone/water (1 : 1, v/v) was treated with potassium carbonate (1.25 g; 9 mmol) and sodium metabisulfite (1.52 g; 8 mmol) according to Lee et al. [37] and Ponpipom et al. [33]. After 45 min stirring at room temperature under argon, the mixture was acidified with 5% (v/v) HCl (100 ml) and extracted with chloroform (100 ml). The chloroform layer was dried (anhydrous sodium sulfate) and evaporated to obtain 1'-deoxy-1'-thioacetic acid-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (compound **4b**) as a slightly yellow oil (TLC: R_f 0.96; ethyl acetate/acetic acid/water, 8 : 2 : 1) which was deacetylated without further purification [37]. To a solution of **4b** (2 g; 4.7 mmol) in 100 ml methanol/water (25 : 85, v/v) was added 5.5 g (40 mmol) potassium carbonate and the mixture was stirred overnight at room temperature. The solvents were then evaporated under vacuo and the resulting 1'-deoxy-1'-thioacetic acid-α-D-mannopyranoside (**5b**) was purified by chromatography on a 25 × 2 cm Dowex 1-X2 (for-

mate form) column. Compound **5b** was obtained, after a washing with 100 ml water, by elution with 2 M formic acid (300 ml; 2 ml/min) and after lyophilization, as a colorless sirup (21% yield). TLC on silica gel plates (butanol/acetone/water, 4:5:1) indicated a single spot (R_f 0.47).

1'-Deoxy-1'-N-(3-amino-2-propanol)thioacetamide-α-D-mannopyranoside (compound 6b). A solution of **5b** (250 mg; 1 mmol) and 1,3-diamino-2-propanol (450 mg; 5 mmol) in 10 ml water, adjusted to pH 5.5 with 0.1 M HCl, was treated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (575 mg; 3 mmol). After 12 h at room temperature, the reaction was essentially complete. Compound **6b** was purified by chromatography on a 20×2 cm Dowex 50-X2 (H^+) column, washed by 100 ml water and then eluted with 0.33 M hydrochloric acid (300 ml; 2 ml/min). Fractions containing **6b**, which were positive for amine [38] and sugar reagents [39], were combined and lyophilized. Compound **6b**, which was obtained as a chlorohydrate (yield 40%), gave a single spot on TLC with R_f 0.21 (pyridine/ethyl acetate/acetic acid/water, 5:5:1:3).

1'-Deoxy-1'-N'-(N-(acetylmercaptoacetyl)-3-amino-2-propanol)thioacetamide-α-D-mannopyranoside (compound 7b). SATA (92.4 mg; 0.4 mmol) was added to a solution of **6b** (145 mg; 0.4 mmol) in 8 ml anhydrous dimethyl formamide containing triethylamine (0.056 ml; 0.4 mmol). After one night at room temperature, under argon, the reaction was complete as judged by the disappearance of the free primary amino group of **6b** measured with fluorescamine [38]. After elimination of the solvent, the reaction product was chromatographed on a 90×1.6 cm column of Bio-Gel P2, 200–400 mesh, eluted with water. The fractions containing the glucidic derivatives were pooled and after lyophilization, 110 mg of an amorphous white solid was obtained (yield 58%). TLC: R_f 0.30 (butanol/acetone/water, 4:5:1).

1'-Deoxy-1'-N'-(N-(mercaptoacetyl)-3-amino-2-propanol)thioacetamide-α-D-mannopyranoside (8b). The compound **8b** was obtained by treating **7b** with a 30-fold molar excess of hydroxylamine in 5 mM Hepes buffer (pH 7.4) containing 0.15 M EDTA, under argon. After 30 min at room temperature, the thiol deprotection was complete, as estimated by sulfhydryl-group determination with 5,5'-dithiobis(2-nitrobenzoic acid) [40]. Compound **8b** was used immediately without further purification.

Preparation of liposomes and coupling of the thiol functionalized α-D-mannosyl residues

Large unilamellar vesicles were prepared from 10 μmol lipids (PC/MPB-PE/cholesterol, at molar ratio 10:2:7) by the reverse-phase evaporation technique [41]. The molecules to be encapsulated, e.g. 50 mM 5(6)-carboxyfluorescein, were in 5 mM Hepes buffer (pH 7.4) and the osmolality of the solutions was adjusted to 290 mosmol/kg with NaCl. For the conjugation of the ligands, freshly prepared vesicles (1 ml) were mixed with 2-fold molar excess of compounds **5a** or **8b** over MPB-PE. Control liposomes, i.e. non-targeted liposomes, were reacted similarly with excess mercaptoethanol. The conjugated liposomes were then separated from reagents, unencapsulated molecules and excess ligands by filtration on a 1×18 cm Sephadex G-75 column equilibrated and eluted with PBS (6.48 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, 136.75 mM NaCl; pH 7.4). After organic extraction of the phospholipids [42], the organic layer (chloroform) was analyzed for its phosphorus content [43] and amount of covalently coupled mannosyl residues [39].

Interaction between concanavalin A and neo-mannosylated liposomes

Neo-mannosylated liposomes (final concentration 19.5 μM) composed of PC/cholesterol/MPB-PE (10:7:1) were incubated in 1 ml (final volume) of 5 mM Hepes buffer (pH 7.6) containing 140 mM NaCl and 0.5 mM $CaCl_2$, at room temperature, with varying concentrations (0–100 μg) of concanavalin A. After rapid mixing, aggregation of the vesicles was estimated by the time-dependent increase in turbidity as measured by absorbance at 360 nm with a Shimadzu spectrophotometer (Model MPS-2000), equipped with a graphic printer (PR-3). The reversibility of the aggregation was assessed by addition of free methyl α-D-mannopyranoside.

Interaction between the neo-mannosylated liposomes and macrophages (Kupffer cells and resident peritoneal cells)

Resident peritoneal macrophages were obtained as described [44] from female Balb/c mice (6 to 8 weeks old) in DMEM-10% (v/v) decomplemented-FCS (30 min, 56°C) containing heparin (5 U/ml). The cell number was adjusted to 10^6 cells/ml and the suspension was plated (final volume 1 ml) in multiwell plates (Falcon). After 2 h at 37°C in a humidified atmosphere of 5% CO_2 in air (final pH 7.4), non-adherent cells were eliminated by rinsing the dishes three times with PBS. The adherent cells presented a viability superior to 95% as checked with the Trypan blue exclusion test and were non-specific esterase positive at 90% (Sigma research kit 90). Mouse Kupffer cells, purified from a collagenase liver digest by centrifugal elutriation techniques [45], were kindly provided by Dr. F. Keller (Laboratoire de Virologie du Professeur A. Kirn; Strasbourg) as adherent cells in multiwell plates ($1 \cdot 10^6$ to $2 \cdot 10^6$ cells/well).

The adherent cells (peritoneal macrophages and Kupffer cells), 24 h after their isolation, were fed with fresh serum-less DMEM and incubated with varying amounts of targeted and control liposomes containing 50 mM 5(6)-carboxyfluorescein or [^{3}H]inulin ($23.4 \cdot 10^3$ dpm/nmol phospholipid). After the incubation time,

the medium was pipetted-off and the cells washed four times with cold PBS (4°C). Carboxyfluorescein associated to the cells was measured fluorometrically (Jobin-Yvon spectrofluorimeter, Model JY-3D) after cell digestion in 1 ml PBS containing 0.1% of emulphogene BC-720, a non-ionic detergent (GAF-France), and scraping with a rubber policeman. A standard fluorescence curve was established under the same conditions with aliquots of the initial liposome preparations [46] in order to correlate the amounts of fluorescent label with phospholipid content [43]. When [^{3}H]inulin-encapsulated vesicles were used, amounts of label associated with the cells were determined and related to liposomal phospholipid. Control experiments indicated that adsorption of targeted and control liposomes to the plastic dishes, in the absence of cells, was negligible. The stability of the liposomes was also measured by their ability to retain encapsulated carboxyfluorescein [46]; under the different incubation conditions used in this study more than 90% integrity of the vesicles was observed.

The results given in the Figs. 3–5 are that of typical experiments. The points represent amounts of liposomal lipids associated to 10^6 cells; they are means of that least duplicates, which do not differ by more than 5%.

Results

Covalent coupling of α-D-mannosyl residues to preformed vesicles was achieved according to an adaptation of the method of Martin and Papahadjopoulos [22]

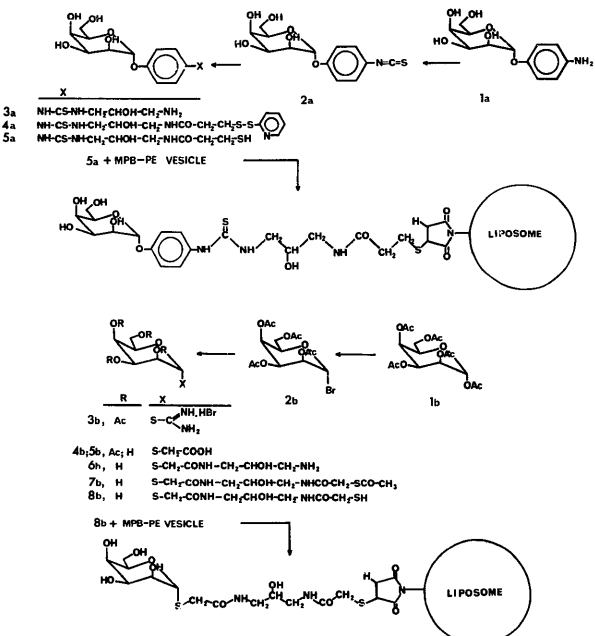

Fig. 1. (a) General scheme for the synthesis of α-D-mannose-derived ligands and conjugation to preformed liposomes. (b) General scheme for the synthesis of α-D-thiomannose-derived ligands and conjugation to preformed liposomes.

to small molecular weight compounds. It involves the reaction, under mild conditions, between preformed liposomes containing the reactive MPB-PE, i.e. an electrophilic maleimido moiety linked through a spacer arm to phosphatidylethanolamine, and ligands possessing a thiol group. As ligands we have selected two α-D-mannosyl derivatives which were linked to a hydrophilic spacer and functionalized with a sulfhydryl group (compounds **5a** and **8b**; Figs. 1a and 1b). The two compounds differ essentially by their linkage to the spacer arm. In compounds **5a** the mannosyl residue is coupled through an α-O-linkage to an aromatic residue; this is the classical approach adopted e.g. for the synthesis of neo-glycoproteins [29,30]. In contrast, in compound **8b** the anomeric carbon of the mannosyl residue is linked through an α-thioether to an alkyl chain, as previously advocated by Ponpipom et al. [47] because of the greater chemical and metabolic resistance of this glycosidic bond. In our work we have put more emphasis on this latter approach, although it was more demanding from a synthetic point of view.

Synthesis of the functionalized mannosyl ligands and coupling to liposomes

The synthetic routes for the preparation of **5a** and **8b** have been outlined in Figs. 1a and 1b and described under Materials and Methods. We will discuss here only the chemical steps which are original compared to the published procedures in this area. Compound **2a** was conveniently obtained, in high yields, from the commercially available *p*-aminophenyl-α-D-mannose by action of *N,N'*-thiocarbonyldiimidazole [31]; this reagent circumvents utilization of the agressive thiophosgene which has been traditionally used for that purpose [29,30]. Derivative **2a** was grafted to a hydrophilic spacer arm consisting of 1,3-diamino-2-propanol. If needed, this arm could easily be further lengthened by successive treatment with succinic anhydride and coupling of an additional 1,3-diamino-2-propanol [32]. For the step leading to the introduction of the sulfhydryl function of the ligand, compound **3a** was reacted with the heterobifunctional reagent SPDP. The pH which gave the best yields for this step was 7.0, the coupling being complete in minutes. The reduction of the disulfide linkage of compound **4a**, which generates the thiol function of **5a**, was accomplished with sodium borohydride, the excess reductant being destroyed in presence of acid. This reagent avoids usage of dithiothreitol which is generally taken for that purpose [48] and which necessitates lengthy separation of the generated thiol from the excess reducing agent.

In order to prepare compound **8b**, commercially available penta-*O*-acetyl-α-D-mannose (**1b**) was first converted into the known [33] intermediary compound **4b** using methods adapted from literature. Briefly, the per-acetylated derivative of D-mannose was converted into the corresponding bromide **2b**, which in presence of thiourea gave the thiopseudourea hydrobromide derivative **3b**. After reduction of **3b** by sodium metabisulfite, the generated active thiol group was reacted in situ with iodoacetic acid to yield compound **4b**. Compound **5b** was finally obtained, in an overall yield of about 20%, after deacetylation of **4b** by potassium carbonate in methanol-water. As above, the latter compound was conjugated to the hydrophilic spacer arm 1,3-diamino-2-propanol. For the final step leading to the introduction of the sulfhydryl function of the ligand, compound **6b** was reacted with SATA, an other heterobifunctional reagent [25,49], in dimethyl formamide to give **7b**. The ligand **8b**, with the free thiol group, was ultimately obtained by deprotection with aqueous hydroxylamine. The final products 5a and 8b were handled in acidic (pH 4.0) deoxygenated media, under argon, in order to minimize disulfide exchange and/or reoxydation reactions.

Coupling of the functionalized ligands (**5a** and **8b**) to preformed liposomes (REV), composed of PC/cholesterol and containing 10 or 15 mol% of MPB-PE, was carried out essentially as described [22]. The extent of the reaction was estimated by analysis of the sugar moieties found associated, after extraction, with the liposomal phospholipids. An average of 80% and 97% of total MPB-PE were found conjugated, respectively, to **5a** and **8b**, indicating that these functionalized ligands had also access to the lumen of the liposomes.

Concanavalin A-mediated aggregation of the neo-mannosylated liposomes

In order to evaluate the validity of our approach we have investigated if concanavalin A was able to bind to neo-mannosylated liposomes and mediate their aggregation. Concanavalin A is a tetrameric protein which has four binding sites specific for terminal non-reducing α-mannosyl residues [50,51]. By cross-linking the vesicles the lectin can promote their agglutination. In Fig. 2 are given the turbidity (light-scattering) increases, measured at 360 nm, which are observed after addition of increasing concentrations of free concanavalin A to a preparation of liposomes containing 15 mol% 1-thio-α-mannosyl (**8b**) residues. The apparent rate of agglutination is dependent on the lectin concentration. When concanavalin A concentration was above 50 μg/ml the extent of aggregation was decreased indicating a saturation of the binding sites. Cases have been reported where this lectin was able to induce aggregation and fusion of liposomes constituted of only phospholipids [52]; this was not the case with our unconjugated vesicles (not shown). Moreover, on addition of a 60-fold molar excess of free methyl α-D-mannopyranoside over liposomal mannosyl residues, the aggregation of the neo-mannosylated vesicles by concanavalin A could be reversed (Fig. 2; inset), providing evidence for the specific nature of the

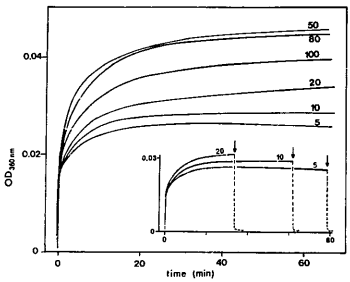

Fig. 2. Aggregation of the neo-mannosylated liposomes by concanavalin A. Liposomes (20 μM phospholipids) composed of PC/cholesterol/MPB-PE (10:7:1.5) and conjugated to **8b** were incubated in 1 ml 5 mM Hepes, 140 mM NaCl, 0.5 mM $CaCl_2$, (pH 7.6) at room temperature. Time course of turbidity changes, at 360 nm, were recorded after addition of indicated amounts (μg) of lectin. Inset: at the arrow, free methyl α-D-mannose (0.2 mM) was added after aggregation with given amounts (μg) of lectin.

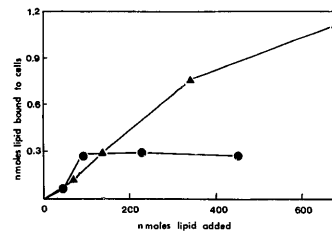

Fig. 3. Association of neo-mannosylated liposomes to peritoneal macrophages. Mouse resident peritoneal macrophages ($2.9 \cdot 10^6$ cells/well containing 2 ml Dulbecco's modified Eagle's medium) were incubated at 37 °C for 90 min with increasing amounts of neo-mannosylated liposomes (▲) composed of PC/cholesterol/MPB-PE (10:7:1) conjugated to **5a** and containing 50 mM 5(6)-carboxyfluorescein. The unconjugated liposomes (●) were composed of PC/cholesterol (10:7) containing 50 mM 5(6)-carboxyfluorescein. After three washes with cold PBS, the cells were scraped with a rubber-policeman into 1 ml PBS containing 0.1% emulphogene BC-720. The fluorescence was measured and converted into nmoles of phosphate associated to 10^6 cells as described under Materials and Methods.

interaction studied. These results, which are comparable to those obtained by other authors on the interaction of lectins with synthetic glycolipids incorporated into liposomes [53–56], indicate that our synthetic scheme yields vesicles whose mannosyl residues are well exposed at their surface and are effective and specific ligands of concanavalin A.

In vitro interaction between the neo-mannosylated liposomes and macrophages

The interaction of neo-mannosylated large unilamellar vesicles with mannose-specific receptors was studied with mouse phagocytic cell types, i.e. Kupffer cells and resident peritoneal macrophages, which present such a lectin at their surface (see Introduction). The liposomes were prepared from egg phosphatidylcholine and cholesterol; such a composition was chosen to minimize their non-specific interactions with cells and to increase their stability. Binding of the targeted and control vesicles to cells was quantified by a fluorometric method, using liposomes which had encapsulated high concentrations of 5(6)-carboxyfluorescein [46], or a radiolabel method using encapsulated [^{3}H]inulin [57].

Interaction of targeted liposomes with macrophages at 37 °C. Mouse resident peritoneal macrophages and Kupffer cells were incubated, at 37 °C, with increasing concentrations of neo-mannosylated and control liposomes having encapsulated 50 mM 5(6)-carboxyfluorescein. At this temperature the macrophages can bind and phagocytose the liposomes. Vesicles conjugated to **5a** (Fig. 3) and to **8b** (Fig. 4) were found associated, respectively, to peritoneal macrophages and Kupffer cells in about 2 to 4-fold greater amounts than the nontargeted liposomes. It has been shown recently that macrophages possess a transporter for organic anions, such as carboxyfluorescein, which allows their

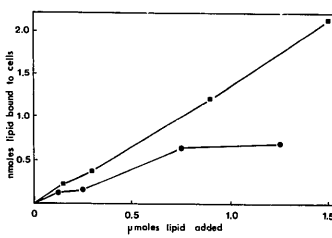

Fig. 4. Association of neo-mannosylated liposomes to Kupffer cells in vitro. Mouse Kupffer cells (2×10^6 cells/well containing 2 ml Dulbecco's modified Eagle's medium) were incubated at 37 °C for 2 hours with increasing amounts of neo-mannosylated liposomes (■) composed of PC/cholesterol/MPB-PE (10:7:1.5) conjugated to **8b** and containing 50 mM 5(6)-carboxyfluorescein. The unconjugated liposomes (●) were composed of PC/cholesterol/MPB-PE (10:7:1.5) reacted with mercaptoethanol and containing 50 mM 5(6)-carboxyfluorescein. After three washes with cold PBS, the cells were scraped with a rubber policeman into 1 ml PBS containing 0.1% emulphogene BC-720. The fluorescence was measured and converted into nmoles of phosphate associated to 10^6 cells as described under Materials and Methods.

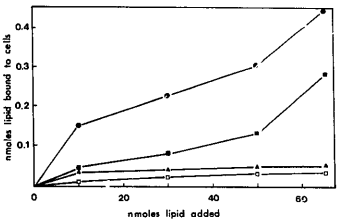

Fig. 5. Association of neo-mannosylated liposomes to peritoneal macrophages. Influence of the encapsulated marker on the observed results. Mouse resident peritoneal macrophages ($0.9 \cdot 10^6$ cells/well containing 2 ml Dulbecco's modified Eagle's medium) were incubated at 37°C for 2 hours with increasing amounts of neo-mannosylated liposomes composed of PC/cholesterol/MPB-PE (10:7:1.5) conjugated to **8b** and containing [^{3}H]inulin (●) (23400 dpm/nmol phosphate) or 50 mM 5(6)-carboxyfluorescein (■). The unconjugated liposomes were composed of PC/cholesterol/MPB-PE (10:7:1.5) reacted with mercaptoethanol and containing [^{3}H]inulin (▲) or 50 mM 5(6)-carboxyfluorescein (□). After three washes with cold PBS, the cells were scraped with a rubber policeman into 1 ml PBS containing 0.1% emulphogene BC-720. The fluorescence was measured and converted into nmoles of phosphate associated to 10^6 cells as described under Materials and Methods.

efficient release into the extracellular medium after their injection into the cells [58]; since this might lead to an underestimation of the specific binding of the liposomes to these cell types, when using the fluorescent dye as label, we have conducted similar experiments with targeted vesicles having encapsulated the non-permeant [^{3}H]inulin. This latter marker is only slowly released from cells, probably by exocytosis during membrane recycling [59]. In Fig. 5 are represented the association of liposomes conjugated to **8b** (15 mol%) to peritoneal macrophages using both methods. Compared to unconjugated liposomes, the neo-mannosylated vesicles, at the highest concentration given, were found associated about 7.5-fold more when using carboxyfluorescein as marker, and 9-fold more when using inulin as marker. The association of the targeted liposomes to the macrophages could not be inhibited by high concentrations (0.1 and 0.5 mg/ml) of mannan (results not shown). This contrasts with the inhibition of neo-mannosylated proteins binding to mannose receptors by this polymannosyl derivative [60].

Interaction of targeted liposomes with macrophages at 4°C. In order to better define the specificity of the neo-mannosylated liposomes interaction with the surface of the macrophages, we have tried competition experiments at 4°C, a temperature which precludes any uptake of the vesicles by the cells. The association of targeted vesicles, composed of PC/cholesterol/MPB-PE (10:7:2) conjugated to **8b**, to mouse resident peritoneal macrophages as well as to Kupffer cells, could not be inhibited by addition of mannan even with concentrations as high as 0.5 mg/ml (results not shown). The binding of the carboxyfluorescein-labelled neo-mannosylated liposomes to peritoneal macrophages could, however, be inhibited by excess empty targeted liposomes. The cells were incubated, at 4°C for 16 hours, with three different concentrations (10–30 nmol phospholipid/2 ml DMEM) of neo-mannosylated vesicles having encapsulated 50 mM 5(6)-carboxyfluorescein and the amount of cell-associated phospholipids was determined fluorometrically. An inhibition of this binding was observed in presence of increasing concentrations (0–100 μM phospholipid) of unlabelled neo-mannosylated liposomes. A value of $I_{50} = 36 \pm 5$ μM ($n = 3$) was obtained from classical displacement curves (not shown). These results indicate that the binding of the targeted vesicles to the cell surface is reversible under these experimental conditions and that the two liposome populations, i.e. containing carboxyfluorescein or not, compete for a same binding site. In contrast, when empty unconjugated liposomes were used (0–400 μM phospholipid), no direct competition was observed for the binding of the targeted liposomes (not shown).

Discussion

In the present paper we have reported a new approach for the preparation of neo-mannosylated liposomes in order to exploit the known interaction of mannose derivatives with their receptor expressed at the surface of macrophages. The flexible strategy developed allows a chemically well defined neo-glycosylation of preformed vesicles with the sugar headgroup extended away from the bilayer surface. It represents an extension to small ligands of the method of Martin and Papahadjopoulos [22], which was described for the coupling of antibodies to the surface of liposomes.

In a first step, a derivative of *p*-isothiocyanatophenyl-α-D-mannose was chosen as ligand; this compound was previously utilized in immunochemistry for the coupling of mannosyl moieties to proteins [29]. These glycoconjugates (i.e. neo-glycoproteins) were also described as potent ligands in binding and uptake studies with macrophages [21]. Even if this first mannosyl derivative presented, when grafted on liposomes, a good affinity for peritoneal macrophages, we synthesized, in a second step, mannosyl ligands presenting a sulfur atom, instead of oxygen, at the anomeric position. This was to avoid the hydrolysis of the coupled mannosyl residues by e.g. serum glycosidases. It is known that thiomannosyl derivatives keep a high affinity for the mannose receptor of macrophages [24] and that they are more resistant to glycosidases and to acidic media [23]. This latter consideration is particularly important for in vivo

applications of these neo-mannosylated vesicles. In order to provide a good accessibility to the ligand, the thiomannosyl moieties were coupled to a hydrophilic spacer arm whose length can be easily extended. The results obtained in the aggregation experiments with concanavalin A, a lectin selective for α-D-mannosyl residues, confirm that the sugar ligands introduced by our technique at the surface of the targeted vesicles are perfectly available to the lectin and present the right anomeric configuration.

If a relatively large number of studies dealing with the in vivo fate fo glycolipid-containing liposomes are found in literature [61–68], only a very few publications report the in vitro interaction of such vesicles with their putative target cells [9,56,61,69,70]. Our aim was to study the interaction of neo-mannosylated liposomes with isolated cells in order to demonstrate the existence of a specific binding due to the mannose receptor present at the surface of macrophages. Compared to nontargeted vesicles, a 4- to 9-fold preferential association, depending on the cell type and the liposome label (i.e., 5(6)-carboxyfluorescein or [^{3}H]inulin) of liposomes containing 15 mol% mannose-ligand was observed at 37°C. At 4°C, a temperature which precludes phagocytosis [71], a specific binding was also observed. At both temperatures, and in contrast with neo-glycoproteins [72], the specific binding of the neo-mannosylated liposomes could not be inhibited by high concentrations (up to 0.5 mg/ml) of a free polymannose derivative (mannan). These results indicate that the mannosyl residues, introduced at the surface of the targeted liposomes, present a conformational mobility which allows their interaction with the mannose receptor of the peritoneal macrophages and of the Kupffer cells. The lack of binding inhibition by the mannan might reflect the occurrence of high affinity cluster binding structures for the mannose receptor [72] in these cells.

Related to the present work, Barratt et al. [9] had studied the interaction, with murine peritoneal macrophages, of liposomes composed exclusively of manosylated myo-inositol, extracted from the cell wall of microorganisms. Although the type of liposomes was different, i.e. multilamellar vesicles, they observed a similar high affinity and preferential uptake of the targeted liposomes by these cells. In this context, it should be noted that interaction of liposomes with macrophages can also be markedly increased by incorporating negatively charged (phospho)lipids into the vesicles (see, for example, Refs. 57, 73–75). However, since similar results could be observed with other cells types such as fibroblasts [76] lymphoma or myeloma [77], this type of interaction does not seem specific for macrophages.

In conclusion, our results indicate that it is feasible to obtain, in vitro, a binding of neo-mannosylated liposomes to peritoneal macrophages, and also to Kupffer cells, via a specific interaction with their mannose receptors. Work has been done in vivo showing that such targeted liposomes, having entrapped endotoxin (LPS), induce through their interaction with macrophages, an improved regression of experimental solid tumors in mice (EMT6 sarcoma, 3LL carcinoma) and were especially effective on lung metastasis (Dumont, S. et al., submitted).

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