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Preparation of neo-galactosylated liposomes and their interaction with mouse peritoneal macrophages

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In order to target liposomes to cells expressing at their surface a galactose-binding site we have prepared liposomes containing new synthetic galactolipids. Neo-galactosylated liposomes were prepared by covalently coupling β-D-1-thiogalactopyranoside residues, substituted with a hydrophilic spacer-arm and functionalized with a sulfhydryl group, to preformed large unilamelar vesicles containing 4-(*p*-maleimidophenyl)butyryl phosphatidylethanolamine. The vesicles, having a galactose content above a threshold value of about 5 mol%, could be aggregated with *Ricinus communis* agglutinin. This aggregation was reversed by addition of excess free methyl β-D-galactopyranoside, indicating that the surface glucidic moieties of these liposomes were accessible to the lectin. Compared to the control vesicles, the neo-galactosylated liposomes (containing 15 mol% galactose) presented in vitro an increased binding to cells possessing a β-D-galactose specific receptor, i.e. resident mouse peritoneal macrophages. At 4°C, the specific binding was about 2-fold, whereas at 37°C it was increased to about 4–5-fold. This differential binding was largely unaffected by serum and, interestingly was much dependent on the degree of galactosylation of the liposomes, i.e. a threshold value of 5 mol% was needed to observe an increased binding of the targeted vesicles to the macrophages.

Introduction

A Gal/GalNAc-specific lectin is present at the surface of mammalian hepatocytes [1]; it mediates the binding and the uptake of glycoproteins such as asialoglycoproteins. This receptor was also considered as a potential target to direct selectively liposomes to liver cells (for review, see Ref. 2). Vesicles presenting at their surface β-galactose residues were prepared by incorporation of natural [3–5] or synthetic glycolipids [6,7]; it was found that in vivo these targeted liposomes are rapidly cleared by the liver [3,8] and in some cases this could be accounted for by a preferential uptake by the parenchymal cells [4,9]. This receptor, however, is not restricted to the hepatocytes, since it was established later that a lectin of similar binding specificity existed on the surface of Kupffer cells [10–12] and of murine peritoneal macrophages [13–15]. The hepatocyte and macrophage galactose-specific binding sites nevertheless differ

Abbreviations: Gal, D-galactose; GalNAc, *N*-acetylgalactosamine; 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; REV, reverse phase evaporation; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's modified Eagles' medium.

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in their structure and their mode of association to the outer cell membrane [12,14]. Interestingly in view of targeting, it was found by Spanjer et al. that liposomes which had incorporated a triantennary galactose structure were preferentially taken up by the Kupffer cells, presumably because of the preclustering of the receptors on these macrophages [16].

Since incorporation into liposomes of ligands capable of interacting with macrophage surface receptors can markedly promote liposome uptake [16–18] we have now studied the interaction of neo-galactosylated liposomes with murine peritoneal macrophages. The interaction of glucidic residues present at the surface of vesicles with soluble or membrane-bound lectins is sensitive to e.g. (i) steric factors, it can be optimized by using an adequate spacer-arm length [19,20], and to (ii) the ligand concentration per surface area, the binding requiring an above-threshold ligand density [19]; at a cellular level these threshold responses [21] probably reflect a clustered receptor arrangement [22]. In order to control such parameters we have adopted a new synthetic strategy which allows a direct coupling of terminal non-reducing β-D-thiogalactosyl residues to preformed liposomes following the versatile method of Martin and Papahadjopoulos [23]. The glucidic residue was conjugated to the vesicles via a hydrophilic arm whose length can be easily extended. A thioether linkage was chosen for the galactosyl moiety for its chemical and metabolic stability. The results obtained in this study indicate that the targeted liposomes prepared by this method are susceptible to aggregation by the *Ricinus communis* lectin and present a higher affinity for the macrophages than the unconjugated vesicles.

Materials and Methods

Cholesterol (recrystallized in methanol), phosphatidylethanolamine (egg yolk), fluorescamine, 5,5′-dithiobis(2-nitrobenzoic acid), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, ninhydrin (spray), *Bandeiraea simplicifolia* agglutinin (BS-I), methyl α-D-galactopyranoside, methyl β-D-galactopyranoside and β-D-galactose pentaacetate were purchased from Sigma Chemical Co. Lectin from *Ricinus communis* (RCA I) was obtained from Boehringer Mannheim. SMPB was from Pierce Chemical Co. and MPB-PE was prepared according to Martin and Papahadjopoulos [23]. SATA was synthesized according to Duncan et al. [24]. Phosphatidylcholine was extracted from egg yolk and purified according to Nielsen [25]. 5(6)-Carboxyfluorescein was obtained from Eastman Kodak Co. and was purified according to Ralston et al. [26]. Polycarbonate filters were from Nuclepore. Dulbecco's modified Eagle's medium, fetal calf serum were obtained from Gibco and the culture dishes from Costar. All other reagents were of analytical grade.

Synthesis of the thiol functionalized β-D-galactosyl residue

1-Deoxy-1-thio-[2-hydroxy-3-(2-thioacetate) aminopropyl]carbamoylethyl-β-D-galactopyranose (compound **8**) was synthesized by a procedure outlined in Fig. 1. The known intermediary 1-deoxy-1-thio-(2-carboxyethyl)-β-D-galactopyranose (**5**) [27] was obtained from penta-*O*-acetyl-β-D-galactopyranose (**1**) by procedures adapted from literature [28–30]. In brief, **1** (10 g; 25 mmol) in acetic anhydride (50 ml) was converted into 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**2**) by successive addition, at 0°C, of PBr_3 (16 ml; 170 mmol) and water (22 ml) according to Kempen et al. [30]. The bromide (yield: 84%) was recrystallized from diisopropyl ether/hexane (m.p. 79–81°C; lit. [30] m.p. 77–78°C). **2** (8.66 g; 21 mmol) in anhydrous acetone (25 ml) was then heated under reflux for 3 h in the presence of thiourea (1.6 g; 21 mmol) and transformed into 1-deoxy-2,3,4,6-tetra-*O*-acetyl-1-(2-thiopseudourea hydrobromide)-β-D-galactopyranosyl (**3**), which was recrystallized (yield: 71%) in isopropanol; m.p. 172–174°C (lit. [28] m.p. 171–171.5°C). A mixture of **3** (7.5 g; 15 mmol) and freshly recrystallized 3-iodopropionic acid (3.2 g; 16 mmol) in 40 ml acetone/water (1:1, v/v) was treated with sodium carbonate (2.58 g; 17.4 mmol) and sodium metabisulfite (2.58 g; 13.6 mmol) [29]. After 2 h stirring at room temperature, the mixture was acidified with 10 ml HCl (5%), and extracted with ethyl acetate. 1-Deoxy-2,3,4,6-tetra-*O*-acetyl-1-thio-(2-carboxyethyl)-β-D-galactopyranosyl (compound **4**) was obtained (yield: 92%) as a colorless oil which was homogeneous on TLC: ^{1}H-NMR

(200 MHz; C^2HCl_3) δ(ppm) 1.99(s), 2.07(s), 2.08(s), 2.17(s) (4 × CH_3CO-), 2.78 (t, 2 H, -S-CH_2-CH_2-COOH), 2.97 (m, 2 H, -S-CH_2-), 3.91–4.2 (m, 3 H, H-5, 2 × H-6), 4.56 (d, 1 H, H-1, $J_{1,2}$ = 9.86 Hz, β configuration), 5.02–5.45 (m, 3 H, H-2, H-3, H-4).

Compound **4** (5.4 g; 13.8 mmol) was then deacetylated [29] in 45 ml of methanol/water/triethylamine (5:4:1, v/v). After 24 h at room temperature the solvents were evaporated under vacuum and compound **5** was purified by chromatography on a 38 × 2.2 cm Dowex 1-X2 (formate form) column. **5** was obtained as a colorless oil (quantitative yield) after elution with 2 M HCOOH. Thin-layer chromatography on silica gel plates (ethyl acetate/acetic acid/water (8:2:1, v/v)) indicated a single spot (R_f 0.15 compared to 0.83 for compound **4**). Analytical data are in agreement with literature [27]: $[\alpha]_D^{22} = -19°$ (c = 0.016; water).

1-Deoxy-1-thio-(2-hydroxy-3-aminopropyl)carbamoylethyl-β-D-galactopyranoside (**6**). A solution of **5** (1.0 g; 3.6 mmol) and 1,3-diamino-2-propanol (1.6 g; 18 mmol) in 40 ml water, adjusted to pH 5.5 with HCl, was treated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.2 g; 10.8 mmol). After 24 h at room temperature, the reaction was essentially complete. Compound **6** was purified by chromatography on a 40 × 2.4 cm Dowex 50-X2 (H^+) column eluted with 0.33 M HCl. Fractions containing **6**, which were positive for amine [31] and sugar [32] reagents, were combined and lyophilized. Compound **6**, which was obtained (yield: 65%) as a white amorphous solid, gave a single spot (R_f 0.13) on thin-layer chromatography eluted with ethyl acetate/acetic acid/water (6:4:2, v/v).

1-Deoxy-1-thio-[2-hydroxy-3-(2-*S*-acetylthioacetate)aminopropyl]carbamoylethyl-β-D-galactopyranose (**7**). SATA (69.3 mg; 0.3 mmol) was added to a solution of **6** (109 mg; 0.29 mmol) in 15 ml anhydrous dimethylformamide containing triethylamine (45 μl; 0.3 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 **6**, measured with fluorescamine [31]. After elimination of the solvent, the reaction product was chromatographed on a 80 × 2.8 cm column of Bio-Gel P-2, 200–400 mesh, eluted with water. The fractions containing the glucidic derivatives were pooled and after lyophilization 91 mg of an amorphous white solid was obtained. Analysis of the reaction product by mass spectrometry showed the presence of **7**, m/z: 457 ($M^{+\cdot}$), of the thiol **8**, m/z: 415 ($M^{+\cdot}$), of the *N*-acetylated derivative of **6**, m/z: 383 ($M^{+\cdot}$) and of higher molecular weight compounds corresponding to homologous mono- and poly-acetylated derivatives. ^{1}H-NMR ($C^2H_3O^2H$) corroborated this analysis and indicated the presence of several acetyl peaks.

1-Deoxy-1-thio-[2-hydroxy-3-(2-thioacetate) aminopropyl]carbamoylethyl-β-D-galactopyranose (**8**). The compound **8** was obtained by treating **7** with a 50 molar excess of hydroxylamine in 5 mM Hepes (pH 7.4) under argon. After 45 min at room temperature, the thiol deprotection reaction was complete, as estimated by sulfhydryl-group determination with 5,5′-dithiobis(2-nitrobenzoic acid) [33]. **8** was used immediately without further purification. Analysis of the reaction products by mass spectrometry, indicated only the presence of **8**, m/z: 415 ($M^{+\cdot}$) and of the *N*-acetylated derivative of **6**, m/z: 383 ($M^{+\cdot}$).

Preparation of liposomes and coupling of the thiol functionalized β-D-galactosyl residue

Large unilamellar vesicles were prepared from 15 μmol lipids (PC, MPB-PE and cholesterol at molar ratios indicated under Results) by the reverse-phase evaporation technique [34]. The molecules to be encapsulated, e.g. 40 mM 5(6)-carboxyfluorescein, were in 5 mM Hepes buffer (pH 7.4) and the osmolarity 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 4-fold molar excess of compound **8** over MPB-PE content. The mixture was left for 45 min at room temperature and under argon. Control liposomes (i.e. nontargeted liposomes) were reacted similarly with 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 a 5 mM Hepes buffer (pH 7.4) containing 150 mM NaCl. The liposomes were analyzed for their phosphorus content [35] and amount of cova-

lently coupled galactosyl residues [31] after prior hydrolysis of the thioether bond in the presence of mercuric acetate [36].

Interaction between ricin and neo-galactosylated liposomes

Neo-galactosylated liposomes (final concentration 60 μM) composed of PC/cholesterol/MPB-PE (8 : 5 : 2) were incubated, with increasing concentrations of *Ricinus communis* agglutinin, in a cuvette containing 1 ml (final volume) of 5 mM Hepes buffer (pH 7.4) and 150 mM NaCl. After rapid stirring, aggregation of the vesicles was estimated, at 22°C, by the time-dependent increase in turbidity as measured by absorbance at 360 nm with a Shimadzu, Model MPS-2000, spectrophotometer equipped with a graphic printer (PR-3). The reversibility of the aggregation was assessed by addition of free methyl-D-galactosides.

Interaction between the neo-galactosylated liposomes and macrophages

Resident peritoneal macrophages, obtained as described [37] from female Balb c mice (between 6 and 12 weeks old) in DMEM were plated in wells (1 cm diameter) of microtiter plates (Costar) at $1 \cdot 10^6$ cells per 1 ml (final volume) and maintained for 2 h at 37°C in a humid atmosphere of 5% CO_2 in air (final pH 7.4). Non-adherent cells were then eliminated by rinsing the dishes three times with DMEM. The adherent macrophages, whose viability was superior to 95% as checked with the Trypan blue exclusion test, were fed with DMEM containing, when indicated, 10% fetal calf serum and incubated for 2 h at 37°C with varying amounts of targeted and non-targeted liposomes (50–500 nmoles lipids) containing 40 mM 5(6)-carboxyfluorescein. After the incubation time the medium was pipetted off and the cells washed six times with phosphate-buffered saline. Carboxyfluorescein associated to the cells was measured fluorimetrically (Jobin-Yvon spectrofluorimeter, Model JY-3D) after cell digestion in 2 ml phosphate-buffered saline containing 1% (v/v) Triton X-100. A standard fluorescent curve was established under the same conditions with aliquots of the initial liposome preparation as described previously [38]. The results indicated in the Figs. 4–6 represent amounts of liposomal phospholipid associated to cells; they are means of duplicates, which do not differ by more than 10%. The stability of the liposomes, under the different incubation conditions, was also measured by their ability to retain encapsulated 5(6)-carboxyfluorescein. At the end of the incubation time, aliquots (about 10 μl) of supernatants were removed and diluted in 2 ml (final volume) of 5 mM Hepes buffer (pH 7.4) containing 150 mM NaCl. The self-quenching coefficient of encapsulated 5(6)-carboxyfluorescein was determined by measuring the fluorescence before and after addition of Triton X-100 (0.5%, v/v). The amount of dye released, compared to the original liposome preparation, was then calculated according to Barbet et al. [38]. Protein concentration was determined according to Schacterle and Pollack [39] using bovine serum albumin as standard.

Results

Covalent coupling of β-D-galactosyl residues to preformed vesicles prepared by the reverse-phase evaporation technique was achieved according to the method of Martin and Papahadjopoulos [23]. It involves the reaction, under mild conditions, between liposomes containing an electrophilic lipid derivative, i.e., MPB-PE, a maleimido moiety linked through a spacer to phosphatidylethanolamine, and a ligand possessing a thiol group. As ligand we have selected a β-D-thiogalactosyl derivative which was attached to a hydrophilic spacer-arm and functionalized with a sulfhydryl group.

Synthesis of the functionalized galactosyl ligand and coupling to liposomes

Fig. 1 outlines the synthetic scheme followed to prepare the functionalized galactosyl ligand **8**. Penta-*O*-acetyl-β-D-galactopyranose (**1**) was first converted, in an overall yield of about 40%, into the known compound **5** [28–30]. In step **3** → **4**, the introduction of a propionate derivative was found preferable to an acetate; i.e., after the deacetylation of **4**, by triethylamine in methanol-water, a shorter arm introduced complications presumably because of interactions with the neighboring hydroxyl group. ^{1}H-NMR analysis of **4** (see Materials and Methods) confirmed that the synthesis

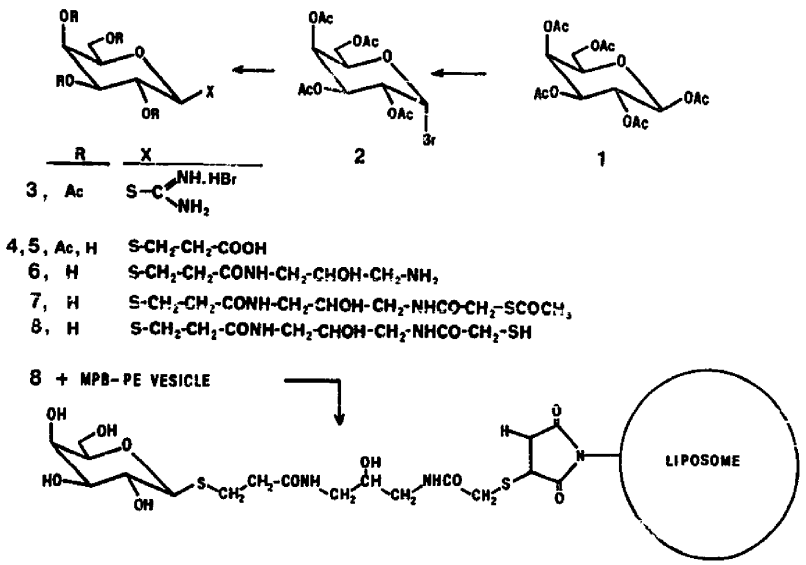

Fig. 1. General scheme for the synthesis of β-D-thiogalactose derived ligands and conjugation to preformed liposomes.

yielded, as expected [29], β-isomers. A hydrophilic spacer-arm consisting of 1,3-diamino-2-propanol was then added by treatment with a water soluble carbodiimide to yield compound **6**. At this step, if needed, the arm could easily be further lengthened by successive treatment with succinic anhydride and coupling of an additional 1,3-diamino-2-propanol [40]. For the final step leading to the introduction of the sulfhydryl function of the ligand, compound **6** was reacted with SATA [24] in dimethylformamide to give **7**. Finally the ligand **8**, with the free thiol, was obtained by deprotection with aqueous hydroxylamine [24]. The use of the new heterobifunctional SATA, instead of other reagents such as SPDP, to introduce thiol groups was advocated for its conveniency [24,41]; i.e., the removal of the protective group is possible under mild conditions with hydroxylamine and the final compound can be used without lengthy purification steps. In our hands however, SATA had some drawbacks; analysis of products **7** and **8** by ^{1}H-NMR and by mass spectrometry revealed that this reagent was also acting, to some extent, as an *O*- and *N*-acylating agent. Thus about 30% of **5** when reacted with SATA became *N*-acetylated, therefore decreasing the final yield of the synthesis. Moreover some unwanted *O*-acetyl groups were also introduced in the glucidic moiety of **7**, but importantly they were easily removed in the hydroxylamine step. Similar results were obtained with SATA reacting either in organic medium, e.g., in dimethylformamide, or in aqueous solution. Thus, the unacknowledged acylating property of the thioester moiety of SATA restricts the use of this reagent. In our case, however, the reaction sequence used limited these drawbacks and, provided a 4-fold molar excess ligand over liposomal activated lipid (i.e. MPB-PE) was used, the coupling occurred readily. Analysis of the glycosidic residues of the conjugated liposomes indicated that virtually all maleimido groups had reacted; this result shows also that the functionalized ligand could penetrate the vesicles.

Ricin-mediated aggregation of the neo-galactosylated liposomes

In order to evaluate the validity of our approach we have investigated if *Ricinus communis* lectin was able to bind to the neo-galactosylated vesicles and mediated their aggregation. Ricin (RCA I) is a dimer and has two binding sites selective for terminal β-D-galactosyl residues; by cross-linking the vesicles it can promote their agglutination. In Fig. 2 are given the turbidity

(light-scattering) increases observed after addition of varying concentrations of ricin to a preparation of liposomes containing 20 mol% β-D-thiogalactosyl residues. The apparent rates of agglutination are dependent on the lectin concentration. When ricin concentration was above 30 μg/ml, the extent of aggregation was decreased indicating a saturation of the binding sites. On addition of a 500 – fold molar excess of free methyl β-D-galactopyranoside (i.e. 6.0 mM) the optical absorbance was reduced by 50%. In contrast, when using 15 mol% neo-galactosylated vesicles, the ricin-induced aggregation could be essentially reversed with 4.5 mM methyl β-D-galactopyranoside (Fig. 2; inset), providing evidence for the specific nature of the interaction studied. *Bandeiraea simplicifolia* agglutinin, which presents a greater selectivity for α-D-galactosyl residues, was less effective than ricin; i.e., at saturating concentration this lectin agglutinated the neo-galactosylated at a reduced rate (about one order of magnitude; not shown). The rates of aggregation of the vesicles by ricin were also sensitive to the surface density of galactosyl residues (Fig. 3). Under a threshold

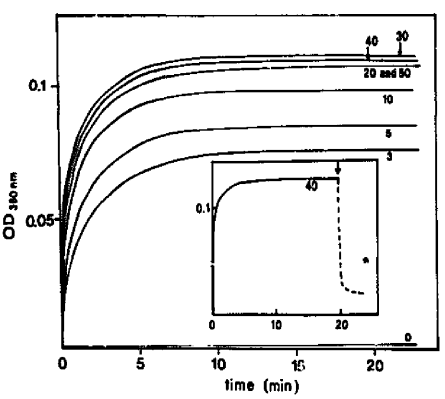

Fig. 2. Aggregation of the neo-galactosylated liposomes by *Ricinus communis* agglutinin. Liposomes (60 μM phospholipid) composed of PC/cholesterol/MPB-PE (8:5:2) and conjugated to **8** were incubated in 1 ml 5 mM Hepes, 150 mM NaCl (pH 7.4) at 22°C. Time course of turbidity changes, at 360 nm, were recorded after addition of indicated amounts (μg) of lectin. Inset: liposomes composed of PC/cholesterol/MPB – PE (8.5:5:1.5) and conjugated to **8** were aggregated with ricin (40 μg/ml), at the arrow methyl β-D-galactopyranoside (4.5 mM) was added.

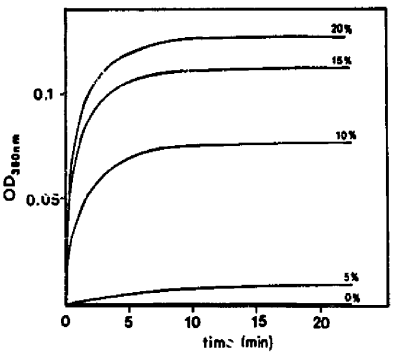

Fig. 3. Influence of galactose surface density on the aggregation of the neo-galactosylated liposomes by ricin. Liposomes (60 μM phospholipid) composed of cholesterol (34 mol%), PC and varying percentages (0–20 mol%) of MPB-PE, such as the total phospholipid content was 66 mol%, were conjugated to **8** and incubated with 40 μg ricin (see legend to Fig. 2).

level of about 5% galactosylation, only a limited aggregation occurred, whereas between 15 and 20% the rates were little affected. Taken together these results, which are comparable to those obtained by other authors on the interaction of lectins with synthetic glycolipids incorporated into liposomes [19,20], indicate that our approach yields vesicles whose galactosyl residues are well exposed and are effective and specific ligands of *Ricinus communis* lectin.

Interaction between the neo-galactosylated liposomes and peritoneal macrophages

The interaction of neo-galactosylated large unilamellar vesicles with galactose-specific receptors was studied with resident mouse peritoneal macrophages which present such a lectin at their surface [13–15]. 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 non-targeted vesicles to cells was quantified by fluorimetric methods using liposomes which had encapsulated high concentrations of 5(6)-carboxyfluorescein [38]. Fig. 4 shows the amounts of lipids bound to cells as a function of added lipid for the control and neo-galactosylated (15 mol%) liposomes at 4°C (panel A) and 37°C (panel B). As

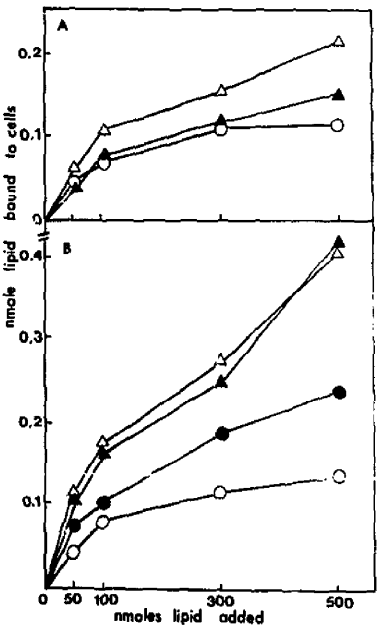

Fig. 4. Binding of neo-galactosylated liposomes to macrophages. Mouse peritoneal macrophages ($1 \cdot 10^6$ cells/well containing 1 ml Dulbecco's modified Eagles' medium) were incubated with increasing amounts of liposomes composed of PC/cholesterol/MPB-PE (8.5 : 5 : 1.5), conjugated to 8 (targeted vesicles) or to mercaptoethanol (○; control vesicles), containing 40 mM 5(6)-carboxyfluorescein. Panel A: The cells were incubated at 4°C for 4 h with targeted vesicles in the absence (△) or presence (▲) of free D-galactose (1 mM). Panel B: the cells were incubated at 37°C for 2 h with targeted vesicles in the absence (△) or presence (▲) of free D-galactose (1 mM). Cells pretreated for 1 h with 10 mM NH_4Cl (●) and incubated with targeted vesicles.

estimated by the fluorescence self-quenching method [38], about 90–95% of carboxyfluorescein remained encapsulated, for both vesicles types, at the term of the incubation periods. At 4°C, the association of the targeted vesicles was 2-fold greater than for controls. To ascertain whether this increased binding was due to a specific interaction between the galactosyl residues at the surface of the vesicles and a galactose-specific receptor we have performed competition experiments. Coincubation of the targeted vesicles with a 100-fold molar excess of free galactose largely abolished the specific interaction. At 37°C, the neo-galactosylated liposomes were found associated to cells in 4-fold greater amounts than the unconjugated liposomes. In this case, however, free galactose did not inhibit the interaction of the targeted vesicles; this could be explained by a different association mechanism, i.e., at this temperature the macrophage can bind and phagocytose the neo-galactosylated vesicles. That this might be the case is shown by the action of NH_4Cl (Fig. 4; panel B); when the cells were preincubated with 10 mM NH_4Cl, the association of the targeted vesicles with the macrophages was reduced by half, indicating that at 37°C a receptor-mediated uptake of these vesicles contributed largely to the measured association. A similar observation was made by other authors who showed that, in contrast to the situation at low temperatures, the association of galactosylated proteins with the galactose receptor of peritoneal macrophages was at 37°C essentially irreversible [15].

Kelm and Schauer have demonstrated a Ca^{2+} requirement for the binding of ligands to the galactose receptor of rat peritoneal macrophages [15]. In agreement with this result, we have found that the association of neo-galactosylated liposomes (15 mol%) with mouse resident peritoneal macrophages (incubation of 4 h at 4°C), which were preincubated for 0.5 h with 10 mM EDTA, was indistinguishable from that achieved with control liposomes (not shown). Since serum was also shown to influence the interaction of glycosylated liposomes with e.g. Kupffer cells [42], we have studied the incidence, at 37°C, of various concentrations of fetal calf serum on the interaction of neo-galactosylated liposomes and the peritoneal macrophages. As indicated in Fig. 5, the serum had an inhibitory action on the association of the targeted vesicles with the cells, however, the differential binding between the conjugated and nonconjugated vesicles remained largely unaffected. Serum proteins are known to adsorb nonspecifically to liposomes, it is therefore possible that the observed inhibition might result from a decrease in free liposome concentration. Under our experimental conditions, at the highest serum concentration used, the liposomes remained intact by more than 85% at the end of the incubation time.

Influence of the galactose surface density on the interaction of the targeted liposomes with macrophages

We have shown above that the agglutination of the liposomes by the *Ricinus communis* lectin was markedly dependent on the surface density of galactosyl residues (Fig. 3). It was therefore interesting to check whether a similar effect could be observed in the interaction between the neo-galactosylated liposomes and the macrophages. Fig. 6 indicates that this was indeed the case. A dramatic increase in the binding and uptake of the targeted vesicles by the cells could be observed when the surface galactosylation of the liposomes was increased from 10 to 15 mol%. In contrast, the interaction of the control liposomes with the macrophages remained unaffected by the increase, in their composition, in the negatively charged MPB-PE derivative with mercaptoethanol. This observation might seem in contradiction with the effect of some negatively charged phospholipids, such as phosphatidylserine, which have been reported to increase the interaction of liposomes with macrophages [17,43,44]; however in the present case the negative charge is much more shielded than in these phospholipids.

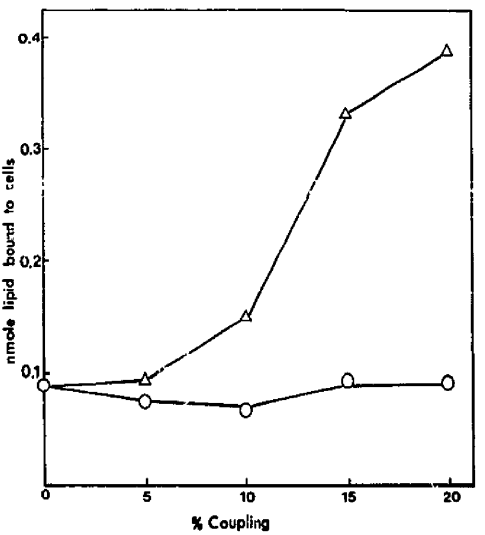

Fig. 6. Influence of galactose surface density on the association of the neo-galactosylated liposomes with macrophages. Liposomes (0.3 μ mol) having a composition similar to those of Fig. 3, conjugated to 8 (△; targeted vesicles) or mercaptoethanol (○; control vesicles) and containing 40 mM 5(6)-carboxyfluorescein, were incubated for 2 h at 37 °C with mouse peritoneal macrophages ($1 \cdot 10^6$ cells in 1 ml Dulbecco's modified Eagles' medium). Results are given as the amount of lipid associated to the cells as a function of the percentage of MPB-PE present in the vesicles.

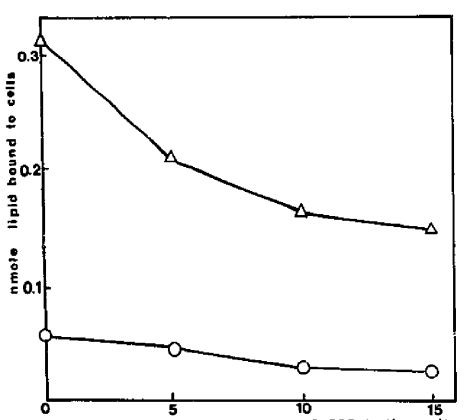

Fig. 5 Influence of serum on the specific-binding of neo-galactosylated liposomes to macrophages. Liposomes (0.3 μmol) composed of PC/cholesterol/MPB-PE (8.5 : 5 : 1.5), conjugated to 8 and containing 40 mM 5(6)-carboxyfluorescein, were incubated for 2 h at 37 °C with mouse peritoneal macrophages ($1 \cdot 10^6$ cells in 1 ml Dulbecco's modified Eagles' medium) in the presence of increasing percentages of fetal calf serum. (△) Binding of targeted vesicles; (○) binding of control vesicles composed of PC/cholesterol (2 : 1).

Discussion

Liposome targeting to specific cells by introducing glucidic ligands onto the surface of the vesicles has been attempted with natural and synthetic glycolipids [2,45,46]. The advantages of using synthetic lipids have been discussed previously [45]; they give access to chemically defined structures, both in the glucidic and the lipophilic anchoring moieties, and allow the control of important parameters such as the spacer-arm length between the liposome surface and the ligands. Synthetic glycolipids which could be incorporated into liposomes have been prepared by chemical coupling between sugar derivatives and cholesterol [45] or PE [46]. We have devised a flexible strategy which allows a chemically well controlled neo-glycosylation of preformed liposomes with the sugar

head-group extended away from the bilayer surface; it represents an extension to small ligands of the method of Martin and Papahadjopoulos [23] which was introduced for the coupling of antibodies to the surface of vesicles.

In the present work we have prepared neo-galactosylated liposomes in order to exploit the known interaction between the galactose receptor, expressed at the surface of macrophages, and non-reducing terminal β-D-galactose residues [10–15]. A derivative of 1-thio-β-D-galactopyranose was chosen as a ligand because it was previously found that a thioether linkage, while not detrimental for the receptor affinity, is chemically more stable and also more resistant to the action of glycosidases [47]; this latter consideration is particularly important for in vivo applications of these neo-galactosylated vesicles. In order to provide a good accessibility of the ligands, the thiogalactose was coupled to a hydrophilic spacer-arm whose length can be easily extended. The results obtained in the agglutination test with *Ricinus communis* agglutinin, a lectin selective for β-D-galactose residues, confirm that the β-D-galactosides introduced, by our technique, at the surface of the targeted vesicles are perfectly available to the lectin and present the right anomeric configuration. The agglutination by the lectin is very sensitive to the surface density of the galactose residues; such threshold phenomenon have been observed previously [19] and might indicate a preference of the lectin for clustered ligands.

If a relatively large number of studies dealing with the in vivo fate of glycolipid-containing liposomes are found in literature [3–9,45,46], only very few publications report the in vitro interaction of such vesicles with their target cells [18,48,49]. Our aim was to study some parameters which are of importance for the specific binding of the neo-galactosylated vesicles with peritoneal macrophages. A 2-fold preferential binding of liposomes containing 15 mol% galactose-ligand was observed at 4°C, which was abolished by an excess of free ligand. Since low temperatures preclude phagocytosis [50], this association must mainly reflect cell-surface adsorbed vesicles. At 37°C, targeted liposomes-cell association was increased more than 4-fold and the specific binding represented about 80% of total binding. Interestingly, a threshold was also found in the specific binding of the neo-galactosylated vesicles to the macrophages; i.e., a galactose density above 5 mol% at the surface of the liposomes was required to observe a specific association. Such a behavior is reminiscent of the properties of the galactose receptor present at the surface of the hepatocytes, which binds with high affinities clustered galactosyl residues; e.g., synthetic bi- or tri-antennary oligo-galactosides present much higher affinities than monovalent galactosides [51,52]. It seems therefore that the galactose receptor of the peritoneal macrophages can also interact simultaneously with great affinity, with a multiplicity of galactose residues which form a binding unit at the surface of the liposomes. Alternatively, due to the size of the vesicles, the possibility exists that the galactosyl head-groups of the targeted liposomes could also interact with several receptors arranged in clusters on the cell surface; such multivalent interactions have been described before for the hepatic galactose receptor [52] and for the binding of targeted liposomes to cells [40,53,54]. The results obtained in this study indicate that the galactosyl residues, introduced at the surface of the targeted liposomes by our technique, present a conformational flexibility which allows, above the threshold concentration, the formation of favorable cluster structures which must bind to the galactose receptors with high affinity. Such a high affinity could explain why, at 37°C, a free monovalent ligand such as D-galactose is unable to prevent the association of the targeted vesicles. In analogy to previous observations [55], the fact that pretreatment of the cells with NH_4Cl resulted in an impairment of the association of the liposomes to the cells is indicative of the uptake of the targeted vesicles by the macrophages. By raising the pH of endosomal compartments, NH_4Cl disrupts their functions and inhibits, in the case of receptor-mediated endocytosis, the recycling of the receptor toward the cell surface and the uptake of their ligands [56].

Related to our work, Wu et al. [48], have studied the interaction of various glycosylated liposomes with macrophages and did not find a preferential binding due to galactosyl residues. Only vesicles bearing 6-aminomannose head-groups showed a strong interaction with these cells; it was found

later, however, that this was unrelated to a carbohydrate receptor but was most probably due to the presence of a positive charge at the surface of the liposomes [50,58]. Since Wu et al. had used vesicles composed of high T_m phospholipids, it is possible that lateral mobility of the ligands in the plane of the membrane, is of importance to allow the clustering necessary for the formation of specific high-affinity complexes. Scherphof et al. using Kupffer cells, also failed to observe a specific uptake of liposomes containing lactosylceramide, presumably because of damages to the receptor due to the proteolytic isolation procedures of these cells [58]. In summary, in contrast to these latter works, our results indicate that it is feasible to obtain, in vitro, a binding of neo-galactosylated liposomes to the macrophages via a specific interaction with their β-D-galactose receptors. Work is in progress to exploit this interaction for bio-active molecules delivery.

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