Biochimica et Biophysica Acta 862 (1986) 153–164
Elsevier

BBA 73340

Preparation and characterisation of liposomes containing mannosylated phospholipids capable of targetting drugs to macrophages

Gillian Barratt, Jean-Pierre Tenu, Alexandre Yapo and Jean-François Petit

Laboratoire 'Immunomodulateurs d'origine bactérienne', CNRS U.A. 1116, Bât 432, Université Paris-Sud, 91405 Orsay (France)

(Received 7 August 1986)

Key words: Mannose receptor; Liposome; Drug targeting; Endocytosis; Mannosylated phospholipids; Drug delivery

We have prepared liposomes from mannosylated phosphatidyl*myo*-inositol, derived from mycobacteria, and cholesterol. The size of the particles so formed could be controlled by membrane filtration. The vesicles encapsulated a significant amount of aqueous phase (about 8 μl per mg phospholipid). Markers of the liposomal membrane and aqueous phase rapidly associated with mouse peritoneal macrophages and, more slowly, with rat alveolar macrophages. The uptake was saturable at high liposome concentrations, although phagocytosis of latex particles of the same mean diameter was not saturable at these concentrations. An excess of unlabelled liposomes composed of phosphatidylcholine and phosphatidylserine, which were also taken up readily by macrophages, did not inhibit the uptake of mannosylated liposomes. The uptake of fluorescent mannosylated bovine serum albumin was inhibited by these liposomes, suggesting a specific interaction with the macrophage mannose-fucose receptor. We conclude that this type of liposome would be useful for the delivery of immunomodulators to reticuloendothelial cells.

Introduction

Over the past 15 years liposomes have attracted considerable interest as potential drug carriers [1]. These phospholipid vesicles are capable of encapsulating both hydrophobic and hydrophilic drugs, are biodegradable and are not normally toxic in vivo [1]. However, the distribution of liposomes in vivo, where they are rapidly removed from the circulation by cells of the reticulo-endothelial system, limits their usefulness. The most fruitful applications of liposomes seem to be those that make use of their uptake into phagocytic cells. One such usage is the delivery of immunomodulators to macrophages. Macrophages can be activated for cytotoxicity against tumour cells by a variety of products [2]; among these is muramyldipeptide a small, water-soluble molecule [3,4]. Fidler and colleagues [5,6] and, later, others [7–9] have shown that the efficacity of this agent is greatly increased by encapsulation in liposomes, particularly if a lipophilic derivative is used. This is presumably because the free product is not readily internalised by macrophages [10] and, in vivo, is rapidly eliminated in the urine [11].

An extensive study by Schroit et al. [12] showed that the optimum phospholipid composition for delivery of liposomes to macrophages is phosphatidylcholine and phosphatidylserine in the molar ratio 7 : 3. In vivo, large PC/PS liposomes are accumulated in the lungs of rats to a greater extent than liposomes of other compositions, however, the major sites of deposition remain the liver and spleen [13]. Similar liposomes containing

Correspondence: Dr. G. Barratt, Laboratoire 'Immunomodulateurs d'origine bactérienne', CNRS U.A. 1116, Bât 432, Université Paris-Sud, 91405 Orsay, France.

muramyldipeptide or a lipophilic derivative, were able to eradicate experimental lung metastases in mice [5,14]. Schroit et al. [12,15] have postulated that macrophages possess a receptor for senescent erythrocytes which recognises phosphatidylserine in the outer layer of the membrane. A number of other membrane receptors have been described for macrophages. Among these is a receptor promoting the uptake of mannose, fucose or glucosamine-terminated glycoproteins [16]. This receptor has been exploited by Roche et al. [17], who delivered muramyldipeptide to macrophages by coupling it covalently to mannosylated bovine serum albumin. Thus, mannose-containing glycolipids might be useful for targetting liposomes to these cells. We therefore decided to prepare liposomes from a mannose-containing phospholipid from the cell wall of mycobacteria. The structure of this component (from *Mycobacterium phlei* and *Mycobacterium tuberculosis*) was determined by Lee and Ballou [18] as phosphatidyl*myo*-inositol substituted with 1 to 6 mannose residues. It seemed likely that this phospholipid would be capable of forming closed vesicles. In this report we describe the preparation of such vesicles and some detailed studies of their phagocytosis by macrophages.

Material and Methods

Preparation of liposomes

Materials. Mannose-containing phospholipids were extracted from the cell wall of *Bacillus* Calmette and Guerin as described by Ballou et al. [19]. The product contained a mixture of mannosylated phosphatidylinositols, substituted with a varying number of sugar residues.

Cholesterol was obtained from Prolabo (Paris, France) and was recrystallised several times before use.

Distearoylphosphatidylcholine (PC) was purchased from Calbiochem and dipalmitoylphosphatidylserine (PS) from Serva (F.R.G.).

Bovine serum albumin conjugated with approximately 20 α-mannose residues and its fluorescent derivative [20] were gifts from Dr. M. Monsigny, Centre de Biophysique Moleculaire, CNRS, Orléans, France.

Bovine serum albumin (Sigma), 20 μg, was iodinated with 2 mCi (1 nmol) of $Na^{125}I$ (C.E.A., Saclay, France), using the Iodogen reagent (Pierce). After 30 min at 4°C the reaction mixture was chromatographed on Sephadex G50 (Pharmacia) equilibrated in phosphate-buffered saline and material eluting in the void volume was used to prepare liposomes.

Cellulose acetate filters, pore size 3 μm, filter diameter 25 mm, and holders were purchased from Millipore SA, Velizy, France. Polycarbonate filters, pore size 3 μm, filter diameter 25 mm, were obtained from Nuclepore (Pleasanton, CA).

Preparation of multilamellar vesicles

Mannosylated phosphatidylmyoinositol and cholesterol were mixed in a 2:1 ratio by weight, dissolved in chloroform and evaporated to dryness in a round-bottomed flask. If labelled liposomes were to be prepared a trace amount of 1,2-[1-^{14}C] dipalmitoylphosphatidylcholine (New England Nuclear, 0.01 mCi/mmol) was added to the chloroform. The film was rehydrated in phosphate-buffered saline, usually 1 ml per 20 mg phospholipids. If double-labelled liposomes were to be prepared a small amount of [^{3}H]sucrose (Amersham International, 9.8 Ci/mmol) was added to the aqueous phase. In some experiments ^{125}I-labelled bovine serum albumin was added in the aqueous phase, to an unlabelled lipid film. The flask was vortexed vigorously to detach the lipid film and to form liposomes.

PC/PS liposomes were formed in a similar manner as previously described by Sponton et al. [7]. PC and PS were mixed in a 7:3 molar ratio. The aqueous phase was heated to 60°C before adding to the lipid film, to allow for the transition temperature of distearoylphosphatidylcholine. After hydration for 2 h at room temperature, the liposomes were centrifuged 5–7 times at 29000 $\times$ *g* for 25 min at 4°C to remove non-entrapped aqueous phase. The pellets were resuspended in a suitable volume of phosphate-buffered saline.

Filtration of liposomes

Filtration was carried out using either cellulose acetate (Millipore) or polycarbonate (Nuclepore) 25 mm diameter filters, in both cases with a pore diameter of 3 μm. These were supported in a 'Swinnex' syringe-fitting filter holder (Millipore).

Liposomes were filtered at a concentration of 2.5–5 mg phospholipids/ml, in a 20-ml plastic syringe.

Determination of lipid yield

For labelled liposomes this was calculated by the recovery of ^{14}C-labelled lipid in the final liposome preparation. Counting was carried out in Aqualuma Scintillation mixture (Luma, France) in a Rackbeta LKB scintillation spectrometer.

In unlabelled liposomes the amount of mannosylated phospholipid could be measured by an estimation of the sugar content by the method of Dubois [21]. Since cholesterol interferes with the assay, a calibration curve was constructed using known amounts of mannosylated phospholipid and cholesterol in a 2 : 1 weight ratio.

The amount of phosphatidylcholine in PC/PS liposome preparations could be determined by the enzymatic method of Takayama et al. [22] using the phospholipid β-test kit (Bio-Lyon).

Size of liposomes

This was measured using a Nanosizer (Coultronics). This apparatus uses a helium-neon laser to analyse the fluctuation of the intensity of scattered light due to Brownian movement of the particles. The translational diffusion coefficient is inversely related to the diameter of the particles. For each preparation we give the mean and standard deviation of five determinations made on the same sample of liposomes diluted in phosphate-buffered saline.

Determination of encapsulation ratio

This is usually expressed as μl of aqueous phase per μmol phospholipid [23]. However, since the mannosylated phospholipid used contained a mixture of oligomannosides we have expressed this parameter as μl/mg phospholipid. This ratio was calculated for double-labelled liposomes, to which trace amounts of 1,2-[1-^{14}C]dipalmitoylphosphatidylcholine and [^{3}H]sucrose had been added during preparation, knowing the specific radioactivities of the aqueous and lipid phases.

Determination of aqueous phase leakage

Liposomes (approximately 1 mg lipid, double-labelled, non-filtered) were incubated in 4 ml of phosphate-buffered saline containing 5% foetal calf serum for 30 min at 37°C. They were then centrifuged at 29000 $\times g$ for 25 min at 4°C and the radioactivity in the pellet and supernatant was measured.

Preparation of mouse inflammatory macrophages

Female (DBA/2 × C57BL/6) F_1 mice were obtained from CSEAL (Orléans, France) or IFFA Credo (L'Arbresle, France). They were injected with 1.5 ml of thioglycolate-containing medium 4 days before collection of peritoneal exudate cells. The peritoneal cavity was washed out with Earle's Minimum Essential Medium (Institut Pasteur, Paris) and the cells were collected by centrifugation at 200 $\times g$ at 4°C for 10 min. They were resuspended in medium containing 5% complement-inactivated foetal calf serum and antibiotics. The number of macrophages (80–90% of the total cell content) was estimated by neutral red uptake, and the suspension was adjusted to $2 \cdot 10^6$ macrophages per ml. This suspension was plated in 35-mm diameter plastic petri dishes (Nunclon) at 2 ml per dish, and incubated at 37°C in a 5% CO_2-humidified atmosphere for 2–3 h to allow adherence of macrophages. Non-adherent cells were removed by washing three times with phosphate-buffered saline and the monolayers were then used for uptake studies.

Rat alveolar macrophages

Female WAG rats, aged 8–10 weeks, were obtained from CSEAL (Orléans, France). They were anaesthesised with 100 mg/kg of Imalgène i.p. (Rhone-Mérieux, France) and exsanguinated via the abdominal aorta. The lungs were carefully removed and lavaged via the trachea with 6 aliquots of 5 ml sterile 0.9% (w/v) sodium chloride solution. These washings were pooled and centrifuged in the presence of 5 ml of Earle's Minimum Essential Medium supplemented with 5% foetal calf serum and antibiotics at 200 $\times g$ at 4°C for 15 min. The pellet, which was 90% alveolar macrophages, was resuspended in the same medium and adjusted to 10^6 macrophages per ml. These were plated in 35-mm dishes (2 ml per dish, hence $2 \cdot 10^6$ macrophages) and allowed to adhere, as for mouse peritoneal macrophages, for 3–4 h. They were then washed and used as for mouse macrophages.

Measurement of liposome uptake by macrophages

Radioactively labelled liposomes, either alone or with unlabelled liposomes, were diluted to suitable lipid concentrations in medium supplemented with 5% foetal calf serum and antibiotics. The macrophages were incubated with 2 ml of medium containing liposomes for 2–20 h at 37°C with a 5% CO_2-humidified atmosphere. The medium was removed at the end of the incubation period and the macrophages were washed four times with phosphate-buffered saline. 0.6 ml of phosphate-buffered saline containing 1% (w/v) Triton X-100 was added and the macrophages were scraped off with a rubber policeman. This operation was repeated with another 0.6 ml of Triton X-100 solution and the two lysates were pooled. Suitable volumes of the supernatant and lysate were counted for radioactivity. ^{125}I was counted by β scintillation spectroscopy using the windows for ^{14}C. The amount of lipid or aqueous phase associated with the macrophages could be calculated from the specific activities of the different components.

Measurement of uptake in the presence of inhibitors, or of latex beads

Latex beads (Estapor, Rhône-Poulenc, Paris-La Défense, France) 0.4 μm or 1.1 μm in diameter, chloroquine or sodium fluoride dissolved in sterile water were added in the medium at the same time as liposomes. Cytochalasin B was from Aldrich and was dissolved in 10% ethanol. 100 μl of this solution was added to 2 ml of medium containing liposomes. In a control experiment, 0.5% ethanol alone did not inhibit the internalisation of liposomes.

In order to study uptake at 4°C, precooled medium containing liposomes was added to the monolayers, and they were incubated at 4°C in a refrigerator (without CO_2). After all these treatments the viability and attachment of the cells was confirmed using an inverted light microscope after trypan blue staining.

Chromatography of liposome-encapsulated bovine serum albumin

A 6 mm × 200 mm column of Sephadex G50 (Pharmacia), in phosphate-buffered saline, was used. 400 μg of mannosylated phospholipid in the form of liposomes containing ^{125}I-labelled bovine serum albumin were mixed with 100 μg of unlabelled bovine serum albumin, disrupted with Triton X-100 (final concentration 1% (w/v) and loaded onto the column. 0.5 ml fractions were eluted with phosphate-buffered saline and assayed for radioactivity. A sample of liposomes which had been incubated for 19 h in medium containing 5% foetal calf serum at 37°C was treated in the same way. Finally, 1 ml of a detergent lysate of macrophages (approx. $4 \cdot 10^6$ cells) which had been incubated with similar liposomes for 19 h was loaded.

Uptake of fluorescent neoglycoproteins

This was studied according to the technique of Tenu et al. [10]. Mouse inflammatory macrophages were incubated with 50 μg/ml of fluorescent mannosylated bovine serum albumin either alone or in the presence of 2.5 mg/ml of mannosylated bovine serum albumin or liposomes, in medium consisting of Earle's Minimum Essential Medium diluted 1 : 1 with phosphate-buffered saline containing 1% (w/v) fatty acid-free bovine serum albumin (Sigma) and antibiotics, for 2 h at 37°C (as for liposome uptake). The macrophage monolayers were then washed three times with phosphate-buffered saline containing 0.5% bovine serum albumin. The cells were then recovered in 2 aliquots of 0.6 ml of phosphate-buffered saline (pH 8.5) containing 1% (w/v) Triton X-100 in the manner described above for liposome uptake. The lysates were centrifuged at 200 × g for 10 min and the fluorescence of the supernatants was measured at $\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm. A calibration curve constructed from the fluorescence readings given by standard solutions of fluorescent mannosylated bovine serum albumin in phosphate-buffered saline (pH 8.5) containing 1% (w/v) Triton X-100 (0–4 μg/ml) allowed the amount of cell-associated glycoprotein to be calculated. Parallel experiments were performed using radioactively-tagged liposomes (both mannosylated phospholipids/cholesterol and PC/PS) under the same conditions and these showed that the modified incubation conditions (necessary for accurate assessment of glycoprotein uptake) did not affect the capacity of the macrophages to take up liposomes.

Results

Size of liposomes prepared by filtration

Before filtration, the liposome preparations were extremely heterogenous, and frequently had a mean diameter larger than 3 μm (the uppermost limit of the Nanosizer). After filtration on 3 μm pore diameter Millipore cellulose acetate membranes the resulting populations were more homogeneous and had mean diameters between 400 and 700 nm. The exact mean diameter of the preparation used is given for each experiment. This applied to both mannosylated phospholipids/cholesterol and PC/PS liposomes. After filtration on 3 μm pore diameter Nuclepore polycarbonate membranes a larger mean diameter was obtained. Two different mannosylated phospholipid/cholesterol preparations had mean diameters of 2365 ± 955 nm and 1395 ± 145 nm. It thus appears that the filtration process is perhaps closer to extrusion, as described by Olson et al. [24]. However, a significant proportion of the lipid was lost on filtration (Table I). This was probably due to larger liposomes blocking the membrane and allowing only particles much smaller than the pore size to pass. This process provided a rapid method for obtaining a near-homogeneous preparation of liposomes of defined size without the possible lipid degradation invoked by sonication.

Encapsulation ratio

Table I shows the yield of phospholipid ([^{14}C]dipalmitoylphosphatidylcholine) and aqueous phase ([^{3}H]sucrose) markers in typical preparations of mannosylated phospholipid/cholesterol liposomes. The recovery of the lipid phase was confirmed by measurement of the sugar content of the liposomes by the method of Dubois [21], taking advantage of mannose present in the mannosylated phospholipid. ([^{3}H]Sucrose was added in only trace quantities and thus did not affect the assay). The yield after centrifugation, i.e. in large multilamellar vesicles, was 52%, which is low compared to that of the phospholipids usually employed to prepare liposomes, such as phosphatidylcholine. The recovery of cholesterol was not measured, but, since it was present in a molar excess in the starting mixture, we expected to obtain a 1 : 1 molar ratio, the maximum possible [25].

The encapsulation ratio (μl of aqueous phase per mg phospholipid) was larger than that observed for 'conventional' liposomes; e.g. 2.4 μl/mg phospholipids reported by Sponton et al. [7] for PC/PS/cholesterol liposomes; and approached values described for reverse-phase vesicles [26].

The encapsulation ratio was increased slightly by filtration, which might represent leakage of aqueous phase from the liposomes. When PC/PS liposomes, labelled with ^{14}C-phospholipid, were filtered on cellulose acetate membranes the recovery was similar to that of mannosylated phospholipid/cholesterol liposomes.

TABLE I

PREPARATION OF LIPOSOMES FROM MANNOSYLATED PHOSPHOLIPIDS

50 mg of mannosylated phospholipids was mixed with 25 mg cholesterol and 0.5 μCi of ^{14}C-labelled lipid in 10 ml chloroform and a film formed. This was rehydrated in 5 ml of phosphate-buffered saline containing 150 μCi [^{3}H]sucrose. The suspension was centrifuged seven times at 29000 × *g* for 30 min, and the final pellet was resuspended in 11 ml phosphate-buffered saline (2.5 mg/ml) and filtered on either Millipore or Nuclepore 3 μm pore size filters. The encapsulation ratio (E.R.) is expressed as μl of aqueous phase per mg of phospholipid. The table shows the means ± S.D. for three typical preparations.

Stage	mg of phospholipid	μl of aqueous phase recovered	% yield of phospholipid	% yield of aqueous phase	E.R.
Starting conditions	50	5000			
After centrifugation	26 ± 3	180 ± 33	52 ± 5	3.6 ± 0.8	6.9 ± 1.0
After filtration Millipore filter	17 ± 2	158 ± 28	33 ± 4	3.2 ± 0.6	9.5 ± 1.0
After filtration Nuclepore filter	16 ± 2	132 ± 24	32 ± 4	2.6 ± 0.5	8.3 ± 1.0

Stability of mannosylated phospholipid/cholesterol liposomes

In a preliminary experiment with non-filtered liposomes, double-labelled vesicles prepared from mannosylated phospholipid/cholesterol (2:1, by weight) were compared to similar liposomes made from mannosylated phospholipid alone. The leakage of [^{3}H]sucrose was measured after incubation for 30 min at 37°C in medium containing 5% foetal calf serum. The percentage leakage from mannosylated phospholipid liposomes was 67% of the total aqueous phase whereas that from mannosylated phospholipid/cholesterol was only 6%. Therefore cholesterol was able to reduce leakage, as reported for other liposome types [27], although the cholesterol-rich liposomes still lost entrapped material on incubation. To minimise this loss, cholesterol was always included in these liposomes, although we focused mainly on the uptake of lipid phase markers.

Uptake of mannosylated phospholipid/cholesterol liposomes by mouse inflammatory macrophages

(I) Time-course. Fig. 1 shows the uptake of ^{14}C-lipid label from mannosylated phospholipid/cholesterol liposomes, mean diameter 500 nm, presented at 50 μg of mannosylated phospholipid per ml of culture medium. The uptake was linear with time up to 20 h. At this point about 19% of the available lipid had been taken up representing 4.7 $\mu g/10^6$ macrophages. Within a single experiment, i.e., one macrophage preparation, the levels of uptake between duplicate dishes never varied by more than 20%. However, quantitative differences were seen between preparations of macrophages harvested on different occasions.

(II) Uptake of aqueous phase. When the uptake of double-labelled liposomes containing mannosylated phospholipid and cholesterol was studied, the percentage of the aqueous label associated with the cells at the end of the incubation period (between 2 and 16 h) was always about half of the percentage of the lipid label. This may be because, despite the inclusion of cholesterol in the liposomes, some of the aqueous phase was lost by leakage, which might be provoked by contact with the macrophages [28] or with serum components; or because some of the phospholipid is incorporated into the macrophages by fusion, with escape of the encapsulated material. The fact that the ratio of aqueous to lipid label uptake was similar for incubation times up to 16 h suggested that time-dependent leakage of solute was not extensive in this experimental system. A similar

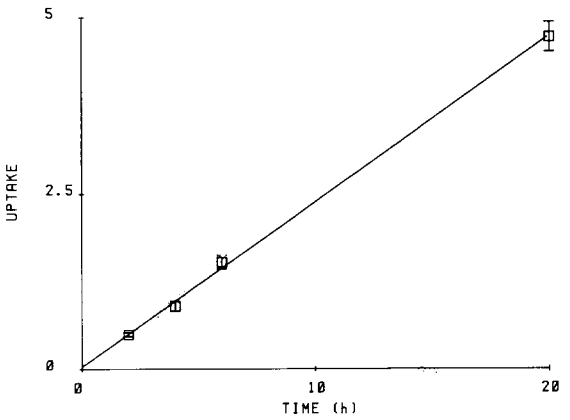

Fig. 1. Time-course of uptake of mannose-containing liposomes by mouse inflammatory macrophages. Liposomes were prepared from mannosylated phospholipids and cholesterol 2:1 by weight, with a trace amount of [^{14}C]dipalmitoylphosphatidylcholine, and filtered to give a mean diameter of 500 nm. Macrophages were plated at $4 \cdot 10^6$ cells/dish and incubated with 50 μg of liposomal phospholipids/ml for the periods stated. Uptake is expressed as μg of phospholipid/10^6 cells. Mean ± range for duplicate dishes.

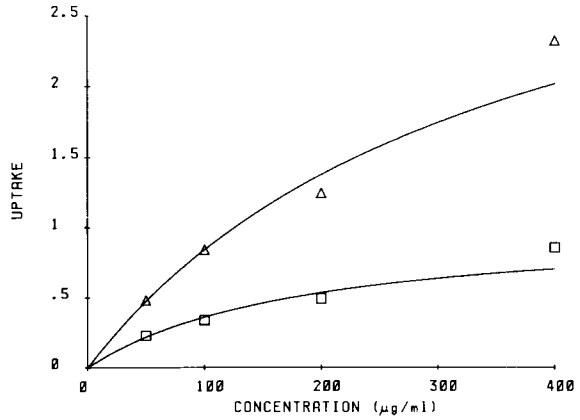

Fig. 2. Uptake of calibrated liposomes by mouse inflammatory macrophages. △, Mannosylated phospholipid/cholesterol, 2:1 liposomes; □, PC/PS, 7:3 molar ratio liposomes. Both preparations were trace-labelled with ^{14}C-labelled lipid and filtered to give a mean diameter of 500 nm. Macrophages were plated at $4 \cdot 10^6$ cells/dish and incubated with liposomes for 2 h. A typical experiment is shown, with each point representing a single dish. Uptake is expressed as μg phospholipid/10^6 cells per h.

result has recently been reported for PC/PS double-labelled liposomes [7].

(III) Concentration dependence of uptake. Fig. 2 shows that the uptake of ^{14}C-labelled lipid from 500 nm diameter mannosylated phospholipid/cholesterol liposomes by mouse inflammatory macrophages was saturable at high liposome concentrations. However, a plateau was not reached at 400 μg/ml of liposomal lipid. Similar results were obtained in five independent experiments.

(IV) Comparison with PC/PC liposomes. Fig. 2 also shows the uptake of PC/PS liposomes of the same diameter under the same conditions. At low phospholipid concentrations the percentage internalisation of PC/PS liposomes was about half that of mannosylated phospholipid/cholesterol liposomes. Also, saturation seemed to occur at a lower level for PC/PS liposomes. Thus, mannosylated phospholipid/cholesterol liposomes compared favourably with the type of vesicles considered optimal for drug delivery to macrophages [12].

Effect of unlabelled liposomes on uptake

The addition of an excess of unlabelled mannosylated phospholipid/cholesterol liposomes (of the same mean diameter) during the uptake of labelled liposomes of the same type inhibited the uptake of label. The uptake of ^{14}C label from liposomes present at 50 μg phospholipid/ml was inhibited by 74% by 400 μg/ml of similar but unlabelled liposomes. This would be expected if the uptake is saturable. Interestingly, an excess of unlabelled PC/PS liposomes of the same size at the same concentration did not inhibit the uptake of liposomes prepared from mannosylated phospholipid. Similarly, excess unlabelled mannosylated phospholipid liposomes did not inhibit the uptake of labelled PC/PS liposomes. Thus the two types of liposomes seemed to be handled by different mechanisms.

The inhibition of uptake by cold particles was used to estimate the concentration of phospholipid necessary for 50% of the maximum uptake. The uptake of ^{14}C-lipid was measured at three concentrations of labelled liposomes, in the presence of different concentrations of analogous unlabelled liposomes. A Dixon plot, i.e. the reciprocal of the rate of uptake of label versus the concentration of inhibitor, for each concentration of labelled liposomes, was constructed (Fig. 3). This shows that the uptake of these mannose-containing liposomes fits a simple Michaelis-Menten model. From the point of intersection of the three

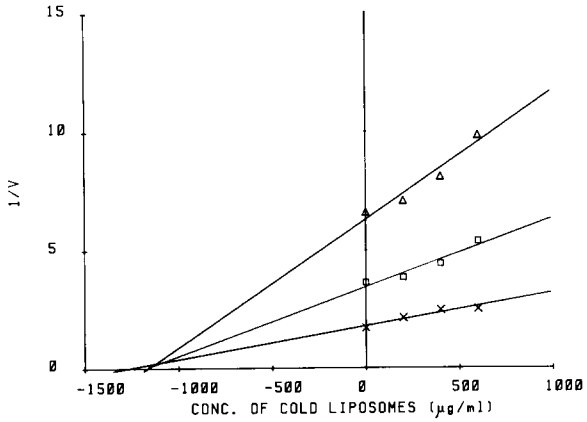

Fig. 3. Inhibition of uptake of mannose-containing liposomes by unlabelled liposomes. Single Petri dishes, containing $4 \cdot 10^6$ macrophages were incubated with one of three different concentrations of ^{14}C-lipid-labelled liposomes prepared from mannosylated phospholipid and cholesterol, 2:1, mean diameter 400 nm; either alone or with unlabelled (cold) liposomes at the concentrations stated. After 16 h, the cell-associated ^{14}C-labelled lipid was determined. △, 25 μg phospholipid as labelled liposomes; □, 50 μg phospholipid as labelled liposomes; ×, 100 μg phospholipid as labelled liposomes; $V = \text{dpm} \times 10^{-4}$. Specific activity of labelled liposomes was 165 dpm per μg phospholipid.

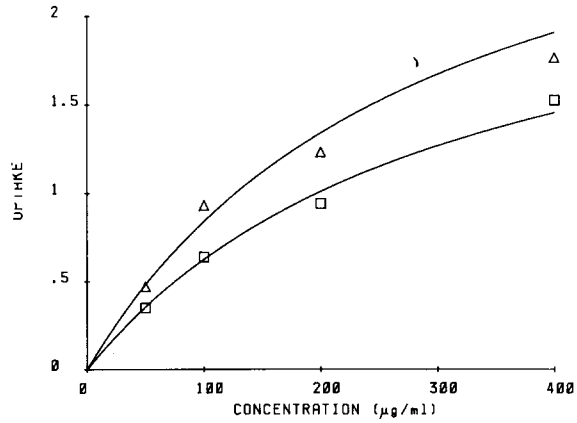

Fig. 4. Uptake of mannose-containing liposomes of different sizes. Mouse inflammatory macrophages ($4 \cdot 10^6$ cells/dish) were incubated with liposomes prepared from mannosylated phospholipids and cholesterol, 2:1 by weight, filtered on either (□) Millipore membranes, giving a mean diameter of 700 nm or (△) Nuclepore, giving a mean diameter of 1400 nm, for 16 h. Each point represents a single dish. Uptake is expressed as μg phospholipid/10^6 cells per h.

straight lines, an estimate of K_i of about 1.1 mg/ml could be obtained (Fig. 3). This confirmed that saturation of uptake occurred at very high liposome concentrations. An approximate estimate of the maximum rate of uptake is 6.5 μg of liposomal lipid per million macrophages per hour.

Effect of liposome size on uptake

^{14}C-labelled mannosylated phospholipid/cholesterol liposomes passed through Nuclepore filters, and having a mean diameter of 1.4 μm, were compared to liposomes of similar composition filtered on Millipore membranes (mean diameter 700 nm). Fig. 4 shows that the larger (Nuclepore) liposomes were taken up more than the smaller (Millipore) ones, and that both uptakes were saturable. The effect of unlabelled liposomes was also tested. Here the unlabelled, larger liposomes (mean diameter 2.4 μm) were less inhibitory than those of a smaller diameter (500 nm).

Uptake by rat alveolar macrophages

The uptake of both mannosylated phospholipid/cholesterol and PC/PS liposomes, 500 nm in diameter and labelled with [^{14}C]dipalmitoylphosphatidylcholine, by rat alveolar macrophages was determined. The rate of uptake was 2–4-times less than that observed for mouse inflammatory macrophages, depending on the particular preparation. The relative rates of uptake of mannosylated phospholipid/cholesterol and PC/PS liposomes were similar; i.e. at a given liposome concentration twice as much marker was associated with the cells when mannose-containing liposomes were used. The uptake was saturable at high liposome concentrations.

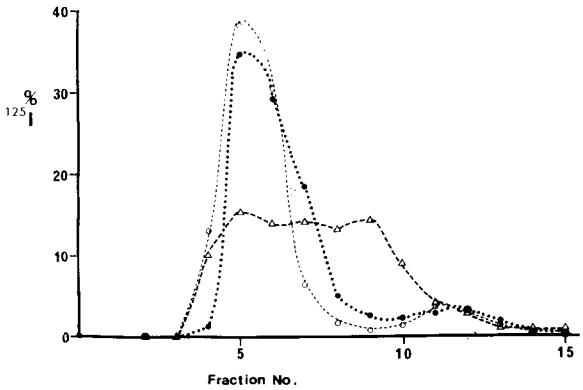

Fig. 5. Chromatography of liposome-encapsulated ^{125}I-labelled bovine serum albumin. O------O, Liposomes lysed with 1% Triton X-100; ●·····●, liposomes incubated in medium, 19 h at 37°C, then lysed; △------△, lysates of macrophages incubated with liposomes, 19 h at 37°C. The radioactivity of each fraction (0.5 ml) is expressed as a percentage of the total activity recovered in the experiment.

Fate of ^{125}I-labelled bovine serum albumin encapsulated in mannose-containing liposomes

Liposomes containing ^{125}I-labelled bovine serum albumin (1.4 μm mean diameter) were incubated for 19 h with $4 \cdot 10^6$ macrophages at 200 μg/ml. After washing, the macrophages were lysed with Triton X-100 and filtered on a Sephadex G50 column. Liposomes suspended in medium without macrophages for the same time were run as a control. Fig. 5 shows that ^{125}I-labelled serum albumin extracted from these control liposomes was eluted as a single peak in the void volume whereas ^{125}I-labelled protein taken up by macrophages was partially degraded to smaller fragments. This proves that these liposomes had been endocytosed by macrophages and had discharged their contents in the lysosomes.

TABLE II

EFFECT OF VARIOUS INHIBITORS AND LATEX BEADS ON UPTAKE OF MANNOSE-CONTAINING LIPOSOMES

Mouse inflammatory macrophages were plated at $4 \cdot 10^6$ cells/dish. Inhibitors were added at the same time as liposomes prepared from mannosylated phospholipids and cholesterol, 2:1 by weight, diameter = 500 nm, labelled with ^{14}C-labelled lipid, at 50 μg phospholipid/ml. Uptake was measured after 2 h, except in (1), when 4 h was used. Each value represents the mean ± S.D. of three dishes. Control uptake at 37°C was 0.39 ± 0.02 μg phospholipid/10^6 cells per h. (2) Concentration equivalent in number of particles to 400 μg/ml of the smaller latex beads.

Treatment	% of control uptake (^{14}C-labelled lipid) ± S.D.
Incubation at 4°C	62 ± 6
Sodium fluoride, 1 mg/ml	61 ± 8
Cytochalasin B, 20 μg/ml	58 ± 9
(1) Latex beads, ∅ = 0.4 μm	
400 μ/ml	135 ± 10
1 mg/ml	161 ± 9
(2) Latex beads, ∅ = 1.1 μm	
8.5 mg/ml	133 ± 9

Effect of inhibitors of endocytosis on uptake of mannose-containing liposomes

Incubation at 4°C reduced the uptake of small, filtered mannosylated phospholipid/cholesterol liposomes by mouse inflammatory macrophages by 58% (Table II). A similar percentage inhibition was observed in the case of latex particle endocytosis (data not shown). The residual 42% could be due to binding of the particles to the surface of the macrophages. Some inhibition was also obtained with cytochalasin B and sodium fluoride (Table II). Thus, at least part of the uptake (50–60%) can be attributed to endocytosis.

We also examined the effect of 40 μM chloroquine on the uptake of 1.4 μm diameter liposomes containing ^{125}I-labelled bovine serum albumin as a single marker, presented at 200 μg phospholipid/ml for 19 h. The apparent uptake was 0.38 ± 0.01 μg/h per 10^6 macrophages in the absence of chloroquine and 0.44 ± 0.03 μg/h per 10^6 macrophages in the presence of the drug. This small increase was significant ($P < 0.02$) and was probably due to an inhibition of degradation of the protein followed by a reduction in the exocytosis of products. The amount of trichloroacetic acid-soluble material in the supernatant was reduced (from 21 to 16% of the total radioactivity) in the presence of chloroquine. In the absence of macrophages, liposomes suspended in culture medium at 37°C for 19 h contained only 9% acid-soluble material, which corresponded to the percentage of free iodide revealed by Sephadex G50 chromatography (Fig. 5).

Effect of latex beads on mannose-containing liposome uptake

The addition of latex particles, which in our system were taken up rapidly in a non-saturable fashion, did not inhibit uptake of liposomes; in contrast, the uptake was stimulated (Table II). This might have been because liposomes are co-endocytosed with latex particles in the same phagocytic vesicle.

Effect of liposomes on uptake of neoglycoproteins by macrophages

Table III shows the result of a typical experiment on the uptake of fluoresceinylated mannosylated bovine serum albumin by mouse inflammatory macrophages under various conditions. The uptake was 74% inhibited by excess unlabelled mannosylated bovine serum albumin showing that the interaction was almost completely specific. A similar inhibition was observed with 500 μg/ml of mannosylated phospholipid/cholesterol liposomes (Table III). PC/PS liposomes were much less inhibitory. This would seem to indicate that mannose-containing liposomes interact with the macrophage mannose receptor. Attempts to inhibit the endocytosis of radioactive mannosylated phospholipid/cholesterol liposomes (50 μg/ml) with mannosylated bovine serum albumin (50–2000 μg/ml) were completely unsuccessful (data not shown). It might be expected that the liposomes, which contain at least one available mannose residue on every phospholipid molecule on the surface would be difficult to displace from receptors with the smaller albumin molecule bearing 20 mannose residues. In fact the situation is analogous to the poor inhibi-

TABLE III

UPTAKE OF NEOGLYCOPROTEINS BY MOUSE INFLAMMATORY MACROPHAGES

Macrophages were plated at $4 \cdot 10^6$ cells/dish. Fluorescent mannosylated bovine serum albumin was added at 50 μg/ml, together with other additions as stated. Liposomes were prepared from mannosylated phospholipids and cholesterol 2:1 by weight or PC/PS 7:3 molar ratios and were filtered to give a mean diameter of 500 nm. After 2 h incubation the cells were washed and cell-associated fluorescence was measured as described in Methods. Percentage inhibition is defined as 100× (control uptake − uptake in the presence of inhibitor)/(control uptake). The table describes a typical experiment. Each point represents the mean ± S.D. of three dishes.

Addition	Concn. (mg/ml)	Uptake of neoglycoprotein (ng/10^6 cells per h)	Inhibition (%)
None	–	110 ± 6	–
Mannosylated bovine serum albumin	2.5	28 ± 5	75 ± 8
Mannosylated phospholipid/cholesterol liposomes	0.1	103 ± 5	6 ± 8
	0.5	25 ± 5	78 ± 8
PC/PS liposomes	0.1	98 ± 5	11 ± 8
	0.5	74 ± 5	33 ± 8

tion of labelled glycoprotein uptake by free mannose.

Discussion

In this study we have described the preparation of a new type of liposome, composed entirely of mannosylated phospholipids and cholesterol. Other workers [29–33] have incorporated glycolipids into liposomes but these are usually a minor component with other non-glycosylated phospholipids. The particles that we have prepared would seem to be closed vesicles because they encapsulate an aqueous label, in amounts which were consistent between different experiments. The encapsulation ratio is larger than that normally observed for multilamellar vesicles (1–4 l/mol), and is more similar to that of reverse-phase vesicles [26]. It may be that the bulky mannose head groups cause larger inter-bilayer spaces than normal. The mannose residues would also be solvated by a considerable number of water molecules. If the phospholipid molecules are not tightly packed, this would explain why liposomes prepared from mannose-containing phospholipids without cholesterol lose their entrapped material rapidly at 37°C.

Many groups of workers have studied the interaction of liposomes with mammalian cells in vitro (see Pagano et al. [34] for a review). In particular, several reports have dealt with the uptake of liposomes by macrophages and macrophage-like cell lines. Hsu and Juliano [35] studied the uptake of dipalmitoylphosphatidylcholine/cholesterol (1 : 1) vesicles of various types and saw saturation of uptake at high liposome concentrations. They also observed that coating the vesicles with IgG or fibronectin increased the uptake. The effect of surface immunoglobulin, causing endocytosis via the Fc receptor, was also noted by Petty and McConnell [36]. Baldeschwieler and co-workers [32] have studied liposome targetting to reticuloendothelial cells using 6-aminomannose covalently linked to cholesterol. This synthetic glycolipid greatly increased phagocytosis of the liposomes. Studies of various sugar analogues led to the conclusion that the uptake was not receptor-mediated, since surface charge seemed to be more important than 3-dimensional configuration [32]. Schroit et al. [12] have shown that the incorporation of 30 mol% of phosphatidylserine into phosphatidylcholine liposomes increased uptake by a factor of 10. Here we have shown that liposomes containing mannosylated phospholipids are taken up by macrophages even more readily than PC/PS liposomes of the same size.

We have shown that the size of the liposomes is important for uptake, since the larger 'Nuclepore' liposomes, 1.4 μm in diameter, were taken up more quickly than those of 400–700 nm diameter prepared using 'Millipore' filters. This may be because the number of particles that can be taken up in a given time is the limiting factor. Differences in the initial rate of uptake of PC/cholesterol multilamellar, reverse-phase and small unilamellar vesicles were also seen by Hsu and Juliano [35] and were related to liposome number.

We performed some experiments in order to distinguish between absorption of liposomes onto the surface of the macrophages and endocytosis. We saw that the uptake was 60% inhibited at 4°C as compared to 37°C (Table II). The residual 40% was probably binding at the surface, whereas over half the uptake was endocytosis, perhaps supplemented with some fusion of liposomes with the plasma membrane. The fact that inhibitors of endocytosis produced almost as great a reduction in uptake as incubation at 4°C (Table II) is further support for this interpretation. A similar percentage inhibition was observed with latex particles, which are known to be phagocytosed. In addition, we have followed the fate of a degradable marker of the aqueous phase, ^{125}I-labelled bovine serum albumin. Chromatography of the cell lysate after overnight incubation with liposomes containing this marker showed that the encapsulated protein had undergone considerable degradation to low molecular weight material. Liposome-encapsulated albumin which was incubated in medium alone for a similar period and released by detergent prior to chromatography was not degraded in this way; we therefore interpret this result as indicating that liposomes and their contents are degraded by lysosomal enzymes. Some low molecular weight material was also found in the supernatant of macrophage cultures after incubation with liposomes, suggesting an export of degraded material. This release was re-

duced by treatment with 40 μM chloroquine, a concentration which did not cause cell detachment as determined by light microscopy. At the same time, chloroquine slightly but significantly reduced liposome uptake, as measured by the association of the aqueous phase marker. All this evidence, which is similar to observations made on phosphatidylcholine liposome uptake by Kupffer cells [37], points to the endocytosis of these liposomes followed by processing in the lysosomal compartment.

The fact that unlabelled liposomes of the PC/PS type do not inhibit the uptake of labelled mannose-containing vesicles and vice-versa suggests different receptor mechanisms dealing with the two liposome compositions used here. The data in Table III are clear evidence that liposomes prepared from mannosylated phosphatidylinositol interact specifically with the macrophage mannose receptor, whereas PC/PS liposomes do not interact to any great extent. The internalisation of PC/PS liposomes may occur via a receptor specific for phosphatidylserine, as suggested by Schroit et al. [12,15].

The rapid uptake of mannose-containing liposomes by macrophages makes them ideal carriers for immunomodulators. If possible, a lipophilic derivative would be preferable to a soluble molecule, since the former is incorporated more efficiently into liposomes, and the leakage of aqueous phase material may be a problem even in cholesterol-rich liposomes of this type. We have shown, in a preliminary study, that the lipophilic immunomodulator muramyldipeptide-L-alanyl-cholesterol, incorporated into liposomes prepared from mannosylated phospholipids and cholesterol as described in this paper, is capable of activating rat alveolar macrophages for cytotoxicity against a syngeneic tumour target, both in vitro and in vivo [38]. Since phosphatidylserine-containing liposomes are apparently handled by a different route, it might be possible to deliver more encapsulated drug to macrophages by using a mixture of the two liposome types at non-saturating concentrations. Alternatively, the two preparations could be used to deliver two different immunomodulators simultaneously to macrophages. This is of interest since we have recently demonstrated a synergism between muramyldipeptide and bacterial lipopolysaccharide for activating rat alveolar macrophages [39].

Calibrated liposome preparations might also be useful in studying macrophage phagocytic mechanisms in detail. Since mannose-containing liposomes seem to have a high affinity for the macrophage mannose receptor and can be used to encapsulate a wide range of radioactive or other markers they could replace or complement modified glycoproteins in this field. In particular, they might be useful in determining the role of membrane receptors in the entry of micro-organisms into macrophages.

Acknowledgements

This work was supported by C.N.R.S. (U.A. 1116) and by grants from the Ligue Nationale contre le Cancer and the Royal Society, U.K. (G.M.B.).

References

1 Ryman, B.E. and Tyrrell, D.A. (1980) Essays in Biochemistry (Campbell, P.N. and Marshall, R.D., eds.), Vol. 16, pp. 49–98, Academic Press, London
2 Hibbs, J.B., Chapman, H.A., Jr. and Weinberg, J.B. (1980) in Mononuclear Phagocytes: Functional Aspect, Part II (Van Furth, J.R., ed.), pp. 1681–1720, Martinus Nijhoff Medical Division, The Hague
3 Ellouz, F., Adam, A., Ciorbaru, R. and Lederer, E. (1974) Biochem. Biophys. Res. Commun. 59, 1317–1325
4 Juy, D. and Chedid, L. (1975) Proc. Natl. Acad. Sci. USA 72, 4105–4109
5 Fidler, I.J., Sone, S., Fogler, W.E. and Barnes, Z.L. (1981) Proc. Natl. Acad. Sci. USA 78, 1680–1684
6 Sone, S. and Fidler, I.J. (1981) Cell. Immunol. 57, 42–50
7 Sponton, E., Drouin, D., Delattre, J., Puisieux, F., Tenu, J.P., Yapo, A., Barratt, G. and Petit, J.F. (1985) Int. J. Pharmacol. 23, 299–313
8 Lopez-Berestein, G., Mehta, K., Mehta, R., Juliano, R.L. and Hersh, E.M. (1983) J. Immunol. 130, 1500–1502
9 Phillips, N.C., Moras, M.L., Chedid, L., Petit, J.F., Tenu, J.P., Lederer, E., Bernard, J.M. and Lefrancier, P. (1985) J. Biol. Resp. Mod. 4, 464–474
10 Tenu, J.P., Roche, A.C., Yapo, A., Kieda, C., Monsigny, M. and Petit, J.F. (1982) Biol. Cell 44, 157–164
11 Parant, M., Parant, F., Chedid, L., Yapo, A., Petit, J.F. and Lederer, E. (1979) Int. J. Immunopharmacol. 1, 35–41
12 Schroit, A.J., Galligioni, E. and Fidler, I.J. (1983) Biol. Cell. 47, 87–94
13 Fidler, I.J., Raz, A., Fogler, W.E. and Poste, G. (1980) Rec. Res. Cancer Res. 75, 246–251
14 Fidler, I.J. (1985) Cancer Res. 45, 4714–4726

15 Schroit, A.J., Tanaka, Y., Madsen, J. and Fidler, I.J. (1984) Biol. Cell. 51, 227–238
16 Stahl, P. and Gordon, S. (1982) J. Cell Biol. 93, 49–56
17 Roche, A.C., Bailly, P. and Monsigny, M. (1985) Invasion Metastasis 5, 218–232
18 Lee, Y.C. and Ballou, C.E. (1965) Biochemistry 4, 1395–1404
19 Ballou, C.E., Vilkas, E. and Lederer, E. (1963) J. Biol. Chem. 238, 69–76
20 Kieda, C., Roche, A.C., Delmotte, F. and Monsigny, M. (1979) FEBS Lett. 99, 329–332
21 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350–356
22 Takayama, M., Itoh, S., Nagasaki, T. and Tanimizu, I. (1977) Clin. Chim. Acta 79, 93–98
23 Hunt, C.A. and Szoka, F. (1982) in Liposome Methodology (Leserman, L.D. and Barbet, J., eds.), pp. 157–159, Les Editions de l'INSERM, Paris
24 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23
25 Ladbrooke, B.D., Williams, R.M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333–340
26 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198
27 Kirby, C. and Gregoriadis, G. (1983) Biochem. Pharmacol. 32, 609–615
28 Dijkstra, J., Van Galen, M., Regts, D. and Scherphof, G. (1985) Eur. J. Biochem. 148, 391–397
29 Hoekstra, D., Tomasini, R. and Scherphof, G. (1980) Biochim. Biophys. Acta 603, 336–346
30 Heath, T.D., Macher, B.A. and Papahadjopoulos, D. (1961) Biochim. Biophys. Acta 640, 66–81
31 Nicolau, C., Soriano, P., Le Pape, A., Londos-Gagliardi, D., Legrand, A. and Grosse, E. (1983) Biol. Cell 47, 121–130
32 Baldeschweiler, J.D. (1985) Ann. N.Y. Acad. Sci. 446, 349–367
33 Latif, N. and Bachhawat, B.K. (1984) Immunol. Lett. 8, 75–79
34 Pagano, R.K., Schroit, A.J. and Struck, D.K. (1981) in Liposomes: from Physical Structure to Therapeutic Applications (Knight, C.G., ed.), pp. 323–348, Elsevier/North-Holland Biomedical Press, Amsterdam
35 Hsu, M.J. and Juliano, R.L. (1982) Biochim. Biophys. Acta 720, 411–419
36 Petty, H.R. and McConnell, H.M. (1983) Biochim. Biophys. Acta 735, 77–85
37 Dijkstra, J., Van Galen, M. and Scherphof, G.L. (1984) Biochim. Biophys. Acta 804, 58–67
38 Barratt, G.M., Yapo, A., Nolibe, D., Petit, J.F. and Tenu, J.P. (1986) Cell Biol. Rev. S1, p. 76
39 Barratt, G.M., Tenu, J.P., Nolibe, D. and Petit, J.F. (1986) in Biological Properties of Peptidoglycan (Seidl, P.H. and Schleifer, K.H., eds.), Proceedings of the 2nd International Workshop on the Biological Properties of Peptidoglycan, Munich, May 1985, Walter de Gruyter and Co., Berlin, in the press