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Binding and uptake of liposomes containing a poly(ethylene glycol) derivative of cholesterol (stealth liposomes) by the macrophage cell line J774: influence of PEG content and its molecular weight

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Abstract

The binding and intake of liposomes containing a different molar content and chain length of a PEG-Chol derivative had been studied in cultured macrophage cell line J774. The decrease in binding and endocytosis of the liposomes containing PEG-Chol is dependent on (i) the PEG chain length, (ii) the molar content of the surfactant, (iii) the liposome concentration in the external medium. The best results in reducing the uptake of liposomes were obtained by a PEG-Chol liposome suspension with a high molar content (25%) which presents a non negligible amount of free PEG-Chol. Moreover, we could show an increase by 2 for binding and by about 5 for endocytosis of filtrated-liposomes containing 25 mol% of ^{8800}PEG-Chol, in the absence of free PEG-Chol in the suspension. Binding and intake of control liposomes was also inhibited in the presence of free PEG-Chol. Fluid phase endocytosis of SRh was inhibited up to 45% of control in the presence of liposomes containing PEG-Chol or free PEG-Chol. Based on the comparison of ^{4400}PEG-Chol with the most commonly used PEG derivative ^{5000}PEG-PE, PEG-Chol is more powerful in terms of reducing their binding and endocytosis by J774 cells. Inhibition of the fluid phase endocytic process is attributed to the binding of PEG-Chol to the cells' plasma membrane inducing a decrease in surface hydrophobicity of the cells, resulting in a marked decrease in the extent of phagocytic ingestion.

Keywords: Liposome; Endocytosis; Macrophage; Poly(ethylene glycol); Drug delivery system

1. Introduction

Liposomes have been studied as a drug delivery system in the last two decades [1]. Most of the applications were based on the ability of the liposomes to be taken up by the RES cells in the liver and spleen [1,2]. However, avoiding the mononuclear phagocyte system (MPS) become a new task since the uptake of liposomes by macrophages may be useful for treatments of leishmaniasis [3,4] or systemic fungal infection [5,6], but become an obstacle to the delivery of drugs to other cells of the body. It is generally advantageous to use large liposomes for the delivery of water soluble drugs since a large aqueous phase will accommodate greater quantities of drugs. Unfortunately, even the most stable large liposomes exhibit brief half-life. An interesting relationship had been observed between liposomal stability and rate of vesicles clearance from the circulation; the more stable the vesicles in presence of serum in vitro, the longer their biological half-life is. This was true for vesicles of all sizes (see [7] for review). On the other hand, it had been shown that cholesterol-containing liposomes are eliminated less rapidly from the blood stream and taken up to a lesser extent by the liver macrophages than the cholesterol-free liposomes [8] and that highly hydrophilic bilayer surface will substantially extend the half-life of liposomes [9]. Indeed, a new generation of evasive liposomes, or stealth liposomes, coated

Abbreviations: Chol, cholesterol; G_{M1}, ganglioside; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; PC, egg yolk phosphatidylcholine; PE, phosphatidylethanolamine; PEG, poly(ethylene glycol); ^{m}PEG-Chol, poly(ethylene glycol) with a M_r (m) conjugated to cholesterol or polyoxyethylene cholesteryl ether; PEG-PE, dioleoyl *N*-(monoethoxy poly(ethylene glycol) succinyl)PE; ^{5000}PEG-PE, PEG-PE with PEG M_r of 5000; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HCMF, Hepes calcium magnesium free buffer; RES, reticuloendothelial system; SRh, sulforhodamine B.

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with chain of polyethylene glycol (PEG), 50 to 250 monomers long, are able to stay in the blood 100-times longer than regular liposomes.

From all these latest studies on stealth liposomes, the molecular mechanism of the liposomes longevity in vivo can be summarized by the two following propositions. Papahadjopoulos and collaborators proposed that steric stabilization of liposomes is responsible for their prolonged circulation time [10]. They suggested that stabilization result from local surface concentration of highly hydrated groups that sterically inhibit both electrostatic and hydrophobic interactions of a variety of blood components at the liposomes surface. However, Cevc and collaborators [11] more recently have concluded that hydrophilicity or presence of steric barriers at the lipid bilayer surface was parameters of secondary importance but that it is the mobility of polar surface barriers in combination with a sufficiently large thickness of this barrier which prevents or slows down the adsorption of macromolecules from the blood.

However, little had been done in vitro on the RES avoiding mechanism of PEG-Chol liposomes and more studies need to be done to give details on the role of PEG size, the amount required and more important, may be, the role of the molecule to which it had been bound, allowing the design of carriers with specific physical-chemistry characteristics and properties.

A long life time in vivo corresponds also to a low binding or uptake of liposomes with cells in vitro. As it had been mentioned earlier, cholesterol is known to extend the life time of liposomes in the blood stream. So it seems interesting to investigate the impact of PEG binding to cholesterol on the in vitro behaviour of liposomes as drugs carriers. Earlier research had shown that multilamellar liposomes containing PEG-Chol with chain length of 50, 100 and 200 meres possess a higher life time in blood and a lower accumulation in liver than uncoated control liposomes [12].

In this present report, we investigated, in vitro, the influence of PEG, with different chain lengths covalently bound to cholesterol in position 3, on the liposome binding and intake by macrophage-like cell line J774 and also its effect on pinocytosis process. Finally, the comparison with the most commonly used PEG derivative ^{5000}PEG-PE had been carried out.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (EPC) was kindly provided from Asahi Kasei (Japan), PS from bovine brain was purchased from Sigma (St. Louis, USA), Cholesterol (Chol) was supplied by Wako Pure Chemical (Japan) and was used after double recrystallization from ethanol. Polyoxyethylene cholesteryl ether (PEG-Chol) with an average of PEG unit number 50, 100 and 200 which have an average molecular weight of about 2200, 4400, 8800, respectively (^{2200}PEG-Chol, ^{4400}PEG-Chol and ^{8800}PEG-Chol), were synthesized by Nikko Chemical (Japan). These surfactants have the poisson distribution of ethylene oxide chain length, but were used without purification. ^{5000}PEG-PE was kindly provided by Theromo Pharmaceutical (Japan). All other chemicals from Wako Pure Chemical (Japan) were of special grade. Water was purified by double distillation in a glass still.

2.2. Cell culture and cell preparation

J774 were grown in monolayer in a humidifier incubator (5% CO_2) at 37°C in 75 cm^2-flask (Nunc) containing 20 ml of Dulbecco's modified Eagle's medium (DMEM: Nissui, Japan) supplemented with 10% fetal bovine serum (FBS: Bio-Whittaker, Maryland, USA), L-glutamine, penicillin and streptomycin (Cosmobio, Japan). Cells were harvested after treatment with Hepes calcium magnesium free buffer (HCMF: NaCl 137 mM, KCl 6 mM, glucose 6.1 mM and Hepes 10 mM; pH 7.4) containing 2% of EDTA for 10 min at 37°C, centrifuged, rinsed with medium, and counted. Cells ($3 \cdot 10^5$) were then plated on 9 cm^2 multi-wells dishes, allowed to recover for 24 h in a humidified incubator at 37°C and used in the following experiments unless mentioned otherwise. In our culture conditions, J774 growth rate is of 2.4 ± 0.3 per 24 h, which means the cells density for the experiments is about $7.2 \cdot 10^5$ per ml.

2.3. Liposome preparation

Multilamellar liposomes were prepared by the extrusion method as follows; lipids in chloroform were mixed in presence of 0.2% (mol/mol) of DiI, dried with a rotary evaporator and kept in vacuo for 2 h. The lipid film was then hydrated with HCMF (pH 7.4) to a final lipid concentration of 5 mM and extruded 5 times through Nuclepore filter 0.2 μm (Millipore, USA) using Amicon 8010 system. Phospholipid concentration was assayed by the Bartlett method [13]. The average size of liposomes, measured by dynamic light scattering using a laser particle analyzer LPA-3100 (Otsuka Electronics, Tokyo, Japan), was 218 ± 10, 185 ± 7 and 180 ± 8 for control liposomes, liposomes containing 5 mol% and 25 mol% of PEG-Chol, respectively, no significant difference could be observed in function of the PEG-Chol length [12].

2.4. Treatment of cells with liposomes and determination of liposome uptake

Liposomes were preincubated for 10 min at 37°C in DMEM-10% FBS, prior to contact with the cells. Cells, plated at $3 \cdot 10^5$ per ml, one day before experiments, were

then incubated after removal of the old medium, in a humidified incubator at 37°C, in the presence of liposomes at the desired concentration and period. Cells were then rapidly cooled down and washed with iced HCMF for 1 min, three times. Cells were then resuspended in TX100 0.2% in HCMF to dissolve the cells and fluorescence was recorded at excitation and emission wavelengths 550 nm and 565 nm, respectively. Experiments were carried out in parallel at 5°C to evaluate adsorption and membrane interaction since no endocytosis occurs below 18°C [14]. Calibration curves of the liposomes fluorescence were drawn to allow direct reading of the lipid uptake from fluorescence measurements.

Cellular viability was tested by the Trypan blue exclusion assay, on cells plated on cover-slip 0.12–0.17 mm (Matsunami Glass, Japan) a day before experiment and observed under microscope over a hematocytometre.

2.5. Effect of PEG-Chol on the incorporation of the fluid phase uptake marker SRh

Cells, plated at $3 \cdot 10^5$ per ml of DMEM-10% FBS, one day before experiments, were then incubated after removal of the old medium, in a humidified incubator at 37°C, with or without liposomes at the desired concentration and period. SRh final concentration was 0.2 mg per ml of medium after dilution with a known amount of diluted liposomes. After incubation, cells were then rapidly cooled down, extensively washed with iced HCMF, and 500 μl of 0.2% TX100 in HCMF was added to dissolve the cells for the spectrophotometric measurements. Fluorescence of SRh was measured at excitation and emission wavelengths 565 nm and 580 nm, respectively.

2.6. Separation of free PEG-Chol from liposomes

Aliquot of the 5 mM 25 mol% ^{8800}PEG-Chol containing liposomes labeled suspension was applied to a Sepharose CL-4B column [14] equilibrated in glucose-free HCMF. Fractions of 15 drops were collected with a Mini Collector SJ-1410 (ATTO, Tokyo, Japan). For each fraction DiI fluorescence was measured along with PEG-Chol content by the Bio-Rad Protein assay [15] and phospholipid concentration by the Bartlett method [13]. When PEG-Chol molecules were not completely incorporated in the liposomes, elution profile shows two peaks, one broad, with fractions containing almost pure PEG-Chol and the other, sharp, containing lipids, PEG-Chol and DiI. The 5 fractions containing the highest concentration of liposomes from two filtrations were mixed, phospholipid content measured and liposomes concentration was adjusted to 100 μM total lipids to compare with the original suspension. Due to the filtration process, liposomes were diluted about 10-fold, implying a dilution by a third of the DMEM-10% FBS medium, this was also done for non-filtrated liposomes. Incubations procedures and cells treatments were done as described above.

2.7. Fluorescence microscopy of cells

Cells ($3 \cdot 10^5$) were platted on sterile cover-slips in 9 cm^2 petri dishes and grown for 24 h in DMEM-10% FBS. Liposomes were incubated for 1 h to 3 h at 37°C or 5°C with the cells, then medium was removed and the cells were rapidly cooled by adding ice-cold HCMF and placing the dishes on ice. Cells were then rinsed three times for 1 min with ice-cold HCCM. In the case of cells incubated at 37°C, to avoid interferences in the observation due to the liposomes adsorbed at the cell surface, cells were chased for 1 h in fresh DMEM-10% FBS. Specimens were observed using an Olympus microscope equipped with a phase contrast and epifluorescence filter set: 535–550 nm band pass filter, 580 nm dichroic mirror and 590 nm long pass filter (objective 40 ×). Exposure time was typically 1 s for phase contrast and 30 s for fluorescence. Photography was taken with Neopan 400 Presto film (Fuji Photo, Japan) and developed as 3200 ASA.

3. Results

3.1. Effect of PEG-Chol on liposome binding

Typical binding curves obtained at 5°C had been observed in function of time, as shown Fig. 1A, with a saturation value depending on the lipid concentration except in the case of liposomes containing only 5 mol% of PEG-Chol where binding is constant for the first 2 h of incubation and sudden increase appears at 4 h and reaches the plateau value obtained for the control liposomes.

In the presence of 25 mol% PEG-Chol, liposome binding is drastically reduced compared to the control liposome. However, the biggest discrepancy, shown Fig. 2A, between 5 mol% and 25 mol% PEG-Chol liposomes is observed by increasing the liposomes concentration from 50 to 250 μM (total 50 to 250 nmol lipids per $7.2 \cdot 10^5$ cells) where binding of 25 mol% PEG-Chol liposomes is not dose dependent.

Moreover, 25 mol% PEG-Chol liposomes binding inhibition is not dependent on the chains length. In contrast, increasing doses of 5 mol% PEG-Chol liposomes will have a higher binding that will depend on the number of meres (Fig. 3). However, increasing the concentration of liposomes in the external medium abolish the effects of the 5 mol% ^{2200}PEG-Chol and ^{4400}PEG-Chol on the binding of liposomes (Fig. 4B) but not ^{8800}PEG-Chol. Binding of PS-liposomes is about 3-fold higher than the control without PS (data not shown).

Microphotographs of the fluorescent liposomes binding to J774 cells are shown on Fig. 5. Pictures were taken in

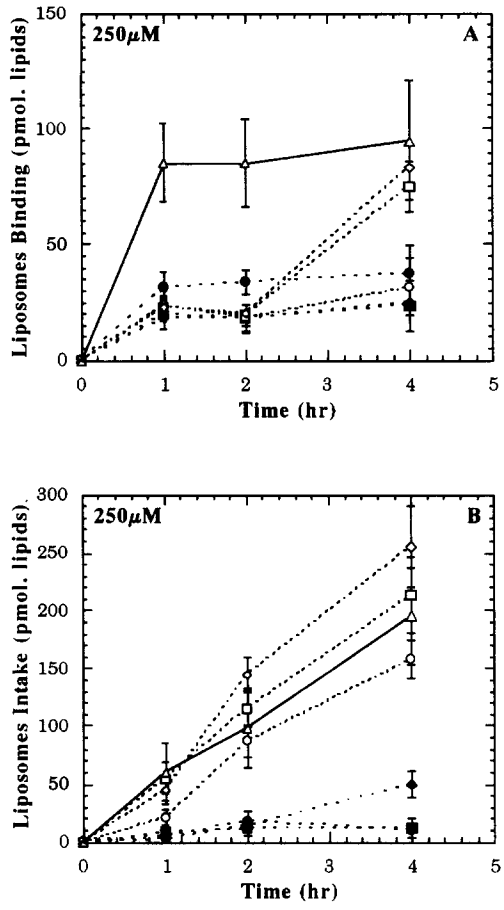

Fig. 1. Kinetics of liposome binding (A) and endocytosis (B) to J774 cells. Liposomes containing various molar amounts of the different PEG-Chol were incubated with J774 cells at 4°C and 37°C in CO_2 incubator. Control liposomes EPC 25 mol% Chol (△) and liposomes composed of EPC containing 5 mol% of ^{8800}PEG-Chol (○), ^{4400}PEG-Chol (□), ^{2200}PEG-Chol (◇) or 25 mol% of ^{8800}PEG-Chol (●), ^{4400}PEG-Chol (■), ^{2200}PEG-Chol (♦) were labeled with 0.2 mol% Dil. All types of liposomes were added at 250 μM lipids concentration to the cells ($\approx 7.2 \cdot 10^5$ cells per Petri dish). Experiments performed at 4°C gave the binding results (A) and endocytosis was calculated from results obtained at 37°C minus those at 4°C (B). Results are expressed in pmol of lipids directly read from the standard curves. Data represent mean ± S.E. for four separates experiments duplicated.

the same conditions of illumination and exposure, and had been printed as well, so direct comparison can be made. One can see that the number of liposomes bound to the cells surface decrease when PEG-coated liposomes are used, indeed, binding of control liposomes (panel A) is higher than 5 mol% and 25 mol% ^{8800}PEG-Chol liposomes (panels B and C, respectively). However, little detail can be seen on these pictures because of the large binding difference with control liposomes as already shown and the chosen printing conditions.

3.2. Effect of PEG-Chol on liposomes endocytosis

Endocytosis is calculated from the data obtained at 37°C, representing the total binding and intake of liposomes by the cells, minus those obtained at 5°C corresponding to the binding only. The effect of the presence of PEG-Chol in the multilamellar liposomes on the endocytosis seems even more interesting. In function of time, the results are presented in Fig. 1B, endocytosis of 25 mol% PEG-Chol liposomes seems delayed, the longer the PEG chain is, the longer time lag is and the higher dose is, the stronger the effect can be seen. This behaviour does not appear in the case of liposomes containing 5 mol% PEG-Chol content. It seems interesting to check a longer incubation time to determine the extension of this phenomenon.

As presented in Fig. 2B in function of the lipid dose after 4 h incubation, one can see that liposomes containing 5 mol% PEG-Chol (with total Chol and PEG-Chol of 25%) does not differ so much from control liposomes.

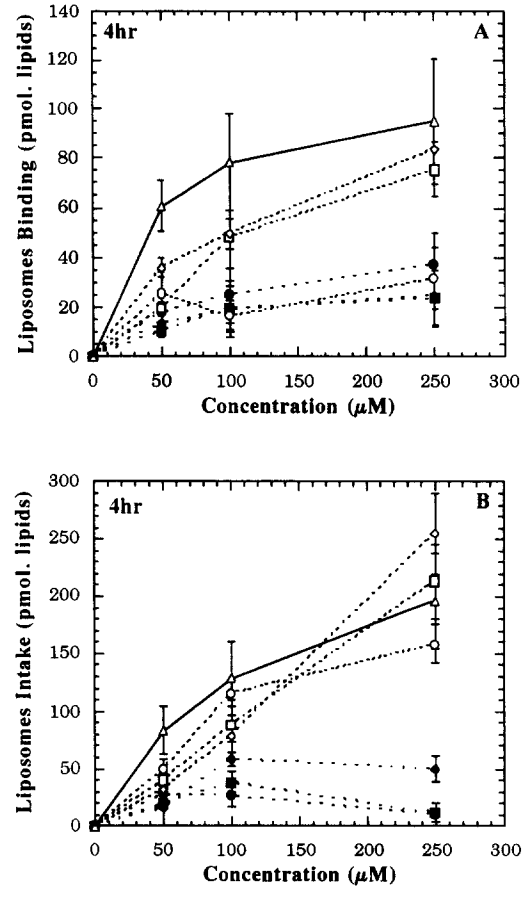

Fig. 2. Liposomes binding and endocytosis in function of lipid concentration. Liposomes containing various molar amounts of the different PEG-Chol were incubated 4 h with J774 cells at 4°C and 37°C in CO_2 incubator. Control liposomes EPC 25 mol% Chol (△) and liposomes composed of EPC containing 5 mol% of ^{8800}PEG-Chol (○), ^{4400}PEG-Chol(□), ^{2200}PEG-Chol (◇) or 25 mol% of ^{8800}PEG-Chol (●), ^{4400}PEG-Chol (■), ^{2200}PEG-Chol(♦) were labeled with 0.2 mol% Dil. All types of liposomes were added at 50, 100 or 250 μM lipids concentration to the cells ($\approx 7.2 \cdot 10^5$ cells per Petri dish). Experiments performed at 4°C gave the binding results (A) and endocytosis was calculated from results obtained at 37°C minus those at 4°C (B). Results are expressed in pmol of lipids directly read from the standard curves. Data represent mean ± S.E. for four separates experiments duplicated.

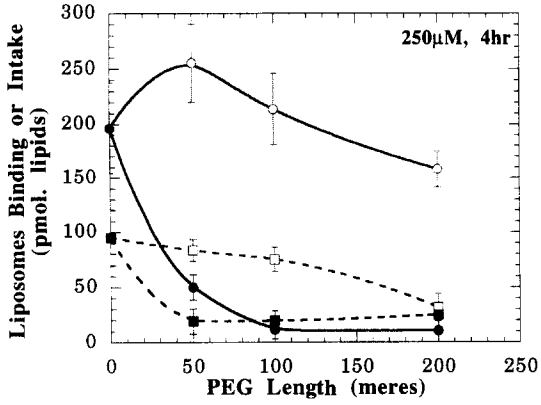

Fig. 3. Liposomes binding and endocytosis in function of PEG length. Liposomes containing various molar amounts of the different PEG-Chol were incubated 4 h with J774 cells at 4°C and 37°C in CO_2 incubator. Liposomes composed of EPC containing 5 mol% of PEG-Chol (○, □) or 25 mol% of PEG-Chol (●, ■) were labeled with 0.2 mol% Dil. All types of liposomes were added at 250 μM lipids concentration to the cells ($\approx 7.2 \cdot 10^5$ cells per Petri dish). Experiments performed at 4°C gave the binding results (□, ■) and endocytosis was calculated from results obtained at 37°C minus those at 4°C (○, ●). Results are expressed in pmol of lipids directly read from the standard curves. Data represent mean ± S.E. for four separates experiments duplicated.

Similar results are obtained for liposomes containing 25 mol% cholesterol, indicating that 5 mol% PEG-Chol does not alter the surface nature of liposomes and it seems that the contribution is owed more to cholesterol than PEG. However, the endocytosis of liposomes drops drastically with the increase of PEG-Chol amount in membrane. Endocytosis of PS-containing liposomes (PC/PS/Chol, 2:1:1) is 3- to 5-times larger than that of control liposomes (data not shown).

Once more at 50 μM total lipid concentration, the effects of PEG chain length on the endocytosis for the two kinds of liposomes are similar (Fig. 4B). But a large discrepancy is arising (see Fig. 3) for long incubation time. Increasing the dose of liposomes shows little effect of the PEG length at 5 mol% PEG content but a complete inhibition was observed for all 25 mol% PEG-Chol liposomes what so ever the PEG length. Moreover, increasing the concentration of liposomes in the external medium abolishes the effects of the 5 mol% PEG-Chol in the liposomes endocytosis process no matter which PEG chain length is used (Fig. 4B).

Microphotographs of the endocytosed liposomes are presented Fig. 6. To avoid any visual interferences from the bound liposomes at the cells surface, samples were chased for 1 h in fresh medium after the 2 h incubation with the liposomes. In this case, the bound liposomes are endocytosed as well. This explained the large difference in the fluorescence intensities between the cells incubated with the control and the 5 mol% ^{8800}PEG-Chol liposomes (panels A and B, respectively) which does not exist in Fig. 1B.

3.3. Effect of free PEG-Chol on liposomes binding and endocytosis

As it had been shown in our previous physical chemistry study on liposomes containing these PEG-Chol derivatives [12], the incorporation of the PEG moiety in the liposomal membrane, with an initial concentration of PEG-Chol at 25 mol%, reached 18, 16 and 14 mol% for PEG unit number 50, 100 and 200, respectively. However, 5 mol% was totally incorporated for all types of PEG-Chol used. Due to its large polar head group, PEG-Chol cannot be incorporated as much as Chol, the larger the head group is, the smaller the incorporation will be.

In this series of experiments, PEG-coated liposomes were separated from free PEG-Chol molecules by gel-

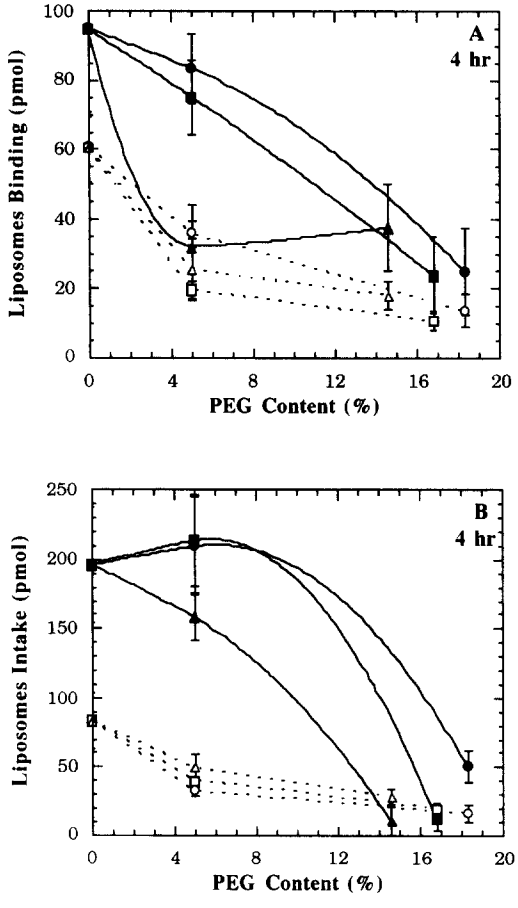

Fig. 4. Liposomes binding and endocytosis in function of PEG content. Liposomes containing various molar amounts of the different PEG-Chol were incubated with J774 cells at 4°C and 37°C in CO_2 incubator for 4 h. Control liposomes EPC 25 mol% Chol and liposomes composed of EPC containing ^{8800}PEG-Chol (○, ●), ^{4400}PEG-Chol(□, ■), ^{2200}PEG-Chol (◇, ◆) were labeled with 0.2 mol% Dil. All types of liposome were added at 50 (open symbols) or 250 μM (closed symbols) lipid concentration to the cells ($\approx 7.2 \cdot 10^5$ cells per Petri dish). Experiments performed at 4°C gave the binding results (A) and endocytosis was calculated from results obtained at 37°C minus those at 4°C (B). Results are expressed in pmol of lipids directly read from the standard curves. Data represent mean ± S.E. for four separates experiments duplicated.

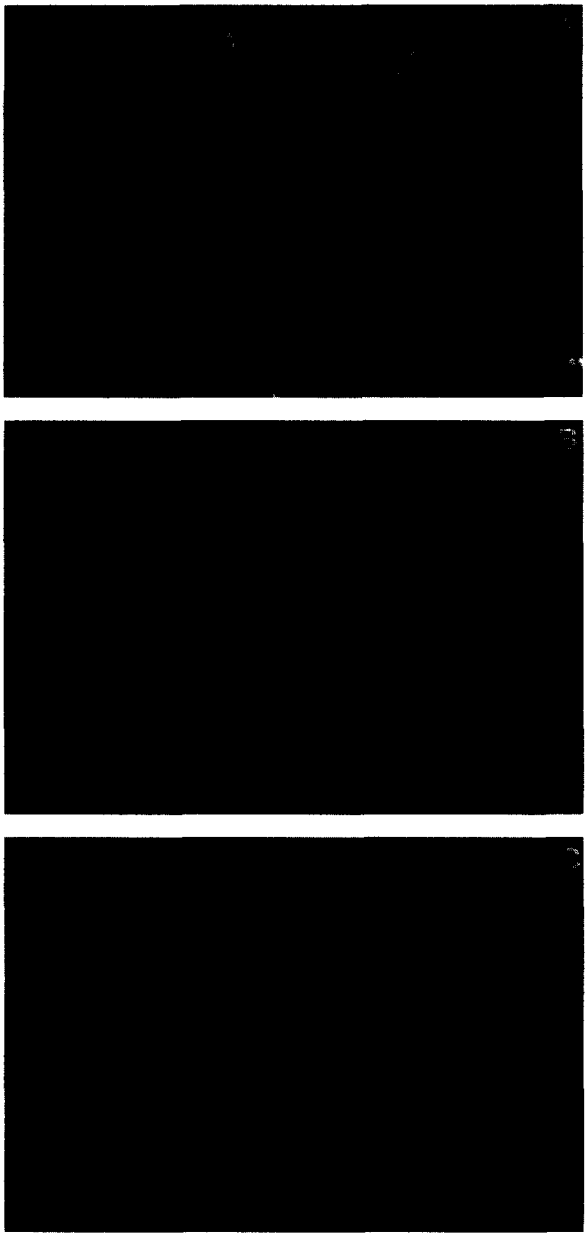

Fig. 5. Fluorescent microphotographs of liposomes bound to J774 cells. Cells were treated with Control liposomes EPC 25 mol% Chol (A) and liposomes composed of EPC containing 5 mol% (B) or 25 mol% ^{8800}PEG-Chol (C) at 100 μM, labeled with 0.2 mol% Dil, for 2 h at 5°C. Pictures were taken in the same conditions of illumination-exposure and had been printed as well, to allow direct comparison. Field enhancement $\times$1540.

filtration on Sepharose CL-4B column [15]. However, to overcome the large liposomes dilution caused by the filtration, non-filtrated liposomes had been diluted in the same extent resulting in a dilution of about a third of the DMEM–10% FBS and the values obtained in these conditions for binding and endocytosis were surprisingly reduced by about 30%. One can see from Fig. 7, that presence of free ^{8800}PEG-Chol in micelles and monomers forms, drastically inhibit both events. After 4 h incubation, the presence of free-PEG-Chol had reduce by 2.4 the binding and about 4 the intake of liposomes. Similar results had been obtained if we add free ^{8800}PEG-Chol at the known concentration to control liposomes in DMEM-10% FBS (data not shown). Moreover, addition of free PEG-Chol affects the control liposomes binding which depends little on the PEG-Chol derivative but more on the concentration, increase of the liposomes concentration and free surfactant reduce the effect, binding 1–4 h was 50–70%, 60–75% or 100% of control for the liposomes concentration of 50 μM, 100 μM and 250 μM, respectively (data not shown). This means that the presence of

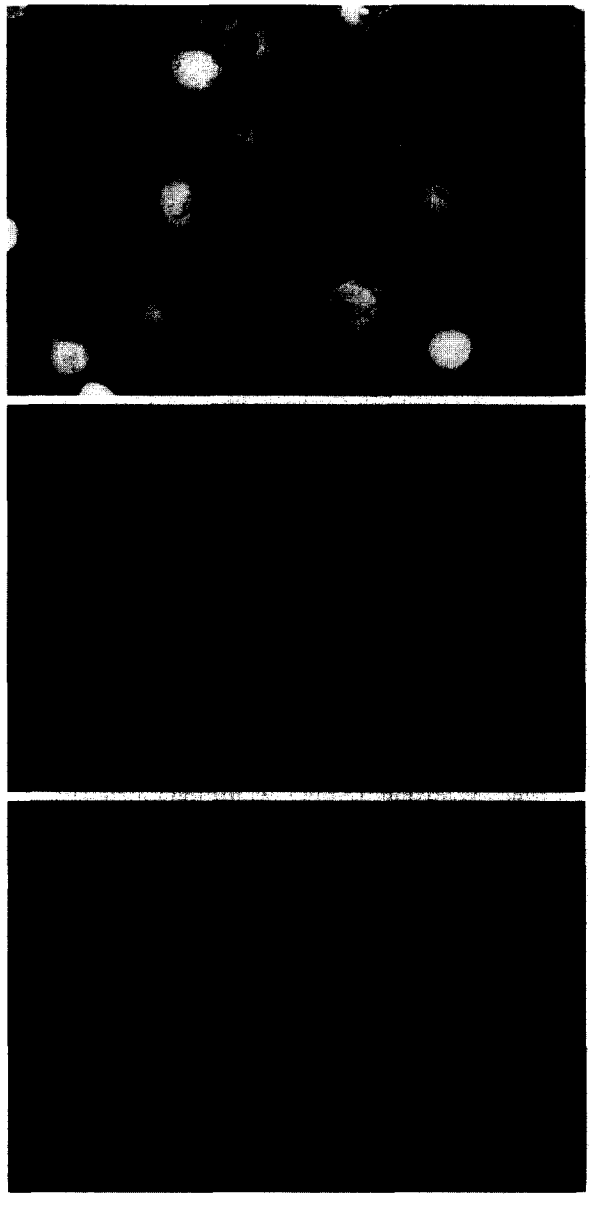

Fig. 6. Fluorescent microphotographs of liposomes endocytosed by J774 cells. Cells were treated with Control liposomes EPC 25 mol% Chol (A) and liposomes composed of EPC containing 5 mol% (B) or 25 mol% ^{8800}PEG-Chol (C) at 100 μM, labeled with 0.2 mol% Dil, for 2 h at 37°C and chased for 1 h in fresh medium liposomes-free. Pictures were taken in the same conditions of illumination-exposure and had been printed as well, to allow direct comparison. Field enhancement $\times$1540.

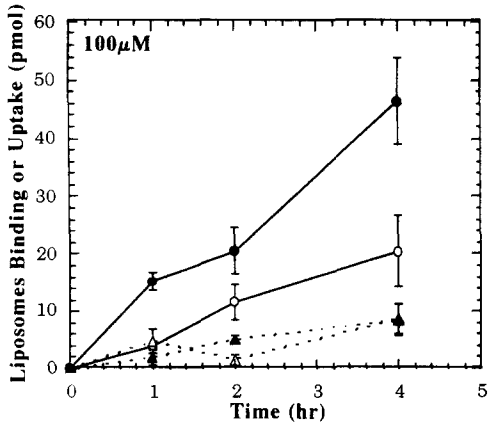

Fig. 7. Effect of free PEG on liposomes binding and endocytosis. Liposomes containing 25 mol% of ^{8800}PEG-Chol, labeled with 0.2 mol% Dil, were filtrated on the a Sepharose CL-4B column to separate free PEG-Chol from liposomes, due to this procedure liposomes were diluted and by consequences the incubation medium too. Filtrated (○, ●) and unfiltrated (△, ▲) liposomes were incubated with J774 cells in DMEM 10% FBS medium at 4°C and 37°C in CO_2 incubator. Liposomes were added at 100 μM lipids concentration to the cells ($\approx 7.2 \cdot 10^5$ cells per Petri dish). Experiments performed at 4°C gave the binding results (open symbols) and endocytosis was calculated from results obtained at 37°C minus those at 4°C (closed symbols). Results are expressed in pmol of lipids directly read from the standard curves. Data represent mean ± S.E. for three separates experiments duplicated.

free ^{8800}PEG-Chol had an effect on both binding and endocytic process beyond the experimental differences in the procedures.

3.4. Effect of PEG-Chol on pinocytosis process and on the cell viability

To assess a direct effect of free PEG-Chol on the endocytic process, we had used a soluble marker sulforhodamine (SRh) which allows to quantify the extent of pinocytosis in the cells. Results, expressed in percent of SRh uptake in untreated cells, are partially presented in Table 1. The free PEG-Chol concentration used had been calculated from the results obtained after liposomes filtration which gave us the percentage of PEG-Chol incorporation in liposomes. For example, with a liposome concentration of 250 μM, we have 31.8, 30.4 and 23.3 μM of free ^{8800}PEG-Chol, ^{4400}PEG-Chol and ^{2200}PEG-Chol, respectively, in the external medium. Depending on its concentration, free PEG-Chol has an effect on the SRh pinocytosis, which is similar for the three types of PEG-Chol molecules. Saturation is observed at about 35 μM, reaching a value of 45% of control, corresponding to an inhibition of 55% of the process (data not shown). Increasing the external concentration of PEG-Chol does not change this maximum value. Poly(ethylene glycol) with M_r 2000 and 6000 had no effect on the SRh pinocytosis on the same range of concentration (data not shown).

As we can see Table 1, control liposomes containing 25 mol% of Chol have no effect on the SRh pinocytosis, same as for liposomes containing 25 mol% of PS or smaller Chol content (data not shown). However, the presence of PEG-Chol in the liposomal membrane alters slightly the uptake of SRh since we can observe an effect for liposomes containing 5 mol% of PEG-Chol even if no free PEG-Chol had been detected. More interesting are the results obtained with liposomes containing a higher molar ratio of PEG-Chol. Indeed, a large inhibition of the SRh pinocytosis, from 25% up to 55% of control, can be observed with increase of the liposome concentration. Similar results were observed using control liposomes, by adding free PEG-Chol in such amount that it corresponds to the concentration of free PEG-Chol measured for liposomes containing 25 mol% PEG-Chol.

Toxicity of the surfactant had been shown in vitro for small chain length PEG-Chol (25 to 50 meres long) which have hemolytic activity toward red blood cells [16,17]. Maximum hemolytic activity had been observed for

Table 1
Effect of PEG-Chol on sulforhodamine fluid phase endocytosis

Liposomes	Sulforhodamine uptake (% of control)		
liposomes concentration:	50 μM	100 μM	250 μM
Control 25 mol% Chol	94.2 ± 10.7	100.2 ± 5.2	98.4 ± 2.5
5 mol% ^{8800}PEG-Chol	83.5 ± 10.7	88.6 ± 7.0	81.3 ± 10.9
25 mol% ^{8800}PEG-Chol	74.3 ± 8.8	61.2 ± 10.1	52.3 ± 9.6
Control + ^{8800}PEG-Chol	64.2 ± 4.1	59.0 ± 5.2	44.8 ± 3.2
5 mol% ^{4400}PEG-Chol	86.0 ± 10.6	80.4 ± 10.7	68.7 ± 8.0
25 mol% ^{4400}PEG-Chol	66.7 ± 17.7	53.0 ± 11.0	45.4 ± 5.6
Control + ^{4400}PEG-Chol	63.7 ± 4.2	63.5 ± 7.0	48.0 ± 3.5
5 mol% ^{2200}PEG-Chol	90.4 ± 5.3	85.2 ± 20.3	87.2 ± 11.0
25 mol% ^{2200}PEG-Chol	72.4 ± 10.5	46.8 ± 6.1	47.2 ± 3.7
5 mol% ^{5000}PEG-PE	104.1 ± 2.5	94.7 ± 2.4	95.7 ± 2.5
25 mol% ^{5000}PEG-PE	87.6 ± 2.8	73.4 ± 7.0	75.4 ± 2.5

Cells were incubated at 37°C for 4 h in presence of SRh 0.2 mg in DMEM–10% FBS containing various concentrations of liposomes. Results are expressed in % of control cells untreated. Data represent mean ± S.E. for three separate experiments duplicated.

derivatives with 25–30 oxyethylene units. These effects were drastically reduced for the derivative containing 50 oxyethylene units (^{2200}PEG-Chol), since the surfactant concentration to induce 50% hemolysis was higher by almost 2 order magnitude.

Experiments had been performed with the cells grown on coverslip allowing an easy counting under hematocytometre. Control cells without any treatment have usually a viability of 95–96% of total cells. In presence of liposomes containing PEG-Chol slight toxicity appears at high liposomes concentration (250 μM) reaching, at most, a viability of 80% for liposomes containing ^{8800}PEG-Chol and the largest amount of free surfactant. However, these results cannot explain the strong effect of PEG-Chol on the pinocytic process. In our conditions, long chain PEG-Chol does not appear to be drastically toxic as the short chain was [17].

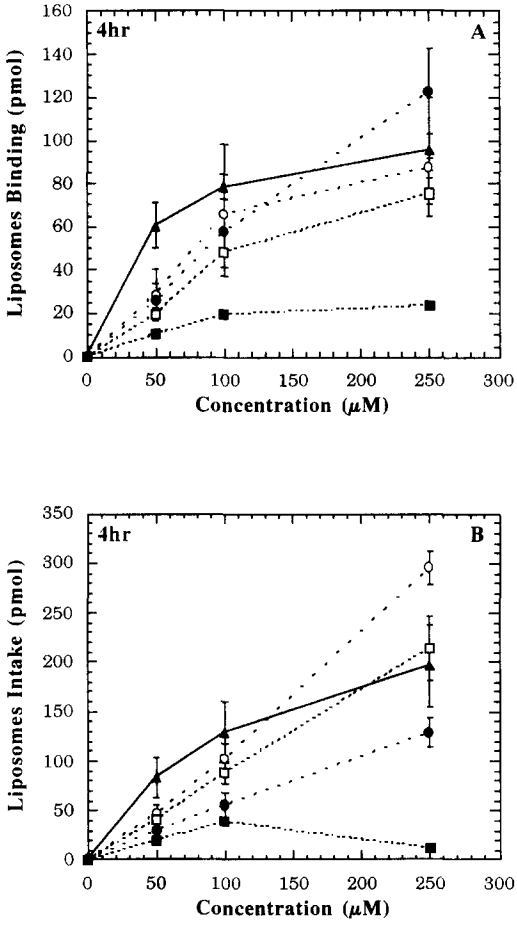

Fig. 8. Comparative effect of PEG-Chol and PEG-PE on the binding and endocytosis. Liposomes containing various molar amounts of the different ^{4400}PEG-Chol (□, ■) or ^{5000}PEG-PE (○, ●) were incubated 4 h with J774 cells at 4°C and 37°C in CO_2 incubator. Control liposomes EPC 25% Chol (▲) and liposomes composed of EPC containing 5 mol% (open symbols) or 25 mol% (closed symbols) of PEG derivative were labeled with 0.2 mol% Dil. All types of liposomes were added at 50, 100 or 250 μM lipids concentration to the cells ($\approx 7.2 \cdot 10^5$ cells per Petri dish). Experiments performed at 4°C gave the binding results (A) and endocytosis was calculated from results obtained at 37°C minus those at 4°C (B). Results are expressed in pmol of lipids directly read from the standard curves. Data represent mean ± S.E. for three separates experiments duplicated.

3.5. Comparative effect of PEG-Chol and PEG-PE on the binding and intake of liposomes

As it had so far been said, most of the study on the effect of PEG coating on liposomes in vivo and in vitro had been done with derivatives obtained with a PEG binding to phosphatidylethanolamine. The ^{5000}PEG-PE, with a M_r of 5000, corresponds to a PEG length of about 100 monomers that why we will compare the results observed with ^{4400}PEG-Chol liposomes.

As far as binding experiments are concerned, one can see on Fig. 8A that ^{4400}PEG-Chol has a strongest effect at high molar content. Liposomes containing 5 mol% or 25 mol% ^{5000}PEG-PE behave similarly while the difference between liposomes containing 5 mol% and 25 mol% of ^{4400}PEG-Chol is really strong.

If we expressed the results as percent of the control liposomes, after 4 h incubation, ^{5000}PEG-PE and ^{4400}PEG-Chol behave similarly at 5 mol% but greater differences arise at higher molar content that is ^{5000}PEG-PE representing 55–100% and ^{4400}PEG-Chol between 20–25% of control liposomes.

In Fig. 8B, we can see the liposomes behaviour in function of the lipid concentration. Increasing the concentration means a decrease of the effect of ^{5000}PEG-PE and ^{4400}PEG-Chol on liposomes endocytosis. However, the presence of ^{4400}PEG-Chol 25 mol% inhibits drastically the endocytosis whatever the liposomes concentration is. As for endocytosis in function of time, whatever the concentration is, the amount of endocytosed liposomes lies in the order 5 mol% ^{5000}PEG-PE > 5 mol% ^{4400}PEG-Chol > 25 mol% ^{5000}PEG-PE > 25 mol% ^{4400}PEG-Chol. No inhibitory effect of the PEG moiety is observed on the endocytic process for low molar content liposomes and high concentration.

Finally, as we show in Table 1, PEG-Chol liposomes effect on SRh pinocytosis does not depend on the PEG moiety type. We had observed that 25 mol% of ^{5000}PEG-PE had the same effect than 5 mol% of ^{4400}PEG-Chol. We could not observe a drastic reduction of pinocytosis for ^{5000}PEG-PE as shown for PEG-Chol.

4. Discussion

As it had been shown by Allen and coworker [18], there is a direct correlation between the uptake of liposomes by cultured murine bone marrow macrophages and the uptake of liposomes by the RES in vivo in mice. Using liposomes containing PEG-Chol derivatives that indeed reduced the

RES uptake in rats [12] we had been able to show drastic effects on their endocytosis as well as on their binding toward macrophages J774 cells. Efforts had been made to elucidate the influence of the PEG chain length and the molar content of the PEG-Chol on these processes. As for in vivo results, comparison of the distribution of multilamellar vesicles (70–100 nm) of various compositions of Chol and EPC or DPPC or DSPC containing PG or PS and/or containing G_{M1} or PI or PEG-PE led to a hypothesis on the molecular origin of the extended circulation time [10]. The model of a polymer extending over the liposome surface can explain the finding that increased surface charge does not increase the blood clearance rates. However, this qualitative model cannot explain the size dependence of the clearance of sterically stabilized liposomes.

In vitro, experiments differ greatly, indeed, increasing the liposomes concentration emphasizes the behaviour differences between low and high PEG-Chol content liposomes with the disappearance of the effect due to the presence of 5 mol% PEG-Chol except in the case of ^{8800}PEG-Chol. Actually, increasing the concentration of liposomes containing ^{8800}PEG-Chol emphasizes the differences between low and high PEG-Chol molar content. Both binding and endocytosis of the liposomes are sensitive to the presence of PEG-Chol, indeed, a reduction of the binding may be directly related to a decrease of liposomes intake if the internalization is not due to the pinocytic process but more related to the adsorptive endocytosis. Also, looking at the results obtained for liposomes containing 5 mol% of ^{8800}PEG-Chol, one can see that binding represents only 30% of the value obtained for control and the intake 75%. The large discrepancy between these two values seems to reflect the role of the pinocytic process. However, it had been shown that in the case of liposomes PC/Chol (2:1) with a diameter range between 80 and 110 nm, an intake of these liposomes via fluid-phase pinocytosis in J774 was of little contribution since the rate of uptake was 10-fold higher than that of fluid phase pinocytosis [19] and that the overall rate of uptake was dictated by the binding stage. The differences in our results for control liposomes PC/Chol (3:1) or PC/PS/Chol (2:1:1) between the two studies may be due to the significant difference in size of liposomes, the slight discrepancy in the lipid composition and at last but not at least, the presence of 10% serum in the external medium during all our incubations with cells. Another study by the same authors [20] tries to elucidate the recognition of different liposome types by J774 and CV1 cells. The large spectrum of liposomes types containing negatively charged phospholipids or G_{M1} or PEG-PE allows the authors to determine different mechanisms of liposomes uptake for the two cell lines. Our results obtained with control and PS or PEG-PE-containing liposomes are in good agreement with this study taking in account the presence of 10% FBS in our experiments.

The presence of PEG-Chol in the liposomal membrane inhibits the binding of liposomes to their receptors as PEG-PE and major contribution of electrostatic interaction is unlikely. Two major factors are controlling the PEG-Chol liposomes binding to the cell surface. The surface properties of the liposomes changed in presence of PEG-Chol in the bilayer. The presence of free-PEG-Chol in the medium through its interaction with cells can reduce the binding. The modification of the cell membrane by PEG-Chol is unknown at the present time but seems to be a major factor in controlling both binding and endocytosis. Comparison with the mostly used PEG derivatives, allows us to point out the more powerful action of PEG-Chol derivatives on the reduction of uptake by macrophage that may be directly related on the higher blood life time. The main reason of these big differences in the effect must be due to the difference in anchor group namely Chol or PE and the difference in their physical-chemistry properties. Indeed, the most striking point in our study is the direct effect of PEG-Chol on the pinocytic process. As mentioned above, the uptake of fluid phase marker can be inhibited up to 50% of the uptake of SRh. Moreover, binding of control liposomes is also affected by the presence of added free PEG-Chol. Adverse effects had been shown with the surfactant Pluronic F-68 which directly affect the pinocytic uptake by cultured fibroblast since a supplementation of Pluronic at 0.05% (w/v) enhances the incorporation of 2-deoxyglucose and amino acid [21]. Poly(ethylene glycol) molecules, which do not bind to the membrane in our conditions [22], had no effect on the pinocytic process. However, it had been shown a detergent-like properties of poly(ethylene glycol)s (1000 and 6000) on small unilamellar vesicles at high concentration, 30% w/v [23]. These observations led us to believe that the surfactants interact with the cells and are incorporated in the plasma membrane inducing changes in cells responses. Indeed, the presence of PEG-Chol at the cell surface may introduce a change in the surface hydrophilicity leading to a decrease in endocytic process as those accounted for lectins such as phytohemagglutinin or concanavalin A which causes a decrease in surface hydrophobicity of the phagocytes themselves and consequently a marked decrease in the extent of phagocytic ingestion [24]. Another hypothesis may be related to a more physical approach, actually the decrease of phagocytosis in some cases appears to be due to the suppression of pseudopods formation and an increase of tendency for the cells to assume a spherocytic shape. Such a shape change and particularly the reduced ability to form pseudopods will give rise to an enhanced electrostatic repulsion between the both negatively charged phagocyte and bacteria [24]. However, this second point may not be involved in our case since the liposomes size does not induce the phagocytic process but seems more related to an endocytic one. It is worth to say that preliminary experiments indicate that larger liposomes with a diameter of about 1.5 μm containing PEG-Chol show no increase of intake by the cells comparing to small lipo-

somes while control liposomes were taken up to 10 times more than the smaller ones. This is in good agreement with the study of the effect of amphipathic polymeric surfactant F108 and T908 [25]. The authors show that phagocytosis of polystyrene particles is decreased when coated with poloxamer F108 or poloxamine T908. In addition, suppression of the phagocytic activity was observed when cells were pretreated with surfactant and then challenged with non-coated particles without affecting integrity, viability or functional state of the cell. Authors speculate that the surfactants interact with the cell membrane in a manner that mimics the interaction of hydrophylic surfaces with particulates in general and that steric constraints resulting from either repulsive charge interactions or failure of the particle to interact with surfactant-masked domains of the cell surface were plausible explanations for their results. This, indeed, may be also true for the PEG-Chol effect on such process.

Mechanisms for the decrease in uptake of sterically stabilized liposomes had been proposed too. They are related either to a decrease of opsonin binding on the liposome surface leading to a decrease of endocytosis or the presence of the suppressive substances represented by dysopsonins that will in turn suppress the liposome uptake via the C3 and the Fc receptor [26].

The mechanisms of pinocytosis of fluid phase markers and receptor-mediated endocytosis are basically different since the later involved clathrin cluster and not the former [27]. It will be of great interest to check the effects of PEG-Chol molecules on the receptor-mediated endocytosis process, that is, whether these surfactants inhibit the process or not. Finally, the comparison between in vivo experiments and in vitro ones, revealed that the long life of these surface-modified liposomes should be due to the reduced endocytosis and pinocytosis by free and liposome-incorporated PEG-Chol molecules.

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References

[1] Gregoriadis, G. (1988) Liposomes as Drug Carriers, Recent Trends and Progress (Gregoriadis, G., ed.), Wiley, Chichester.
[2] Hwang, K.J. (1987) in Liposomes: From Biophysics to Therapeutics (Ostro, M.J., ed.) pp 109–156, Marcel Dekker, New York.
[3] Alving, C.R. (1983) Pharmac. Ther. 22, 407–424.
[4] Oliaro, P.L. (1993) Parasitol. Today 9, 323–328.
[5] Bolard, J. (1989) in Bioactive Metabolites from Microorganism (Bushel, M.E. and Gräfe, U., eds.), pp. 151–159, Elsevier.
[6] Legrand, P., Vertut-Doï, A. and Bolard, J. (1993) Biomat. Art. Cells Immob. Biotech. 21, 407–413.
[7] Gregoriadis, G. (1991) J. Antimicrob. Chemother. 28, 39–48.
[8] Rocrdrink, F.H., Regts, J., Handel, T., Sullivan, S.M., Baldeschwieler, J.D. and Scherphof, G.L. (1989) Biochim. Biophys. Acta 980, 234–240.
[9] Lasic, D.D. (1993) in Liposomes: From Physics to Applications (Lasic, D.D., ed.), Elsevier, Amsterdam.
[10] Lasic, D.D., Martin, F.J., Gabizon, A., Huang, S.K. and Papahadjopoulos D. (1991) Biochim. Biophys. Acta 1070, 187–192.
[11] Blume, G. and Cevc, G. (1993) Biochim. Biophys. Acta 1146, 157–168.
[12] Ishiwata, H., Vertut-Doï, A. and Miyajima, K. (1995) Chem. Pharm. Bull. 43, 1005–1011.
[13] Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468.
[14] Besterman, J.M. and Low, R.B. (1983) Biochem. J. 210, 1–13.
[15] Allen, T.M., Hansen, C., Martin, F., Redemann, C., Yau-Young, A. (1991) Biochim. Biophys. Acta 1066, 29–36.
[16] Tasset, C., Goethals, F., Préat, V. and Roland, M. (1990) Int. J. Pharmaceut. 58, 41–48.
[17] Miyajima, K., Baba, T. and Nakagaki, M. (1987) Coll. Polym. Sci. 265, 943–949.
[18] Allen, T.M., Austin, G.A., Chonn, A., Lin, L. and Lee, K.C. (1991) Biochim. Biophys. Acta 1061, 56–64.
[19] Lee, K.D., Nir, S. and Papahadjopoulos, D. (1993) Biochemistry 32, 889–899.
[20] Lee, K.D., Hong, K. and Papahadjopoulos, D. (1992) Biochim. Biophys. Acta 1103, 185–197.
[21] Cawrse, N., De Pomerai, D.I., Lowe, K.C. (1990) J. Pharm. Pharmacol. 42, 153P.
[22] Lentz, B.R. (1994) Chem. Phys. Lipids 73, 91–106.
[23] Sáez, R., Alonso, A., Villenea, A. and Goñi F.M. (1982) FEBS Lett. 137, 323–326.
[24] Absolom, D.R. (1986) Methods Enzymol. 132, 281–318.
[25] Watrous-Peltier, N., Uhl, J., Stell, L. and Merisko-Liversidge, E. (1992) Pharm. Res. 9, 1177–1183.
[26] Allen, T.M. (1994) Adv. Drug Del. 13, 285–309.
[27] Hewlett, L.J., Prescott, A.R. and Watts, C. (1994) J. Cell Biol. 124, 689–703.