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Design of sterile muramyldipeptide-containing oligolamellar liposomes suitable for i.v. injection. Effect of liposome size and lipid composition on their ability to render peritoneal macrophages antitumoral

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Summary

The water-soluble immunomodulator muramyldipeptide has been encapsulated in oligolamellar liposomes (mean diameter 200 nm) of various lipid compositions. These liposomes were sterilized by Millipore filtration without loss of entrapped solute and are therefore suitable for i.v. administration. The amount of encapsulated MDP increased when the volume of aqueous phase (containing a given MDP concentration) used for hydrating the phospholipid film increased and this did not vary for at least 15 days when the liposomes were kept at 4°C.

The presence of cholesterol did not modify the encapsulation results but prevented the leakage of encapsulated water-soluble solute in serum-containing media.

The internalization of various types of liposomes by murine peritoneal macrophages has been studied in order to define the best conditions for activating macrophages. For a given lipid composition, sterile oligolamellar vesicles (suitable for i.v. injection) were more efficient than multilamellar vesicles in rendering macrophages capable of inhibiting the growth of a syngeneic tumor, particularly when these vesicles contained muramyldipeptide in their aqueous space and cholesterol in their lipidic phase.

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Introduction

The treatment of neoplastic diseases is still often unsuccessful because of the appearance of metastases characterized by a wide variability in their susceptibility to conventional therapy (Poste and Fidler, 1980).

There is increasing evidence to suggest an important role for macrophages in the control of tumor cell growth regardless of their phenotypic diversity (Hibbs et al., 1980). Normal macrophages can be activated to become tumoricidal by lymphokines (Cohn, 1978), whole microorganisms and their products such as endotoxin (Taffet et al., 1981) as well as some synthetic compounds such as muramylpeptides (Matter, 1979) hopefully devoid of a number of undesirable side-effects. Probably because of the rapid clearance of muramyldipeptide from the blood in vivo (Parant et al., 1979) and the absence of cell surface receptors for MDP on some types of macrophages (Tenu et al., 1982), the encapsulation of MDP derivatives in liposomes can strongly increase their ability to render macrophages tumoricidal both in vitro (Sone and Fidler, 1981; Sone and Tsubura, 1982) and in vivo (Fidler et al., 1982; Schroit et al., 1983).

In a pioneering work, Fidler et al. (1981) have shown that liposome-encapsulated MDP could efficiently activate macrophages in vivo and eradicate spontaneous pulmonary metastases of syngeneic melanoma in mice. However, relatively little work was done in order to correlate the parameters of liposome–macrophage interaction with their ability to render macrophages tumoricidal. In this field, Mehta et al. (1982) examined the uptake and processing of a liposome-encapsulated muramyldipeptide derivative by human monocytes.

In this study we focused on the preparation and conservation of sterile small oligolamellar liposomes of defined size and controlled MDP content suitable for i.v. injection. We studied their ability to activate macrophages to become antitumoral taking into account the effect of vesicle size and the effect of cholesterol which prevents the destabilizing effect of serum on the liposomal membrane. As MLV are easier to prepare than sonicated vesicles and have been studied in our laboratories for a long period, we have used the results obtained with these liposomes as preliminary experiments in the design of active sterile oligolamellar liposomes.

Materials and Methods

Liposome preparation

Reagents

The lipids used in these studies were egg phosphatidylcholine from Koch-Light Laboratories (Sochibo, Boulogne-Billancourt, France), distearoylphosphatidylcholine (from Calbiochem-Hoechst-Behring, France) and beef brain phosphatidylserine from Sigma Chemicals (Saint Louis, MO, U.S.A.), the cholesterol used was purchased from Prolabo (Paris, France) and was of analytical grade. The absence of lysolecithin in egg phosphatidylcholine was checked by TLC.

N-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP) was purchased from Choay-Chimie, Montrouge, France. 6-Carboxyfluorescein was provided by Kodak (Touzart et Matignon, Vitry, France) and used without further purification.

Preparation of large multilamellar liposomes

The lipids were dissolved in anhydrous chloroform at a concentration of 13 mg/ml or 30 mg/ml. In some preparations, cholesterol was added in appropriate quantities at this stage; the organic solution was placed in a round-bottom flask. The molar ratios were: DSPC/PS/chol 7:3:10; PC/PS/chol 7:3:0 or 7:3:3; DSPC/PS 7:3; DSPC/chol 7:5.

After rotary evaporation to dryness for half-an-hour at 25°C under partial vacuum, an appropriate volume of PBS (0.01 M) or MDP at a suitable concentration in PBS was added. Liposomes were obtained by vortexing, hand-shaking or rotary-shaking at appropriate temperature (25°C when PC was used and 60°C when DSPC was used). Liposomes were allowed to swell at 4°C overnight. Then liposomes were separated from the free solute by repeated centrifugation at 24,150 *g* for 20 min at 4°C. After four centrifugations, the resultant pellet, free of non-encapsulated solute, was resuspended in a suitable volume of PBS.

Preparation of oligolamellar liposomes

Oligolamellar liposomes were prepared by sonicating large multilamellar liposomes obtained by the standard method. Sonication was carried out with a Branson sonifier B30 probe for 50 s (25 s twice with a 30 s cooling period) under nitrogen and in an ice bath to avoid phospholipid degradation.

The size of the liposomes was measured with a Nanosizer (Coultronics, Margency, France). After sonication the size could vary slightly but the average diameter was 0.2 μm. Sonicated liposomes were separated from the excess of free solute by gel filtration on an Ultrogel A6 column (IBF reactifs, Villeneuve-la-Garenne, France). Elution was carried out with PBS 0.01 M, pH 7.2 and liposomes, eluted in the void volume, were identified by the presence of turbidity and collected in several fractions. Liposome size was again verified with a Nanosizer (Coultronics).

Sterilization of oligolamellar liposomes

Preparation of sterile liposomes below 0.2 μm in diameter has been attempted by sterilizing filtration. We used a Millipore Swinnex Cell (Millipore S.A., Velizy, France) with polycarbonate filters. The sterilizing filtration was performed through a 0.2 μm (pore diameter) filter under nitrogen pressure of 2×10^6 Pa and was preceded by a passage of the liposomes through a 0.45 μm (pore diameter) filter.

Determination of encapsulated MDP and phospholipids

MDP determination within liposomes was achieved by using a HPLC method (Postaire et al., submitted). The stationary phase was a Microbondapak C-18 Waters column, while the eluent consisted of phosphate buffer/methanol (93/7) supplied at a flow rate of 2 ml/min under a pressure of 138×10^6 Pa. Under these conditions, the liposomes are destroyed and encapsulated MDP can be detected. The detection

was performed with a UV spectrophotometer at 218 nm. The internal standard used was sodium acetate (10 mg/ml); the integrator (Schimadzu CR1B) was calibrated with a MDP solution at 100 μg/ml. The two anomers of MDP characterized by their retention time are sequentially eluted (Halls et al., 1980). No interference of the phospholipid fragments with the HPLC method was observed. Phospholipids in liposomes were determined either by adding a trace amount of radioactive lipid or by the enzymatic method of Takayama et al. (1977) using a phospholipid B-test (Bio-Lyon, Dardilly, France). No interference with the colorimetric reaction by MDP was detected.

Phospholipid integrity was checked by TLC using the eluent system: $CHCl_3/MeOH/CH_3COOH/H_2O$ (100 : 60 : 25 : 10). The detection of the spots was performed by spraying with vanillin sulphate followed by 10 min at 100°C.

Permeability and stability measurements

The technique of Weinstein et al. (1977) was used. The fluorescent marker carboxyfluorescein (100 mM in PBS) was entrapped within the aqueous space of liposomes and non-encapsulated solute was removed by centrifugation or gel filtration as appropriate. Since carboxyfluorescein is self-quenching at this concentration, a newly prepared liposome preparation gives only a low quantum yield of fluorescence. This is increased when carboxyfluorescein leaks from liposomes and is diluted in the surrounding medium, or when liposomes are lyzed by detergent.

The percentage of quenching is defined as $Q = 100\ (1 - I/I_0)$ (Barbet, 1982). In this formula I stands for the fluorescence intensity of the self-quenched liposomes suspension and I_0 for the fluorescence intensity after the addition of Triton X-100 (0.1% final concentration) which induces complete lysis. Continuous monitoring of carboxyfluorescein leakage from liposomes in a given medium was carried out with a Jobin-Yvon JY 3 spectrofluorimeter (Longjumeau, France), equipped with a thermostatic cuvette, a home-made magnetic stirring device and a chart recorder (Sefram, Paris, France) $\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm.

Aqueous space volume, encapsulation ratio and entrapment efficiency determination

These parameters were measured either by determining the MDP content by HPLC and the phospholipids by an enzymatic method (method used for PC/PS and PC/PS/chol liposomes) or by the classical method using double-labelled liposomes (method used for DSPC/PS and DSPC/PS/chol liposomes). [^{3}H]Sucrose (from Amersham, 9.8 Ci/mmol, 1 mCi/ml) was diluted with unlabelled sucrose (from Merck) in PBS so that the final specific radioactivity was 0.19 Ci/mmol (sucrose concentration = 151 μM, 0.029 μCi/μl).

L-Alpha-dipalmitoyl-1-[^{14}C]phosphatidylcholine from NEN (0.01 mCi/mmol) was added to phospholipids in the chloroform solution (0.043 μCi/mg of phospholipid) as a tracer.

The dry film of [^{14}C]-labelled phospholipids was hydrated by using the radioactive sucrose solution as aqueous phase. After extensive washing by centrfugation (until the supernatant was free of radioactivity), the ^{3}H and ^{14}C radioactivities retained to the pellet allowed us to calculate an encapsulated volume of the aqueous phase of (2.4 $\pm$ 0.1 μl)/mg phospholipids.

In vitro experiments

Mice

Female (DBA/2 × C57BL/6) F1 mice (8–12-week-old) were obtained from CSEAL (Orléans, France).

Macrophages

Inflammatory macrophages used throughout this study were obtained from the peritoneal cavity of mice injected 4 days earlier with 1.5 ml thioglycolate medium (Institut Pasteur, Paris) (0.05% thioglycolate).

Mice were killed by cervical dislocation and peritoneal cells were harvested in MEM medium (Institut Pasteur, Paris), centrifuged 10 min at 200 *g* and resuspended in MEM medium containing 5% complement-inactivated foetal calf serum and antibiotics. Thioglycolate exudates contained $(17 \pm 4.7) \times 10^6$ cells per mouse; 80–90% were macrophages as judged by neutral red uptake.

Assay for endocytosis

Peritoneal exudate cells were diluted to the appropriate macrophage concentration, plated in 35 mm diameter Nunclon (Denmark) Petri dishes (2 ml per dish) and incubated at 37°C in a 5% CO_2 humidified atmosphere to allow macrophages to adhere. Non-adherent cells were removed 4 h later by washing 3 times with PBS: the monolayers, which were confirmed to consist entirely of macrophages by examination with an inverted microscope were then ready for use. Unless otherwise stated, 4×10^6 thioglycolate-elicited macrophages were plated in a 35 mm diameter Nunclon Petri dish. In our conditions, more than 90% of thioglycolate-elicited macrophages adhere to the plastic and are not detached by washing. After removal of non-adherent cells by washing with PBS, macrophages were incubated at 37°C in 2 ml of MEM medium containing appropriate radioactive material (free solute or liposomes). At the end of the incubation period, the medium was removed and macrophage monolayers washed 4 times in PBS at 4°C: the last wash was free of any detectable radioactivity. Triton X-100 (1%, 0.5 ml) was added and the monolayer of macrophages was scraped with a rubber policeman: this operation was repeated and the two homogenates were pooled and counted in Aqualuma scintillation mixture (Luma, France) in a Rackbeta LKB scintillation spectrometer.

Assay for cytostasis

Peritoneal exudate cells from (DBA2 × C57BL/6)F1 mice were diluted to the appropriate macrophage (Mϕ) concentration (2×10^6 Mϕ/ml) in MEM medium with 5% FCS and were plated (0.25 ml/well) in microtest II plates (Falcon Plastics, Oxnard, CA) producing a Mϕ density of 17,800 Mϕ/mm^2. Four hours later, non-adherent cells were removed by washing three times with PBS. Macrophages were then incubated at 37°C in the MEM medium containing appropriate activating agents (free MDP, MDP-containing liposomes ...). At the end of the incubation period these agents were removed by washing three times with PBS. 0.25 ml of suspension of P815 mastocytoma cells (0.3×10^6 cells/ml) in MEM medium supple-

mented with antibiotics and 5% FCS, was added to the macrophage layer producing an initial effector to target ratio of 7. P815 mastocytoma cells were passaged as ascites in syngeneic DBA2 mice.

Inhibition of tumor cell growth was measured by the [^{3}H]TdR cumulative incorporation assay. In this assay, 1.2 μM [^{3}H]TdR (spec. radioact. 1 Ci/mmol) was added to the macrophage cultures at the same time as the P815 cells. Tumor cells were cultured in the presence of macrophages for 24 h and afterwards collected on glass fiber filters with a cell harvester (Skatron, Norway). The radioactivity incorporated was measured in a beta-scintillation LKB spectrometer. The cytostatic activity induced by MDP-containing liposomes was calculated according to the following formula (Juy and Chedid, 1975): % G.I. = 100 (1 − X/R) where X is the [^{3}H]TdR incorporation in P815 target cells cultivated on macrophage layers previously treated with liposomes containing MDP and R the same parameter when macrophages have been previously incubated in the medium alone. Possible sources of artefacts that could affect label uptake were explored by Lepoivre et al. (1982). In fact, by using this method, the incorporation of [^{3}H]TdR in P815 cells was proportional to the increased number of tumor cells measured after trypsination.

Results

(1) Liposome properties

Separation of free from liposome-encapsulated solute

Large multilamellar liposomes were separated from the free solute (CF or MDP) by repeated centrifugations. The fourth supernatant did not contain any detectable amount of free solute. This method induced a definite but moderate increase in the average size of MLV from 0.8 μm to 1.3 μm. However, when used for the preparation of oligolamellar liposomes, it caused a marked increase in size from 0.18 μm to 0.54 μm (average particle sizes of the initial liposomal suspension, and of the resuspended pellet, after the fourth centrifugation, respectively). Consequently, oligolamellar liposomes obtained by sonication could not be prepared satisfactorily by this method and gel chromatography was employed to separate non-entrapped solute from these vesicles.

Different types of gels were studied under the same experimental conditions: Sephadex G15, Sephadex G50 (coarse and fine). Sephacryl S300, Trisacryl GF05, Ultrogel ACA202 and Ultrogel A6. The MDP content of the effluent fractions was measured by HPLC. The best separation was achieved with Ultrogel A6, free MDP was completely separated from liposomes. Thus Ultrogel A6 was chosen for further experiments. This method did not result in an increase of the liposome size. The only problem was the sample dilution: 1 ml of sample was collected in 3 fractions (10.5 ml).

Sterilization by filtration

The assay was performed as described in Materials and Methods. This method could only be used for liposomes that were smaller than 200 nm. This filtration did

TABLE 1

AMOUNT OF MDP ENCAPSULATED IN OLIGOLAMELLAR LIPOSOMES

(A) Influence of the total volume of the liposome suspension

Phospholipids (PL) initial concentration 30 mg/ml	PC/PS 7:3			
Initial MDP concentration (mg/ml)	15			
Volume of the liposome suspension (ml)	1	2	3	4
Entrapment Efficiency (EE) mg MDP/100 mg PL	1.78	2.15	2.60	3.50
Encapsulation Ratio (ER) μl/μmole PL	0.94	1.15	1.38	1.86

(B) Influence of the initial MDP concentration

Phospholipids (PL) initial concentation 30 mg/ml,	PC/PS = 7:3 total volume of liposome suspension: 1 ml		
Initial MDP concentration (mg/ml)	15	30	60
mg MDP/100 mg (PL) EE	1.78	2.71	3.62
μl/μmol (PL) ER	0.94	0.77	0.48

EE = entrapment efficiency: amount of drug entrapped in the liposome preparation per 100 mg of phospholipids (see Barbet 1982).

ER = encapsulation ratio: ratio of the total volume of entrapped aqueous phase to the total moles of lipid used.

Assays were carried out with liposomes prepared by the standard method, sonicated and separated by gel chromatography (oligolamellar liposomes). No difference in the average size of liposomes was observed when the volume or MDP concentration were changed.

not change the average size of liposomes (200 nm before and after filtration as measured with the Nanosizer), but made the suspension more homogenous. The amount of phospholipids lost during filtration could be determined by an enzymatic method (see Materials and Methods), and depended on the liposome size. The losses were about 40% for liposomes of 200 nm and 20% for liposomes of 150 nm. We checked by using entrapped CF at a self-quenching concentration that the sterilizing filtration resulted in only a very slight escape of the solute encapsulated in the aqueous space. Sterility controls were performed according to the 'Pharmacopée Française' and no microbial contamination was detected.

Amount of MDP encapsulated in oligolamellar liposomes suitable for injection

As shown in Table 1, the amount of encapsulation was in agreement with those reported in the literature (Schroit and Fidler, 1982). However, large differences among batches existed and the results were not readily reproducible. Two factors which could affect encapsulation were studied: the influence of the total volume of the liposome suspension (Table 1A) and the influence of the initial MDP concentration (Table 1B) as shown in Table 1A, when the volume of liposome suspension was increased (MDP concentration being kept constant), the entrapment efficiency and the ratio of encapsulation (defined in the legend of Table 1) increased. When the

volume of the aqueous phase is increased, it is probable that the phospholipid film is more readily detached from the round-bottomed flask and this makes for a more efficient formation of liposomes and encapsulation of MDP.

As shown in Table 1B, when the initial concentration of MDP was increased (the total volume of liposome suspension being kept constant), the entrapment efficiency increased but the encapsulation ratio decreased.

The presence of cholesterol in PC/PS/chol 7 : 3 : 3 liposomes did not seem to modify the encapsulation results. The encapsulation ratio varied from 1.15 to 1.5 μl/μmol PL and the entrapment efficiency varied from 2.15 mg to 2.80 mg MDP/100 mg PL (for PC/PS 7 : 3 liposomes and PC/PS/chol 7 : 3 : 3 liposomes respectively).

The amount of encapsulated MDP did not vary for at least 14 days, when the liposomes were kept unseparated from free MDP at 4°C, phospholipids and MDP were undegraded as checked, respectively, by TLC or HPLC.

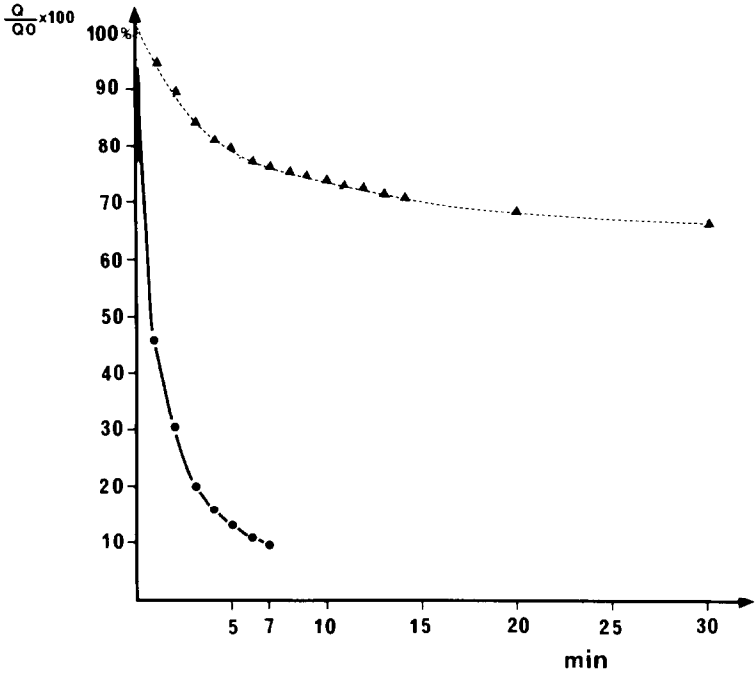

Fig. 1. Stability of liposomes in the presence of FCS. Large multilamellar liposomes containing 100 mM carboxyfluorescein (100 μg of phospholipids) were introduced into the cuvette of a spectrofluorimeter (see Materials and Methods) containing 3 ml of PBS with 5% FCS at 37°C. The suspension was magnetically stirred and the fluorescence intensity continuously recorded. Q(t) is the percentage of quenching at time t and Q_0, the initial percentage of quenching. $Q_0 \equiv 90\%$. ▲- - -▲, DSPC/PS/Chol (7 : 3 : 10) liposomes; ●———●, DSPC/PS (7 : 3) liposomes. The figure shows a typical experiment. Two similar experiments have been performed giving comparable results. Similar data have been obtained with oligolamellar PC/PS and PC/PS/chol oligolamellar liposomes (two experiments).

Stability in FCS containing medium

In order to estimate the escape of solute entrapped in the aqueous space, we used liposomes containing carboxyfluorescein at a self-quenching concentration (100 mM). Figure 1 shows a typical experiment. As it is well known (Black and Gregoriadis 1976; Hunt, 1982; Delattre and Vasson 1983), liposomes are rapidly destroyed in the presence of serum. Cholesterol dramatically increased their stability.

(II) Liposome–macrophage interactions

Endocytosis studies

Kinetics

Experiments to determine endocytosis kinetics were performed at a phospholipid/macrophage ratio slightly lower than the one proposed by Schroit and Fidler (1982). As pointed out by these authors, phosphatidylserine strongly increased the endocytosis of liposomes. It also enhanced the transfer of aqueous solute into macrophages although the fraction of hydrosoluble solute internalized was lower than the fraction of lipid taken up. This observation might suggest that the uptake of this kind of liposome is not a purely phagocytic process but that some fusion with the plasma membrane occurs. The kinetics of liposome uptake slowed down progressively. It seemed that a plateau could be reached after overnight incubation (Table 2).

Efficiency of uptake

Table 3 illustrates the efficiency of uptake of liposome-entrapped molecules

TABLE 2

KINETICS OF LIPOSOME ENDOCYTOSIS

Time (h)	Fraction of [^{3}H]-label internalized		Fraction of [^{14}C]-label internalized	
	DSPC/PS/chol liposomes	DSPC/chol liposomes	DSPC/PS/chol liposomes	DSPC/chol liposomes
2	0.09	0.035	0.15	0.037
4	0.13	0.041	0.22	0.05
6	0.25	0.069	0.43	0.055
20	0.25	0.061	0.40	0.063

Double-labelled large multilamellar liposomes were used: L-α-dipalmitoyl-1-[^{14}C]phosphatidylcholine = 0.043 μCi/mg phospholipids, [^{3}H]sucrose: 0.029 μCi/μl. The aqueous space volumes were: (2.4 ± 0.1) μl/mg phospholipids and (1.1 ± 0.1)-μl/mg phospholipids for DSPC/PS/chol liposomes and DSPC/chol/liposomes, respectively. 4×10^6 macrophages were plated in 35 mm Nunclon petri dishes (cell-density: 4160 Mϕ/mm^2). After 2 h adherence, Mϕ layers were washed 3 times with PBS and then incubated in 2 ml of MEM medium supplemented with antibiotics, FCS and liposome suspension (0.1 mg phospholipids/10^6 Mϕ). At the time chosen, supernatants were removed, layers were washed 4 times with PBS and cells were lyzed by adding Triton X-100 (1% final concentration) in 1 ml PBS. Supernatant and lysate radioactivities were measured with a LKB Beta Spectrometer programmed for double labelling. The fraction of label internalized is the ratio of the radioactivity found in cell lysate to the total radioactivity introduced in the assay.

TABLE 3

EFFECT OF LIPOSOMES CONCENTRATION ON THE INTERNALIZATION EFFICIENCY OF AN AQUEOUS SOLUTE BY MACROPHAGES

mg phospholipids per 10^6 Mϕ	nanomoles sucrose		Fraction internalized B/A	F
	Introduced A	Internalized B		
0 (free sucrose)	3.00	0.0065	0.0022	1
0.025	0.05	0.018	0.36	163
0.062	0.12	0.038	0.30	136
0.124	0.25	0.048	0.20	91
0.185	0.37	0.064	0.17	77
0.250	0.50	0.075	0.15	68

Experimental conditions are as described in the legend of Table 2. DSPC/PS/chol large multilamellar liposomes were used. [^{3}H]Sucrose = 0.029 μCi/μl. The aqueous space volume was (2.4 ± 0.4)-μl/mg phospholipids. Macrophages were incubated for 20 h in the presence of free sucrose or sucrose-containing liposomes. The fraction internalized, B/A, is the ratio of the nanomoles of internalized sucrose to the nanomoles of sucrose introduced in the medium at the beginning of the experiment. F = ratio of the fraction internalized by the liposomal route to the fraction of internalized free sucrose.

compared to the uptake of free molecules available in the same volume of medium bathing the macrophage layer. The uptake of solute encapsulated within liposomes was apparently a saturable phenomenon since the efficiency of internalization of solute decreased as the liposome/macrophage ratio was increased. The ratio F of the fraction internalized by the liposome route to the fraction internalized when the molecules were free in the medium allowed us to quantitate the process. The probability of internalizing one molecule of solute was F-fold higher when the solute was encapsulated in liposomes than when it was in free state in solution, when the volume of medium containing the solute and in contact with the macrophages was kept constant.

Taking into account these data, for activating macrophages, we chose a liposome: macrophage ratio resulting in both a good efficiency of uptake and a large amount of internalized solute. We used a ratio slightly lower than the one proposed by Sone and Fidler (1981), typically close to 0.1 mg phospholipids/10^6 Mϕ. We assumed that a similar ratio would be appropriate for preliminary studies of oligolamellar liposomes. A higher macrophage : lipid ratio could lead to an increasing toxicity for macrophages.

Macrophage activation for cytostatic activity

Kinetics

This assay was carried out as described in Materials and Methods. Macrophages were incubated with DSPC/PS/chol large multilamellar liposomes prepared in PBS containing 50 μg/ml of MDP (0.1 mg phospholipids/10^6 Mϕ). The cytostatic activity increased during 20 h of incubation. The percent of growth inhibition (% G.I. defined in Materials and Methods) was 30 at 3 h and 70 at 20 h. Taking into

account these data and the kinetics of endocytosis, 20 h was chosen for further experiments.

Effect of size and liposome composition

In preliminary experiments, we found that some liposome preparations, per se, activated macrophages without the presence of MDP. Several groups of workers have shown that the size and lipid composition of liposomes is critical for determining the biological response. For example, Jeannin et al. (1983) obtained activation with small unilamellar empty liposomes, whereas Schroit and Fidler (1982) demonstrated that large, multilamellar liposomes containing PS are optimal for the delivery of muramyl peptides to alveolar macrophages. Recently Phillips (personal communication) has activated macrophages with liposomes composed of short-chain phospholipids. Therefore, we investigated the influence of the size and composition of liposomes on their ability to activate macrophages in our system (Table 4). For liposomes containing PC either with or without cholesterol, we observed that oligolamellar liposomes (0.2 μm) were more efficient in activating macrophages than the large ones (1–1.5 μm). This result was observed both with MDP-containing liposomes or with empty liposomes. Comparing PC/PS liposomes and PC/PS/Chol liposomes of the same size, we noticed that cholesterol induced a decrease in the activating ability of empty liposomes. Also cholesterol led to an increase of the efficiency of MDP-containing liposomes. The specific activating effect of MDP was

TABLE 4

EFFECT OF SIZE AND LIPID COMPOSITION OF LIPOSOMES ON MACROPHAGE ACTIVATION MEASURED BY %G.I.

Lipid compositions	Liposome size (μm)			
	1–1.5		0.150–0.200	
	Empty liposomes	MDP liposomes	Empty liposomes	MDP liposomes
PC/PS 7:3	10± 7	18±16	39±9	30±19
PC/PS/chol 7:3:3	−17±19 *	21±14	5±7	49± 2
DSPC/PS/chol 7:3:10	13±19	65± 7 **	N.D. ***	N.D.

Macrophages were plated at a density of 17,800 Mϕ/mm^2 (500,000 Mϕ/well) in microtest II plates (as described in Materials and Methods) and incubated with different types of liposomes as indicated on the table (the corresponding amount of phospholipids added was 0.1 mg/10^6 Mϕ); the amount of encapsulated MDP was about 2.5 μg/0.1 mg phospholipids for PC/PS and PC/PS/chol liposomes and 0.016 μg/0.1 mg phospholipids for DSPC/PS/chol liposomes. After 20 h incubation at 37°C in MEM medium supplemented with 5% FCS and antibiotics, macrophage layers were washed 3 times with PBS and the target P815 mastocytoma cells added at an initial effector-to-target ratio of 7 (75,000 P815 cells per well). The cystostatic activity was determined as described in Materials and Methods section. Results (mean ± S.D. of triplicate cultures) are expressed by % G.I. as defined in Materials and Methods.

* A negative value means that the presence of Mϕ increases the tumor cell growth.

** In this experiment, free MDP at a dose of 160 μg/ml, i.e. 5000 times the amount encapsulated in the DSPC/PS/chol liposomes gave a %G.I. of 30 ± 5.

*** N.D. = not determined.

clearly shown in this experiment because of the low ability of empty liposomes with this composition to activate macrophages. Liposomes made with PC were prepared in PBS containing a high MDP concentration in order to measure the MDP content of liposomes by HPLC. To allow for their expected higher efficiency (Schroit and Fidler, 1982), DSPC/PS/chol liposomes were prepared in PBS containing a lower concentration of MDP (50 μg/ml). In this case, the encapsulated MDP amount was estimated by using the aqueous space volume measured by double-labelling (see Materials and Methods). Large multilamellar liposomes made with DSPC, per se, did not activate macrophages. Compared to the other large MLV preparations used in this study, MDP-containing DSPC/PS/Chol liposomes were the most efficient at activating macrophages. Oligolamellar DSPC/PS/chol are currently being investigated.

Discussion

The aim of the present work was to design MDP-containing sterile liposomes suitable for i.v. injection. We studied their ability to render macrophages cytotoxic in vitro taking into account their size and lipid composition.

The first step was to prepare stable and sterile liposomes. Large multilamellar vesicles (1 μm) were separated from the free drug by centrifugation. This method, when applied to oligolamellar liposomes, induces a large increase in size (see above). Oligolamellar liposomes (0.2 μm) had to be separated by gel chromatography using Ultrogel A6.

Oligolamellar liposomes (0.2 μm) were satisfactorily sterilized by filtration since no change in size occurred and there was no escape of entrapped drug. The sterilization of large MLV is a problem that remains to be solved.

Cholesterol did not influence the MDP encapsulation efficiency (EE) by oligolamellar liposomes at least as far as PC/PS liposomes are concerned. The encapsulation ratio (E.R.) decreases when increasing MDP concentration has already been observed for other hydrophilic drugs (Benita et al., in press). The E.R. was typically around 1 μl/μmol phospholipids for these oligolamellar vesicles. Some differences in the encapsulation efficiency from batch to batch means that it is necessary to determine the exact MDP content of each preparation so that the amount of active drug used in a particular experiment is known.

Using oligolamellar liposomes separated from free MDP, we checked that over a conservation period of one month under nitrogen at 4°C in the dark, no degradation of MDP (monitored by HPLC) or phospholipids (monitored by TLC) occurred.

The amount of encapsulated MDP remained constant at least for 15 days when oligolamellar liposomes were kept unseparated from the free drug. This may be explained by a dynamic balance between the inside and the outside of liposomes. We are currently studying the putative MDP escape from oligolamellar liposomes separated from the free drug.

Using liposomes containing carboxyfluorescein at a self-quenching concentration we confirmed that those composed of PC/PS or DSPC/PS are made leaky very

quickly at 37°C in the presence of serum (FCS). This fact is generally believed to be due to the action of high density lipoproteins and/or phospholipases in serum (Delattre and Vasson, 1983). The escape of entrapped solute was very rapid regardless of the size of the liposomes in the range 1.5–0.15 μm mean diameter. Cholesterol incorporation in the lipid phase resulted in a dramatic decrease of liposome permeability. This result obtained with carboxyfluorescein seems to be true for most hydrophilic molecules. For example, it has been shown that the rate of escape were identical for carboxyfluorescein and methotrexate (Barbet, 1982).

The liposomes obtained were then tested for their ability to activate macrophages.

MDP-containing PC/PS oligolamellar liposomes were not more efficient than their empty counterparts at activating macrophages, probably because in the presence of serum, the escape rate of entrapped MDP is faster than that of liposome endocytosis and macrophage activation. It must be noted, however, that using DSPC instead of egg PC to form MLVs, Sone and Fidler (1981) were able to activate macrophages with encapsulated MDP. Incorporating cholesterol has at least two effects. The intrinsic activating ability of empty liposomes is decreased and the entrapped MDP is prevented from escaping before liposome endocytosis by macrophages. The combination of these two effects results in a maximal difference between empty and MDP-containing liposomes, providing a clearcut demonstration of the intrinsic activating ability of encapsulated MDP.

DSPC/PS/chol MLV liposomes were more efficient that the PC/PS/chol liposomes at activating macrophages. This could be due to the higher capacity of the former for transferring their aqueous space contents to the interior of the macrophages (Schroit et al., 1983).

Taking into account the effect of the size on the liposome ability for activating macrophages, our results agree well with both those of Sone and Fidler (1981) and Jeannin et al. (1983). Large MLV (without encapsulated MDP) used as a control by Fidler were not able to activate macrophages. By contrast SUV liposomes used by Jeannin were very effective in activating macrophages. However, the experimental system of Jeannin was different from ours in that SUV were used instead of oligolamellar liposomes and those SUV remained present during the co-culture of macrophages and target cells. Unlike our results, macrophage activation was no longer observed if SUV were removed before adding target cells.

Bearing in mind that the murine peritoneal macrophages responsiveness to MDP-like structures is rather weak (Lemaire et al., in press) compared to that of other types of macrophages (e.g. rat alveolar), we believe that the results presented here represent a significant modification of the immune system in response to entrapped MDP. It is thus possible that sterile MDP-containing oligolamellar PC/PS/chol liposomes, suitable for i.v. injection, as described here, could activate alveolar macrophages considerably. The results obtained with the different types of liposomes studied allow us to classify them according to their activating ability: MDP-containing multilamellar DSPC/PS/chol liposomes > MDP-containing oligolamellar PC/PS/chol liposomes > empty oligolamellar PC/PS liposomes = MDP-containing oligolamellar PC/PS liposomes > MDP-containing multilamellar PC/PS/chol liposomes > empty multilamellar PC/PS liposomes = MDP-containing

multilamellar PC/PS liposomes = empty multilamellar DSPC/PS/chol liposomes > empty oligolamellar PC/PS/chol liposomes > empty multilamellar PC/PS/chol liposomes.

MDP-containing oligolamellar DSPC/PS/chol liposomes would probably be still more efficient. This hypothesis is currently under investigation in our laboratory. Recently, lipophilic MDP derivatives have generated increasing interest because of their improved retention in liposomes (Kleinerman et al., 1983; Lopez-Berestein et al., 1983). Therefore we are currently studying the design of sterile liposomes containing a new lipophilic derivative of MDP (MDP-L-alanyl-cholesterol, (Bernard et al., submitted)) and their use for preventing metastases of the Lewis Lung Carcinoma in vivo.

Abbreviations

MDP N-acetyl-muramyl-L-alanyl-D-isoglutamine
CF carboxyfluorescein
DSPC distearoylphosphatidylcholine
PC egg lecithin
PS beef brain phosphatidylserine
Chol cholesterol
PBS phosphate-buffered saline
%G.I. percent of tumor growth inhibition
FCS foetal calf serum
MLV multilamellar vesicles
EE entrapment efficiency
ER encapsulation ratio
PL phospholipid
SUV small unilamellar vesicles

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