ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1996, p. 146–151 Vol. 40, No. 1
0066-4804/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Effect of Composition and Method of Preparation of Liposomes on Their Stability and Interaction with Murine Monocytes Infected with *Brucella abortus*

ANA I. VITAS,[1] RAMÓN DÍAZ,[1,2] AND CARLOS GAMAZO[1]*

Dpto. de Microbiologia, Universidad de Navarra,[1] and Clinica Universitaria de Navarra,[2] 31080 Pamplona, Spain

Received 15 June 1995/Returned for modification 12 October 1995/Accepted 6 November 1995

The success of the use of liposomes as drug carriers depends on both their formulation and the method of preparation. We have carried out a series of in vitro studies using different formulations and preparation methods, with the aim of obtaining a type of liposome which is efficient in the treatment of brucellosis. On the basis of results obtained in studies of stability at 37°C in the presence of serum lipoproteins and of the activation of phagocytic cells and antibiotic transport to the interior of monocytes infected with *Brucella abortus*, we conclude that the most suitable vesicles are positively charged, stable plurilamellar vesicles (phosphatidylcholine, 30% cholesterol, and 10% stearylamine). Gentamicin incorporated into these cationic liposomes completely eliminated all of the intracellular *Brucella* organisms (4.6 logs), while free gentamicin was capable of reducing the number of intracellular bacteria by only 0.3 log.

Brucellosis is a zoonosis which causes major economic losses. The intracellular localization of *Brucella* species makes treatment difficult, given that most antibiotics which have been seen to be efficient in vitro do not actively pass through cellular membranes (16). Hence, it has been proposed to use liposomes as an effective vehicle for getting antibiotic into *Brucella*-infected phagocytes. Liposomes are vesicles which are composed of one or several lipid bilayers which enclose an aqueous phase, so they are capable of transporting different substances, with antibiotics being among them. Given that liposomes are particularly foreign to the body, they are captured by the cells of the mononuclear phagocytic system, such as blood monocytes and macrophages of the liver, spleen, and bone marrow (28). This fact, which could be a disadvantage in the treatment of some infections, can be of great benefit in the treatment of those which target the mononuclear phagocytic system for action, as is the case with brucellosis.

Before they can interact with phagocytic cells, liposomes have to overcome another obstacle in the bloodstream: structure destabilization as a result of interaction with certain serum components. High-density lipoproteins (HDL) seem to be mainly responsible for this process (7, 29, 31). Although the mechanism is not clear, it has been suggested that during the interaction of a lipoprotein and a vesicle, some of the lipids are transferred from the liposome to the lipoprotein (21, 29, 34), leaving different-sized pores through which the contents are released (20).

Therefore, for a liposome to be efficient in the treatment of brucellosis and other infections which affect the mononuclear phagocytic system, it should be stable in the bloodstream and interact efficiently with monocytic-macrophagic cells. In our study, we examined how these parameters are affected by variations in both the composition of the liposome and the preparation method of the vesicles. Therefore, we compared a new liposomal formulation (35) with liposomal formulations used by other authors for the experimental treatment of brucellosis (8, 11, 17). The effect of the incorporation of different proportions of cholesterol (CHL) and charged molecules on the stability of vesicles in the presence of HDL was studied. In addition, the effects on the degree of interaction with monocytic cells and on the therapeutic capacity of gentamicin encapsulated in different types of liposomes for monocytes infected with *Brucella abortus* were also studied.

(Some of these data were presented at the 6th European Congress of Clinical Microbiology and Infectious Diseases, Seville, Spain, in 1993 and at the 13th International Symposium of the World Association of Veterinary Microbiologists, Immunologists and Specialists in Infectious Diseases, Perugia, Italy, in 1994.)

MATERIALS AND METHODS

The following freeze-dried compounds (Sigma Chemical Co.) were used to prepare the vesicles: egg yolk phosphatidylcholine (PC; 60% pure), CHL (99% pure), dicetylphosphate (DP; 97% pure), and stearylamine (ST; 99% pure). Bovine HDL and the fluorochrome 5(6)-carboxyfluorescein (CF; 99% pure) were also purchased from Sigma. Sephadex G-50 medium was purchased from Pharmacia-LKB. For the mononuclear phagocytic system studies, the mouse monocyte J774 cell line (ATCC TIB 67) was used. The culture medium used to maintain this line was Dulbecco's modified Eagle medium (DMEM, Whittaker) supplemented with fetal calf serum (Gibco). Other reactive agents used in this analysis were of analytic grade.

Preparation of liposomes. (i) Preparation of MLVs. The preparation technique for multilamellar vesicles (MLVs) described by Dees et al. (8) consisted of the hydration of the suspension of previously dried lipids (10 mg/ml) in Hanks balanced salt solution (Sigma Chemical Co.) for 30 min. The emulsion was then vigorously shaken for 15 min until a homogeneous liposome suspension (multilamellar type) was obtained. The separation of the nonencapsulated material was done by filtration in a Sephadex G-50 column (15 by 1 cm).

(ii) Preparation of freeze-thaw MLVs. Another type of MLV was prepared by following the technique described by Hernández-Caselles et al. (17). Phospholipids (PC) and suitable amounts of CHL, ST, and DP were dissolved in chloroform. The solvent was evaporated under nitrogen current, with the final traces being eliminated by vacuum drying for 3 h. The MLV-type vesicles were prepared by adding the aqueous phase (saline solution, 0.85% NaCl) to the phospholipid film and shaking the suspension vigorously in three heating cycles at 50°C and cooling at 4°C until a homogeneous suspension was obtained. The separation of the nonencapsulated material was done by centrifuging the suspension three times at 15,000 $\times$ *g* (for 15 min each time). The liposomes were finally suspended again at a concentration of 50 mg of PC per ml in saline solution.

(iii) Preparation of SPLVs. Stable plurilamellar vesicles (SPLVs) were prepared by following the method described by Fountain et al. (11). The phospholipid suspension was dispersed in a vesicle rotavapor flask with a spherical base with a volume of 100 ml and was evaporated in a rotavapor at 40°C until it dried completely. Once the suspension had dried, the phospholipids were dissolved in

* Corresponding author. Phone: 34-48-105600. Fax: 34-48-105649. Electronic mail address: cgamazo@mail2.cti.unav.es.

10 mg of diethyl ether per ml, and 0.3 ml of the aqueous phase (HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] buffer [10 mM HEPES, 145 mM NaCl, 0.02% sodium azide [pH 7.4]) was added. The mixture was shaken for 30 s in a mechanical shaker and then placed in a bath-type sonicator, with a nitrogen current being passed through it to facilitate evaporation during the sonication. When no trace of diethyl ether was found to be present, the mass of SPLVs was dispersed in saline solution with a mechanical shaker. The nonencapsulated material was separated by centrifuging the mixture at 15,000 × *g* for 10 min. The process was repeated three times, and the SPLVs were finally suspended in saline solution once more (10 mg of phospholipids per ml).

(iv) Preparation of modified SPLVs. This method was based on that of Fountain et al. (11), with some modifications as described below. First, the suspension of phospholipids was done in chloroform at 10 mg/ml. After the aqueous phase was added (HEPES buffer), the liposome emulsion was obtained by vortex agitation for 30 s and then by sonication in a bath-type sonicator (P-Selecta; 50 W) for 2 min at room temperature. Organic-phase excess was eliminated with a vacuum rotavapor by applying low pressure (200 to 300 mm of Hg) for 5 min at a bath temperature of 40°C. A nitrogen stream was applied to remove any trace of solvent. The liposomes were suspended again in the HEPES buffer with the help of 5-mm-diameter glass beads. The elimination of the nonencapsulated material was done by column filtration (Sephadex G-50), except in cases of positively charged vesicles, as these interact with the column. In these cases, three washes in HEPES buffer were done (15,000 × *g* for 15 min).

Determination of CF and gentamicin. (i) Encapsulation percentage of CF. The fluorescence of each liposome sample was checked before and after being disrupted with 100 μl of Triton X-100 (10%) and then being boiled for 10 min. The quantity of encapsulated CF was calculated by subtracting the initial fluorescence value of the liposomes from that of the lysed ones (total fluorescence) and then extrapolating this value with the linear equation obtained with known concentrations of CF. The percentage of encapsulated CF was calculated with respect to a known concentration of CF included in the aqueous phase during the preparation of the liposomes (see below).

(ii) Determination of gentamicin. To evaluate the bactericidal capacity of gentamicin encapsulated in different types of liposomes, a fixed concentration of gentamicin was used (20 μg/ml; see below). The gentamicin encapsulated in the liposomes was quantified with *o*-phthalaldehyde by the method of Benson and Hare (6). The amine antibiotic reacts with *o*-phthalaldehyde in the presence of β-mercaptoethanol at 21°C, forming a product which can be detected by fluorescence spectroscopy. The sensitivity of the technique is 1 μg of antibiotic per ml. Readings were taken with a Cytofluor 2300/2350 (Millipore) fluorimeter, adjusted to wavelengths of 360 nm for excitation and 460 nm for emission.

Fluorimetric technique for the study of stability and half-life. To study the stability, half-life, and efficiency of encapsulation of the liposomes, the liposomes were charged with 50 mM CF in HEPES buffer, pH 7.4 (36). The stability of the vesicles was studied by continuous follow-up of the increase in fluorescence after the liposomes (diluted in HEPES buffer, pH 7.4, at 50 μg of lipids per ml) were incubated in the presence of HDL (173 μg/ml) for 5 min, with an automatic reading being taken every 0.1 s. The same liposome population suspended in 10 mM HEPES buffer (pH 7.4) was used as a control group to observe the effect of incubation at 37°C. The increase in fluorescence was determined with a Perkin-Elmer LS-50 spectrofluorimeter (Perkin-Elmer Corp., Norwalk, Conn.). The reading conditions were as follows: excitation, 490 nm; emission, 538 nm; and slit width, 10 nm.

The half-life of each liposomal formulation was also studied, i.e., the time interval needed to liberate 50% of the encapsulated CF in the presence of bovine HDLs (173 μg/ml) at 37°C. For each time course, six points in time were taken (1, 1.5, 3, 4, 20, and 50 h). In some experiments (see text), the follow-up was prolonged to 150 h.

In vitro studies. (i) Culture and maintenance of monocytes. The mouse monocytes of the J774 tumor line were kept in sterile and ventilated culture flasks (Costar). The culture medium used was DMEM supplemented with 10% fetal calf serum. The cells were transferred to fresh medium every 3 or 4 days and then were recovered with the aid of a rubber policeman, with the medium being centrifuged at 200 × *g* for 5 min at 4°C.

(ii) Activation of monocytes. The Nitro Blue Tetrazolium test (1) was used to determine the degree of activation of the monocytes following the interaction with the liposomes. In the presence of radical O_2^-, Nitro Blue Tetrazolium (soluble and yellow) is reduced to formazan, an insoluble blue-black material which precipitates intracellularly and can be spectrophotometrically quantified after extraction of the cells with *N*,*N*-dimethylformamide. The technique consists of placing 2×10^6 monocytes in 0.5 ml of medium plus 0.5 ml of liposomes diluted 1:5 in 0.2% Nitro Blue Tetrazolium in saline solution in cone-shaped vials (Eppendorf type). The basal activity of the monocytes was determined by incubating the cells in 0.5 ml of 0.2% Nitro Blue Tetrazolium in saline solution. Zymosan A (*Saccharomyces cerevisiae*; Sigma) at a concentration of 60 mg/ml in saline solution was used as a positive control for activation. The zymosan was deactivated before use by boiling it for 10 min. Before the activators (zymosan and the liposomes) were added to the monocytes, it was necessary to opsonize them with mouse serum (250 μl of the activator was incubated at 37°C for 30 min with 4 μl of serum prediluted to 1/10).

The monocytes were incubated with the opsonized activators for 4 h at 37°C; then they were centrifuged for 3 min at 13,000 × *g*, and the purple precipitate corresponding to the formazan was observed. To extract the precipitate, the supernatant was incubated with 1 ml of *N*,*N*-dimethylformamide at 56°C for 18 h. After the supernatant was centrifuged for 3 min at 13,000 × *g*, the optical density of the supernatant at 515 nm was determined.

TABLE 1. Effect of liposomal CHL content on the stability and the percentage of encapsulation of CF in modified SPLVs

Liposome composition (ratio)	CF release (%)[a]		Half-life (h)	Encapsulation (%)
	5 min	3 h		
PC	45.0	71.6	1–4	13.30
PC-CHL (3:1)	25.6[b]	58.5[b]	<1	2.00[c]
PC-CHL (2:1)	16.7[b]	47.9[b]	1–4	3.83[c]
PC-CHL (1:1)	3.7[c]	28.5[b]	4–20[b]	2.42[c]
PC-CHL (1:2)	0.0[d]	16.3[c]	20–50[c]	8.80[b]

[a] The percentage of CF release was determined after incubation of the liposomes in 173 μg of HDL per ml at 37°C.
[b] $P < 0.05$ for difference from the PC liposomes by Student's *t* test.
[c] $P < 0.01$ for difference from the PC liposomes by Student's *t* test.
[d] $P < 0.001$ for difference from the PC liposomes by Student's *t* test.

(iii) Phagocytosis of *Brucella abortus* and evaluation of the bactericidal capacity of gentamicin encapsulated in liposomes. Monocytes at 10^6 per cup in 0.5 ml of culture medium were placed on 24-well plates (Costar). After the cells were incubated for 1 h at 37°C, 100 μl of saline solution containing 10^6 CFU of *B. abortus* 2308 biotype 1 (for a monocyte-to-*B. abortus* ratio of 1:1) was added. The bacteria used in this experiment were in their log phase after growth on tryptone-soy agar plates, and they were opsonized with serum from mice experimentally infected with *B. abortus* (900 μl of bacterial suspension was incubated for 30 min at 37°C with 100 μl of serum prediluted 1:1,000). The monocytes were kept in contact with the opsonized bacteria for 24 h at 37°C so that phagocytosis could take place. Once this period was over, the noningested bacteria were eliminated by three vigorous washings with DMEM and by centrifuging them in the microplate at 200 × *g* for 5 min at 4°C. Following this, 0.5 ml of fresh DMEM supplemented with 10% fetal calf serum per well was added, along with a further 100 μl of free or encapsulated gentamicin in opsonized liposomes at a concentration of 20 μg/ml. All treatments were performed in duplicate. The antibiotics were allowed to act for 5 h at 37°C. The plates were then washed with DMEM and centrifuged at 200 × *g*, and a sample of the last supernatant was taken to determine the number of extracellular bacteria by culture on tryptone-soy agar plates. Finally, the monocytes were disrupted in three freeze-thaw cycles. After culture on tryptone-soy agar plates and incubation for 4 days at 37°C, the number of surviving intracellular bacteria was calculated by estimating the difference between the number of total bacteria (obtained after disruption of the cells) and the number of extracellular bacteria.

RESULTS

Stability of the liposomes in the presence of HDL. (i) Effect of CHL. Liposomes which carried different molar proportions of PC and CHL (3:1, 2:1, 1:1, 1:2, and 1:0) were studied. The results in Table 1 show how the incorporation of CHL into the liposomes significantly increased their resistance to serum HDL. The continuous fluorimeter readings shown in Fig. 1 illustrate the effect of CHL on the stability of the liposomes. The amount of CF released increased in proportion with a longer incubation period with HDL. The half-life of the vesicles could be determined when this effect was followed up to 50 h after incubation. The liposomes with greater longevity were the ≥50% CHL-enriched ones (up to 50 h), while those with 33% or less CHL showed a shorter half-life (≤4 h) (Table 1).

Furthermore, the incorporation of CHL reduced encapsulation percentages (Table 1). However, although the vesicles formed of PC alone encapsulated a larger quantity of CF, their greater instability meant that the quantity of CF transported after 1.5 h of incubation was higher in those vesicles with CHL (Fig. 2).

(ii) Effect of the incorporation of a charged molecule. Following the incorporation of a negative charge (DP), the liposomes were more resistant to the action of the serum lipoproteins than those formed exclusively of PC and CHL (Table 2). However, the incorporation of a positive charge (ST) did not

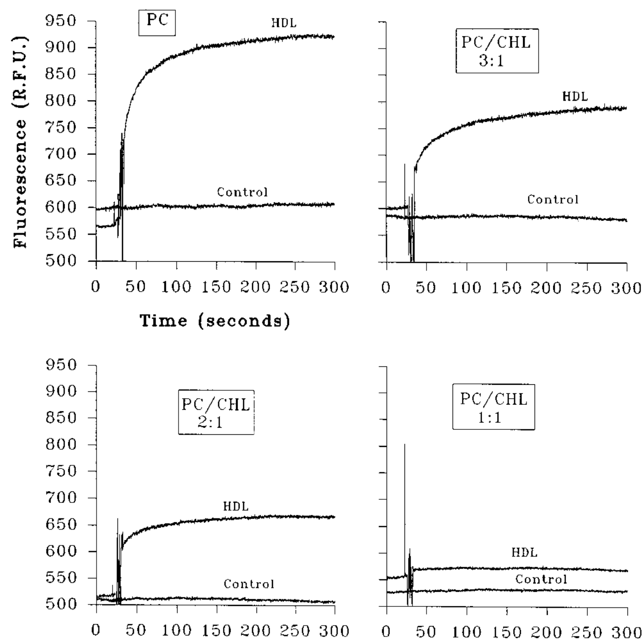

FIG. 1. Effect of liposomal CHL content on the relative release of CF from modified SPLV liposomes. Relative fluorescence (RFU) as a function of the time of incubation in HDL (173 μg/ml) at 37°C is shown graphically.

change the half-life of the liposomes with respect to those formed of PC and CHL. Moreover, the encapsulation percentages were not affected when the vesicles carried a positive or negative charge (Table 2).

(iii) Comparison with other liposome formulations. To carry out the comparative study, we prepared liposomes with the same compositions by following different methods. The analysis revealed that the stabilities of the different types of vesicles were similar, although in the first hours of incubation, the modified SPLVs were generally more unstable (Table 3). Nevertheless, the encapsulation percentages achieved by the methods of Dees et al. (MLVs) (8), Hernández-Caselles et al. (freeze-thaw MLVs) (17), and Fountain et al. (SPLVs) (11) were significantly lower than those obtained by the modified SPLV method described before. In a experiment followed out to 20 h with liposomes prepared by different methods and exposed to HDL at 37°C, we found that with the exception of vesicles formed exclusively of PC, the amount of CF transported by the modified SPLVs was greater than that transported by vesicles formed by the other methods (results not shown).

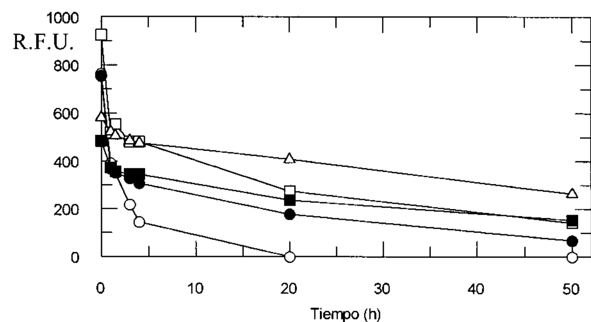

FIG. 2. Effect of liposomal CHL content on the amount of CF carried by modified SPLV liposomes. Relative fluorescence (RFU) as a function of the time (Tiempo) of incubation in HDL (173 μg/ml) at 37°C is shown graphically. ○, PC alone; ●, PC-to-CHL ratio of 3:1; □, PC-to-CHL ratio of 2:1; ■, PC-to-CHL ratio of 1:1; △, PC-to-CHL ratio of 1:2.

TABLE 2. Effect of the inclusion of a charged molecule (DP or ST) on the stability and the percentage of encapsulation of CF in the modified SPLVs

Liposome composition (ratio)	CF release (%)[a]		Half-life (h)	Encapsulation (%)
	5 min	3 h		
PC-CHL (1:1)	3.7	28.5	4–20	2.42
PC-CHL-DP (4:5:1)	0.0[b]	12.0[b]	>150[c]	2.17
PC-CHL-DP (7:8:1)	0.0[b]	7.5[b]	50–150[c]	ND[d]
PC-CHL-ST (4.5:5:0.5)	5.5	24.3	4–20	2.12

[a] The percentage of CF release was determined after incubation of the liposomes in 173 μg of HDL per ml at 37°C.
[b] $P < 0.05$ for difference from the neutral liposomes by Student's t test.
[c] $P < 0.01$ for difference from the neutral liposomes by Student's t test.
[d] ND, not determined.

Activation of monocytes with liposomes with different formulations. After studying the level of formazan production (correlated with the O_2^- yield; see Materials and Methods) in the monocytes of the J774 tumor line, we observed three effects. First, the method of preparation influenced the degree of activation of the monocytic cells. Table 4 shows that empty vesicles that had the same composition (PC) but that were prepared by different methods (MLVs [8], SPLVs [11], and modified SPLVs) produced different levels of formazan. The modified SPLV liposomes appeared to be more efficient than comparable SPLV and MLV liposomes in activating the monocytes, as was indicated by the higher optical density for the modified SPLVs (Table 4). Second, we observed an inhibitory effect of CHL. Vesicles prepared by the modified SPLV method with increasingly higher CHL concentrations (0, 25, 30, and 50%) showed decreasing levels of activation (Table 4). Finally, we also observed an inhibitory effect of the negative charge, while positively charged liposomes gave activation values similar to those of the zymosan positive control group (Table 4).

Effect of gentamicin encapsulated in liposomes on monocytes infected with *B. abortus*. To evaluate the bactericidal capacity of gentamicin encapsulated in liposomes, we used the J774 monocytes experimentally infected with *B. abortus*. These cells were then treated with free gentamicin or gentamicin encapsulated in different types of liposomes. The results (Table 5) showed that the free gentamicin was capable of reducing the number of intracellular bacteria by only 0.3 log in terms of the infected and untreated control group. On the other hand, enhanced gentamicin activity was detected when it was encapsulated in some types of liposomes. Liposomes with a net positive charge were generally the most efficient, regardless of the method used for their preparation. The positively charged MLV liposomes (MLVs +) and freeze-thaw MLVs + led to reductions of 0.59 and 1.58 logs of *Brucella* organisms, respectively, compared with the untreated control group. The positively charged modified SPLVs with 30% CHL completely eliminated all the intracellular *B. abortus*, with a 4.58-log reduction relative to the untreated control group and a 4.13-log reduction compared with the group treated with free gentamicin. The statistical analysis (Student's t test) revealed significant differences between the untreated control group and the one treated with free gentamicin ($P < 0.001$). Significant differences were also observed ($P < 0.01$) for the treatments with

TABLE 3. Effect of the method of liposome preparation on the relative stability and percentage of encapsulation of CF

Liposome	Composition (ratio)	CF release (%)[a]		Half-life (h)	Encapsulation (%)
		5 min	3 h		
Modified SPLV	PC	36.0	71.6	1.0–4.0	13.3
MLV	PC	3.9[b]	70.9	1.0–1.5	0.7[c]
SPLV	PC	8.1[c]	18.6[b]	1.5–4.0[b]	9.2[b]
Modified SPLV	PC-DP (7:2)	25.3	72.0	1.5–4.0	2.4
MLV	PC-DP (7:2)	0.0[c]	22.9[c]	1.5–4.0[b]	0.6[b]
Modified SPLV	PC-CHL-DP (5:1:0.5)	0.0	14.1	20–50	6.9
Freeze-thaw MLV	PC-CHL-DP (5:1:0.5)	0.0	8.2	20–50	2.6[b]
Freeze-thaw MLV	PC-CHL-ST (5:1:0.5)	0.0	10.4	20–50	3.7[b]

[a] The percentage of CF release was determined after incubation of the liposomes in 173 μg of HDL per ml at 37°C.
[b] $P < 0.05$ for difference from the modified SPLV liposomes by Student's *t* test.
[c] $P < 0.01$ for difference from the modified SPLV liposomes by Student's *t* test.

positively charged MLV and SPLV liposomes. The viability studies (exclusion of trypan blue) indicated that the proportion of ST used is not toxic for the cells, as viability levels of more than 93% were obtained (the viability of the untreated control group was more than 95%). The incorporation of CHL also reduced the levels of interaction between liposomes and monocytes. Thus, the reduction in the number of *B. abortus* was greater with the positively charged vesicles with 30% CHL than with the same vesicles with 50% CHL (logarithmic reduction of 4.58 as opposed to 0.84).

DISCUSSION

New methodologies have been developed in recent years to optimize liposomes as the transport system for active principles. In this study, our aim was to obtain a suitable type of liposome for transporting drugs into phagocytic cells infected with *B. abortus*. We have therefore focused on permeability control, liposome half-life, and the in vitro interaction with monocytes.

Given the characteristics of SPLVs (multilamellarity, high encapsulation efficiency, and greater stability), we opted for the encapsulation of antibiotics in this kind of liposome. However, we introduced two changes to the classic preparation methods of Gruner et al. (14) and Fountain et al. (11). First, as a solvent, we used chloroform instead of diethyl ether, because of the higher solubility of some components, such as DP and ST, in chloroform. Second, a vacuum rotovapor was used to evaporate organic-phase excess at a controlled temperature (40°C) and pressure (200 to 300 mm of Hg). We thus ensured the homogeneity of the different liposomal preparations and promoted their drying in a fine and extensive layer on the surface of the flask by using low pressure and a rotating movement, thereby achieving the total elimination of the organic phase. The evaporation of the liposomes by using low pressure is recommended to avoid vesicle deterioration (5).

Once the method of liposome preparation was established, we studied the stability of the liposomes in vitro. We used a fluorimeter method based on the release of encapsulated CF to gauge liposomal stability. This method, introduced by Weinstein et al. (36) and applied by Szoka et al. (33), is based on the principle that CF fluoresces strongly when its concentration is low, so a critical quantity of CF that does not fluoresce (self-quenching) when it is encapsulated in the liposome but does fluoresce when it is released was used. Moreover, stability studies were done at 37°C in the presence of serum components, given that the instability of liposomes in the bloodstream is a well-known phenomenon; in most studies in which encapsulated antibiotics are used in liposomes with a therapeutic aim, they are introduced intravenously (2). The stability of the liposomes in the presence of serum proteins may be enhanced by changes in the composition of the vesicles. Therefore, we studied the effect of the incorporation of CHL and a charged molecule on the stability and half-life of the vesicles.

The results showed that the incorporation of CHL led to a significant increase in the resistance of the liposomes to the serum lipoproteins. These results agree with predictions and with the results of other authors (4, 9, 13, 15, 20, 25). In addition to the effect on stability, another consideration is the effect of CHL on encapsulation performance. Our results indicate that this performance is diminished following the incorporation of CHL. This drop could be related to the fact that CHL can modify the thickness of the lipid bilayer after it enters the lipid membrane (23). Nevertheless, although the CHL-stabilized liposomes showed lower encapsulating efficiency, their greater stability in the presence of HDL meant that the amount of CF transported was higher than that transported by vesicles formed exclusively of PC.

The incorporation of a negative charge made the liposomes more resistant to the action of the serum lipoproteins, while the incorporation of a positive charge did not improve the stability of the vesicles which had previously been stabilized with CHL. These results coincide with those described by

TABLE 4. Activation of monocytes with liposomes and determination of O_2^- production

Liposome[a]	Composition (ratio)	OD_{515}[b]
Modified SPLV	PC	1.060[c]
Modified SPLV	PC-CHL (3:1)	1.050[c]
Modified SPLV	PC-CHL (2:1)	0.883
Modified SPLV	PC-CHL (1:1)	0.621
Modified SPLV −	PC-CHL-DP (6:3:1)	0.400[c]
Modified SPLV +	PC-CHL-ST (6:3:1)	0.696
SPLV	PC	0.502[c]
MLV	PC	0.302[c]
MLV −	PC-DP (7:2)	0.376[c]
MLV +	PC-ST (7:2)	0.155[c]
Freeze-thaw MLV −	PC-CHL-DP (5:1:0.5)	0.272[c]
Freeze-thaw MLV +	PC-CHL-ST (5:1:0.5)	0.781
Freeze-thaw MLV +	PC-CHL-ST (6:3:1)	0.312[c]
Freeze-thaw MLV +	PC-CHL-ST (7:3:0.5)	0.374[c]
Zymosan		0.792

[a] −, negative charge; +, positive charge; Zymosan, activation control.
[b] OD_{515}, optical density of formazan at 515 nm. Formazan was the product from the reaction with O_2^- from the monocyte.
[c] $P < 0.05$ for difference from the control (Zymosan) by Student's *t* test.

TABLE 5. Enhanced intraphagocytic killing of *B. abortus* in murine monocytes by liposomes containing gentamicin

Treatment[a]	Liposome	Composition (ratio)	Reduction of infection (log)
Untreated control[b]			0.00
Free gentamicin			0.34
Gentamicin-containing liposome			
	MLV	PC	0.24
	MLV −	PC-DP (7:2)	0.04
	MLV +	PC-ST (7:2)	0.59
	SPLV	PC	0.08
	Freeze-thaw MLV −	PC-CHL-DP (5:1:0.5)	0.50
	Freeze-thaw MLV +	PC-CHL-ST (5:1:0.5)	1.58[c]
	Modified SPLV	PC	1.00[d]
	Modified SPLV	PC-CHL (3:1)	0.20
	Modified SPLV	PC-CHL (2:1)	0.23
	Modified SPLV	PC-CHL (1:1)	0.00
	Modified SPLV −	PC-CHL-DP (4:5:1)	0.30
	Modified SPLV −	PC-CHL-DP (6:3:1)	0.13
	Modified SPLV +	PC-CHL-ST (4:5:1)	0.84[d]
	Modified SPLV +	PC-CHL-ST (6:3:1)	4.58[e]
	Modified SPLV +[f]	PC-CHL-ST (6:3:1)	0.00

[a] Gentamicin was used at 20 μg/ml.
[b] The level of infection in the untreated monocytes was 3.8×10^4 CFU.
[c] $P < 0.01$ for difference from the free gentamicin-treated group by Student's *t* test.
[d] $P < 0.05$ for difference from the free gentamicin-treated group by Student's *t* test.
[e] $P < 0.001$ for difference from the free gentamicin-treated group by Student's *t* test.
[f] These liposomes were empty.

Allen and Cleland (4). While these authors do not explain the causes of these effects, these effects could be due to the union of some serum proteins, depending on the charge and lipid composition of the liposome (19).

Comparative studies of the stabilities of vesicles prepared by different techniques indicate that in addition to the composition of the liposomes, the method of preparation also has an influence on the physical and chemical characteristics of the vesicles, thus affecting their stability and encapsulating efficiency. These studies showed that the best vesicles for antibiotic transport were modified SPLVs stabilized with CHL.

As was mentioned previously, an effective liposome for the treatment of brucellosis, assuming sufficient encapsulation capacity, half-life and stability in the bloodstream, should interact efficiently with the monocytic-macrophagic cells where *Brucella* organisms are present. The elimination of the liposomes by monocytic-macrophagic cells takes place in two stages: opsonization by serum proteins (immunoglobulins, complement factors, fibronectin, etc.) followed by the phagocytosis of these marked liposomes. During these stages, the cells are activated and release different products. Therefore, in order to measure the capacity of the liposome to interact with these cells, we performed an in vitro study of incubation of monocytes with liposomes, quantifying one of the products of this activation, the O_2^- anion.

The results demonstrated that lower activation values are obtained as the CHL concentration increases. We also observed a lower therapeutic effect of gentamicin when it was encapsulated in liposomes with a high CHL content. These results confirm those of other authors, according to whom an increase in the proportion of CHL leads to a reduction in macrophage capture because of the increased rigidity of the vesicle (3, 26, 27). Moghimi and Patel (24) suggested that after the incorporation of CHL into the vesicles, the distribution of phospholipids in the membrane may be changed, making the union of opsonines and, by extension, the later capture of the vesicles by cells in the monocytic-macrophagic system more difficult. Therefore, despite the fact that concentrations of CHL of 50% or more give greater stability to the liposome in the presence of HDL, we opted for a concentration of 30%, which gives excellent stability without compromising the ability to be captured by the phagocytic cells.

Another factor which plays a major role in the interaction of the liposomes with the monocytic-macrophagic cells is the surface charge. There is strong controversy on this matter. Some authors state that negative charges favor the interaction and later capture (10, 12, 18) and that positive charges do not favor these events (32). On the other hand, others believe that it is precisely the positively charged liposomes which interact better with the cells (17, 22, 30). Our results support the latter position, as the positively charged liposomes led to a greater activation of the monocytes than liposomes with negative charges. Moreover, the positively charged liposomes (regardless of the method of preparation) produced a greater reduction of the infection compared with the untreated monocytes. These results suggest that there is some mechanism which favors the interaction of the positively charged liposomes and the monocytic-macrophagic cells, thus promoting the transport of antibiotic to these cells. This observation is consistent with the hypothesis of Magee et al. (22) that positively charged liposomes interact with the negatively charged cells by electrostatic adsorption and are then internalized by fusion or endocytosis. The different observations by various authors could be accounted for by differences in the methods of preparation, phospholipids, and the cellular line used for the studies of interaction and capture of liposomes.

Our results also suggest that the preparation method may affect liposome capture, as we have observed differences among liposomes with the same compositions but which were prepared by different methods. Thus, the activation values obtained with PC vesicles of the modified SPLV type were higher than those obtained with vesicles prepared according to the methods of Dees et al. (MLV) (8), Fountain et al. (SPLV) (11), and Hernández-Caselles et al. (freeze-thaw MLV) (17). Regardless of differences in size, heterogeneity, and/or morphology (including lamellarity) for vesicles prepared by differ-

ent methods, the differences could be due to the effect of liposomal fluidity on capture (3, 26, 27). Furthermore, and contrary to results reported by other authors, when we used the formulations and methods of preparation of other authors, we obtained very low levels of reduction of infection (8, 11). This disagreement may be due to differences in the cellular line used and the experimental conditions (incubation times, culture media, etc.).

In summary, it is possible to improve the degree of interaction and capture of liposomes by phagocytic cells by using a suitable liposome design. In this study, we have observed that the best results in terms of stability, activation of monocytes, and efficient antibiotic transport were achieved with modified SPLVs stabilized with 30% CHL and positively charged. These liposomes also demonstrated good efficiency as transport vehicles of antigenic extracts of *Brucella melitensis*, producing a protective effect against experimental murine brucellosis (35). Studies on the treatment of experimentally infected animals with gentamicin encapsulated in this type of liposome are now in progress.

ACKNOWLEDGMENTS

This work was supported by a grant from the Ministerio de Educación y Ciencia—Spain (program project research grant PM92-0140-C02-02). Fellowship support for A. Vitas from the Gobierno de Navarra—Spain is gratefully acknowledged.

REFERENCES

1. **Absolom, D. R.** 1986. Phagocytosis study methods. Methods Enzymol. **132:** 95–180.
2. **Allen, T. M.** 1988. Interactions of liposomes and other drug carriers with the mononuclear phagocyte system, p. 37–50. *In* G. Gregoriadis (ed.), Liposomes as drug carriers. Recent trends and progress. John Wiley & Sons, London.
3. **Allen, T. M., G. A. Austin, A. Chonn, L. Lin, and K. C. Lee.** 1991. Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size. Biochim. Biophys. Acta **1061:**55–64.
4. **Allen, T. M., and L. G. Cleland.** 1980. Serum-induced leakage of liposome contents. Biochim. Biophys. Acta **597:**418–426.
5. **Alving, C. R., and G. M. Swartz, Jr.** 1984. Preparation of liposomes for use as drug carriers in the treatment of leishmaniasis, p. 55–68. *In* G. Gregoriadis (ed.), Liposome technology, vol. II. Targeted drug delivery and biological interaction. CRC Press Inc., Boca Raton, Fla.
6. **Benson, J. R., and P. E. Hare.** 1975. *o*-Phthalaldehyde: fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin. Proc. Natl. Acad. Sci. USA **72:**619–622.
7. **Chobanian, J. V., A. R. Tall, and P. I. Brecher.** 1979. Interaction between unilamellar egg yolk lecithin vesicles and human high density lipoprotein. Biochemistry **18:**180–187.
8. **Dees, C., M. W. Fountain, I. R. Taylor, and R. D. Shultz.** 1985. Enhanced intraphagocytic killing of *Brucella abortus* in bovine mononuclear cells by liposomes containing gentamicin. Vet. Immunol. Immunopathol. **8:**171–182.
9. **Demel, R. A., and B. De Kruyff.** 1976. The function of sterols in membranes. Biochim. Biophys. Acta **457:**109–132.
10. **Dijkstra, J., M. van Galen, and G. Scherphof.** 1985. Influence of liposome charge on the association of liposomes with Kupffer cells *in vitro*. Effects of divalent cations and competition with latex particles. Biochim. Biophys. Acta **813:**287–297.
11. **Fountain, M. W., S. J. Weis, A. G. Fountain, A. Shen, and R. P. Lenk.** 1985. Treatment of *Brucella canis* and *Brucella abortus in vitro* and *in vivo* by stable plurilamellar vesicle-encapsulated aminoglycosides. J. Infect. Dis. **152:**529–535.
12. **Gabizon, A., and D. Papahadjopoulos.** 1992. The role of surface charge and hydrophilic groups on liposome clearance *in vivo*. Biochim. Biophys. Acta **1103:**94–100.
13. **Gregoriadis, G., and C. Davis.** 1979. Stability of liposomes *in vivo* and *in vitro* is promoted by their cholesterol content and the presence of cells. Biochem. Biophys. Res. Commun. **89:**1287–1291.
14. **Gruner, S. M., R. P. Lenk, A. S. Janoff, and M. J. Ostro.** 1985. Novel multilayered lipid vesicles: comparison of physical characteristics of multilamellar liposomes and stable plurilamellar vesicles. Biochemistry **24:**2833–2842.
15. **Guo, L. S. S., R. L. Hamilton, J. Goerke, J. N. Weinstein, and R. J. Havel.** 1980. Interaction of unilamellar liposomes with serum lipoproteins and apolipo-proteins. J. Lipid Res. **21:**993–1003.
16. **Hall, W. H.** 1990. Modern chemotherapy for brucellosis in humans. Rev. Infect. Dis. **12:**1060–1099.
17. **Hernández-Caselles, T., A. Vera, F. Crespo, J. Villalaín, and J. C. Gómez-Fernández.** 1989. Treatment of *Brucella melitensis* infection in mice by use of liposome-encapsulated gentamicin. Am. J. Vet. Res. **50:**1486–1488.
18. **Hsu, M. J., and R. L. Juliano.** 1982. Interactions of liposomes with the reticuloendothelial system: nonspecific and receptor mediated uptake of liposomes by mouse peritoneal macrophages. Biochim. Biophys. Acta **720:** 411–419.
19. **Juliano, R. L.** 1981. Pharmacokinetics of liposome-encapsulated drugs, p. 391–407. *In* C. G. Knight (ed.), Liposomes: from physical structure to therapeutic applications. Elsevier Biomedical Press, Amsterdam.
20. **Kirby, C., and G. Gregoriadis.** 1981. Plasma induced release of solutes from small unilamellar liposomes is associated with pore formation in the bilayers. Biochem. J. **199:**251–254.
21. **Krupp, L., A. V. Chobanian, and J. P. Brecher.** 1976. The *in vivo* transformation of phospholipid vesicles to a particle resembling HDL in the rat. Biochem. Biophys. Res. Commun. **72:**1251–1258.
22. **Magee, W. E., C. W. Goff, J. Schoknecht, M. D. Smith, and K. Cherian.** 1974. The interaction of cationic liposomes containing entrapped horseradish peroxidase with cells in culture. J. Cell Biol. **63:**492–504.
23. **McIntosh, T. J.** 1978. The effect of cholesterol on the structure of phosphatidylcholine bilayers. Biochim. Biophys. Acta **513:**43–58.
24. **Moghimi, S. M., and H. M. Patel.** 1988. Tissue specific opsonins for phagocytic cells and their different affinity for cholesterol-rich liposomes. FEBS Lett. **233:**143–147.
25. **Op den Kamp, J. A. F., M. T. Kauerz, and L. L. M. Van Deenen.** 1975. Action of pancreatic phospholipase A2 on phosphatidylcholine bilayers in different physical states. Biochim. Biophys. Acta **406:**169–177.
26. **Papahadjopoulos, D., G. Poste, and B. E. Schaeffer.** 1973. Fusion of mammalian cells by unilamellar lipid vesicles: influence of lipid surface charge, fluidity and cholesterol. Biochim. Biophys. Acta **323:**23–42.
27. **Patel, H. M., N. S. Tuzel, and B. E. Ryman.** 1983. Inhibitory effect of cholesterol on the uptake of liposomes by liver and spleen. Biochim. Biophys. Acta **761:**142–151.
28. **Poste, G.** 1980. The interaction of lipid vesicles (liposomes) with cultured cells and their use as carriers for drugs and macro-molecules, p. 101–151. *In* G. Gregoriadis and A. C. Allison (ed.), Liposomes in biological systems. J. Wiley & Sons, New York.
29. **Scherphof, G., B. Vaneleuwen, J. Wilschut, and J. Damen.** 1983. Exchange of phosphatidylcholine between small unilamellar liposomes and human plasma high-density lipoprotein involves exclusively the phospholipid in the outer monolayer of the liposomal membrane. Biochim. Biophys. Acta **732:** 595–599.
30. **Schwendener, R. A., P. A. Lagocki, and I. E. Rahman.** 1984. The effects of charge and size on the interaction of unilamellar liposomes with macrophages. Biochim. Biophys. Acta **772:**93–101.
31. **Senior, J., G. Gregoriadis, and K. A. Mitropoulos.** 1983. Stability and clearance of small unilamellar liposomes. Studies with normal and lipoprotein-deficient mice. Biochim. Biophys. Acta **760:**111–118.
32. **Senior, J. H., K. R. Trimble, and R. Maskiewicz.** 1991. Interaction of positively-charged liposomes with blood: implications for their application *in vivo*. Biochim. Biophys. Acta **1070:**173–179.
33. **Szoka, F. C., Jr., K. Jacobson, and D. Papahadjopoulos.** 1979. The use of aqueous space markers to determine the mechanism of interaction between phospholipid vesicles and cells. Biochim. Biophys. Acta **551:**295–303.
34. **Tall, A. R., and D. M. Small.** 1977. Solubilization of phospholipid membranes by human plasma high density lipoproteins. Nature (London) **265:** 163–164.
35. **Vitas, A. I., R. Díaz, and C. Gamazo.** 1995. Protective effect of Brucella outer membrane complex included on liposomes against experimental murine brucellosis. FEMS Microbiol. Lett. **130:**231–236.
36. **Weinstein, J. N., S. Yoshikami, P. Henkart, R. Blumenthal, and W. A. Hagins.** 1977. Liposome-cell interaction: transfer and intracellular release of a trapped fluorescent marker. Science **195:**489–491.