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Improved targeting of antimony to the bone marrow of dogs using liposomes of reduced size

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Abstract

A novel liposomal formulation of meglumine antimoniate (MA), consisting of vesicles of reduced size, has been evaluated in dogs with visceral leishmaniasis to determine its pharmacokinetics as well as the impact of vesicle size on the targeting of antimony to the bone marrow. Encapsulation of MA in liposomes was achieved through freeze-drying of empty liposomes in the presence of sucrose and rehydration with a solution of MA. The resulting formulation, with a mean vesicle diameter of about 400 nm, was given to mongrel dogs with visceral leishmaniasis as an i.v. bolus injection at 4.2 mg Sb/kg of body weight. The pharmacokinetics of antimony were assessed in the blood and in organs of the mononuclear phagocyte system and compared to those achieved with the free drug and the drug encapsulated in large sized liposomes (mean diameter of 1200 nm). The targeting of antimony to the bone marrow was improved (approximately three-fold) with the novel liposomal formulation, when compared to the formulation of MA in large sized liposomes. This study provides the first direct experimental evidence that passive targeting of liposomes to the bone marrow of dogs is improved by the reduction of vesicle size from the micron to the nanometer scale.
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Keywords: Liposomes; Dogs; Leishmaniasis; Bone marrow; Antimony; Size

1. Introduction

In the 1970s, a major advance occurred when it was found that liposome-encapsulated antimonial drugs were hundreds of times more effective than unencapsulated ones for the treatment of experimental visceral leishmaniasis (VL) (Alving, 1986). This spectacular effect of liposome encapsulation was attributed to the drug sustained release property of liposomes and to their natural tendency to be cleared from the circulation by the fixed macrophages of the mononuclear phagocyte system (MPS), mainly the liver, spleen and bone marrow, which are the major sites of parasite infection. Similar results were obtained with other antileishmanial agents (Alving et al., 1980; New et al., 1981) and other vesicular systems made from non-ionic surfactants (Baillie et al., 1986), instead of phospholipids. Much effort has also been devoted to the search for effective liposomal formulations in dogs (Chapman et al., 1984; Collins et al., 1993; Valladares et al., 1997, 2001; Nieto et al., 2003; Schettini et al., 2003, 2005), as these animals respond poorly to conventional antimonial therapy and are the main reservoir for VL (Alvar et al., 1994). Nevertheless, no effective therapeutic protocol, achieving consistent parasitological cure in infected dogs, has been reported so far.

Despite the need to improve antimonial chemotherapy and the extremely promising results obtained with liposomes in experimental models of VL, no pharmaceutical formulation associating liposomes and antimonials has reached commercialization so far. This fact can be attributed, at least in part, to the technological

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difficulty of achieving long-term stability in the case of liposomal formulations of water-soluble drugs. This is in contrast with the lipophilic fungicidal and leishmanicidal drug, amphotericin B, whose liposomal formulation, called AmBisome, has recently been approved by the Food and Drug Administration for treatment of VL (Meyerhoff, 1999). On the other hand, the high cost of the latter formulation makes difficult its large-scale use in developing countries.

A novel liposomal formulation (LMA) has recently been proposed for the antimonial drug, meglumine antimoniate, that is obtained by rehydrating freeze-dried empty liposomes with a solution of the antimonial compound (Frézard et al., 2000, 2005; Demicheli and Frézard, 2005). A significant technological advantage of this method over conventional ones (Alving and Steck, 1980; Rao, 1986) is that the liposomal formulation may be stored as freeze-dried empty liposomes and that rehydration may be performed just before use. This formulation has been evaluated in both healthy and *Leishmania chagasi*-infected mongrel dogs for its ability to improve the bioavailability of the antimonial drug and to cure the infected animals (Schettini et al., 2003, 2005). A single intravenous bolus injection of LMA in healthy dogs (about 4 mg Sb/kg of body weight) resulted in high antimony levels in the liver and spleen for a long period of time. On the other hand, the bone marrow showed comparatively lower antimony concentrations (Schettini et al., 2003). Moreover, following a multiple dose-regimen in dogs with VL, LMA resulted in a significant reduction of parasite burden, but was unable to clear *Leishmania* parasites from the bone marrow (Schettini et al., 2005), suggesting that the improvement of antimony targeting to this tissue may be critical for achieving cure with LMA. It was also hypothesized that the large size of liposomes in LMA (mean diameter greater than 1 μm) may be responsible for the low targeting of antimony to the bone marrow.

The present work describes a modification of the dehydration–rehydration method, based on the use of sucrose as cryoprotectant, that allows encapsulation of meglumine antimoniate in vesicles of reduced size. Different physicochemical and pharmacological properties of the resulting formulation have been evaluated, including the kinetics of drug release in vitro, the pharmacokinetics of antimony in the blood and in different MPS organs of dogs with VL and the impact of vesicle size reduction on the concentration of antimony in the bone marrow. Infected dogs, instead of healthy animals, were used in this study because the infection, that affects mainly the MPS organs, is expected to alter significantly liposome pharmacokinetics.

2. Materials and methods

2.1. Animals

Male mongrel dogs (weighing 5–26 kg), naturally infected with *Leishmania chagasi* and destined to euthanasia, were obtained from the Centro de Zoonoses of the Prefeitura Municipal de Belo Horizonte (MG, Brazil). Animals were found to be positive according to the following tests for *L. chagasi*: indirect immunofluorescence (IFAT), complement fixation test (RFC) and enzyme-linked immunosorbent assay (ELISA), demonstration of *Leishmania* amastigotes in Giemsa-stained bone marrow aspirates and polymerase chain reaction using *L. donovani* complex-specific primers (Roura et al., 1999).

2.2. Materials

Cholesterol (CHOL) and dicetylphosphate (DCP) were purchased from Sigma Co. (St. Louis, MO, USA). Distearoylphosphatidylcholine (DSPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). *N*-methyl-D-glucamine and antimony pentachloride ($SbCl_5$, 99%) were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

2.3. Preparation of meglumine antimoniate

Meglumine antimoniate was synthesized, as previously described (Demicheli et al., 2003), from equimolar amounts of *N*-methyl-D-glucamine and pentavalent antimony oxyhydrate. The resulting product contained approximately 30% antimony by weight, as determined by plasma emission spectroscopy (ICP-OES) using a Perkin-Elmer Optima 3000 plasma emission spectrometer.

2.4. Preparation and characterization of meglumine antimoniate-containing liposomes

Small unilamellar vesicles (SUVs) were prepared by ultrasonication of a suspension of multilamellar vesicles in deionized water made from DSPC, CHOL and DCP (molar ratio of 5:4:1), at the final lipid concentration of 55 g/l. After filtration through sterile 0.22 μm membrane, the SUVs suspension was mixed either with water (preparation 1) or with an aqueous sucrose solution (preparation 2) at a sugar/lipid mass ratio of 3:1 and a final sugar concentration of 0.3 M. The resulting mixture was immediately frozen in liquid nitrogen and subsequently lyophilized (Labconco freeze-dryer, 4.5 l).

Rehydration of the dried powder was performed with an aqueous meglumine antimoniate solution (antimony concentration of 80 g/l) as follows: 40% of the original SUVs volume of meglumine antimoniate solution was added to the lyophilized powder and the mixture was vortexed and incubated for 30 min at 55 °C; the same volume of phosphate buffer saline (PBS: 0.15 M NaCl, 0.01 M phosphate, pH 7.4) was then added and the mixture was vortexed and incubated for 30 min at 55 °C. Drug-containing liposomes were separated from the non-encapsulated drug by centrifugation (14,000 × *g*, 30 min). The liposome pellet was then washed twice and finally resuspended in PBS at a final antimony concentration of about 10 g/l. The amount of antimony was determined in the resulting liposome suspension by ICP-OES, after digestion of the sample with nitric acid. Phospholipid concentration was determined in the final liposome suspensions using a colorimetric assay (Stewart, 1980). The size of the vesicles in suspension was determined by photon correlation spectroscopy at 25 °C and a 90° scattering angle using a channel correlator (Malvern Instruments, type 3000HS) in conjunction with a He/Ne laser (wavelength 633 nm, nominal power

output 32 mW). The mean hydrodynamic diameter and polydispersity index were determined.

2.5. Kinetics of release of antimony from liposomes

Liposomal preparations were diluted in PBS at a final lipid concentration of 5 g/l and incubated at 37 °C under constant stirring (microstirring bars). After different times of incubation, samples were centrifuged ($14.000 \times g$, 30 min), supernatants were recovered and antimony was determined by ICP-OES.

2.6. Pharmacokinetics and tissue distribution of antimony in dogs

Animals were divided into three groups. The first group (10 animals) received an intravenous bolus injection of meglumine antimoniate-containing liposomes prepared without sucrose (preparation 1) at 5.7 mg Sb/kg of body weight and bone marrow aspirates were obtained after 96 h. The second group (5 animals) received an intravenous bolus injection of meglumine antimoniate (meglumine antimoniate in distilled water at 0.66 M Sb) at 100 mg Sb/kg of body weight and animals were then sacrificed 96 h after administration. The third group (14 animals) received an intravenous bolus injection of meglumine antimoniate-containing liposomes prepared with sucrose (preparation 2) at 4.2 mg Sb/kg of body weight. Animals were then sacrificed, 24 h (3 animals), 48 h (3 animals), 72 h (3 animals) and 96 h (5 animals) after administration. Before sacrifice at 96 h, blood samples were obtained from animals at the following time intervals: 5, 10, 20, 30, 40, 60, 80, 100, 120, 150 min, 3, 4, 6, 8, 24, 48, 72 and 96 h. In the second and third groups, liver and spleen were recovered, homogenized and frozen at −20 °C. In the case of the bone marrow, two samples were obtained from both the femur and humerus and the uptake values were averaged.

A barbituric drug (sodium thiopental) was used to perform the humane euthanasia of the dogs, as described previously (Smith et al., 1986) and routinely used at the Veterinary Hospital of the Federal University of Minas Gerais. The present research adhered to the Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985) and received approval from the Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais.

Antimony was determined in all tissues as previously described (Schettini et al., 2003, 2005). Tissues were submitted to digestion with nitric acid in a microwave oven (CEM, MDS 200). Antimony was assessed by electrothermal atomic absorption spectrometry, using a Perkin-Elmer Z 5100 graphite furnace atomic absorption spectrometer. All analyses were performed using a continuous background correction. The standard curve for determination of Sb concentrations was linear over the range of concentration tested (0.1–100 μg/ml). The quantification limits of the analytical method were 0.2 μg Sb/ml for blood samples, 0.19 μg Sb/g for bone marrow samples, 0.70 μg Sb/g for liver samples, 0.76 μg Sb/g for spleen samples.

The proportion of total antimony dose recovered from liver and spleen were calculated using the actual weight of the organs. The bone marrow was estimated to be 2.2% of the total body weight in dogs (Hussain et al., 1989).

2.7. Pharmacokinetic analysis

Pharmacokinetic parameters were determined for liposomal meglumine antimoniate using compartmental analysis. Experimental blood concentration–time data were best fitted by a two-compartment open model with i.v. bolus input. Iterative weighted nonlinear least-squares regression with the Rstrip 4.03 computer program was used and model selection was guided by Akaike's information criterion (Yamaoka et al., 1978). Fitted parameters included the disposition phase half-life ($t_{1/2\alpha}$), the terminal phase half-life ($t_{1/2\beta}$), the area under the blood concentration–time curve projected to infinity ($AUC_{0-\infty}$), the volume of distribution at steady state (V_{ss}), the total body clearance (CL) and the mean residence time projected to infinity ($MRT_{0-\infty}$).

2.8. Statistical analysis

Comparisons between the tissue levels of antimony at different times and in different organs were performed by analysis of variance (one-way ANOVA, with Tukey's multiple comparison post test). The difference between bone marrow antimony levels after large and small liposomes was evaluated using Student 's unpaired *t*-test. A two-tailed *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Novel method for encapsulation of meglumine antimoniate in liposomes of reduced size

We previously reported that the rehydration of freeze-dried empty liposomes with a solution of meglumine antimoniate allows encapsulation of the drug in liposomes with a high trapping efficiency (Frézard et al., 2000, 2005; Demicheli and Frézard, 2005). However, the large size of these liposomes (mean diameter greater than 1 μm) presents a problem for intravenous administration. In addition, this formulation promoted relatively low antimony levels in the bone marrow of dogs with VL and did not eliminate parasites from this tissue (Schettini et al., 2005). In an attempt to overcome these limitations, a modification was introduced in the process of liposome preparation (Frézard et al., 2004), so as to obtain liposomes of reduced size, taking advantage of the ability of cryoprotective sugars to control membrane fusion during freeze-drying (Womersley et al., 1986).

Table 1 shows the influence of two disaccharides on the size and drug encapsulation efficiency of liposomes. Accordingly, reduction of liposome mean hydrodynamic diameters was achieved with both sugars, with no significant change of drug encapsulation efficiencies (35–50%) and final Sb/lipid ratios (about 0.25:1, w/w). Sucrose was more effective at a sugar/lipid mass ratio of 3:1 than at 1:1 ratio in promoting particle size reduction. Strikingly, the polydispersity index of the liposome population was also significantly reduced in the presence of

Table 1
Influence of cryoprotective sugars in the process of liposome preparation on the characteristics of meglumine antimoniate-containing liposomes

Process[a]	Initial sugar/lipid ratio (w/w)	Mean diameter (nm) ± S.D.[b]	Encapsulation efficiency (% Sb) ± S.D.[b]
Without sugar	0	1200 ± 450	39 ± 3
With sucrose	1:1	510 ± 100	43 ± 4
	3:1	410 ± 75	40 ± 4
With trehalose	3:1	460 ± 180	53 ± 9

[a] Small unilamellar vesicles made from DSPC, CHOL and DCP (molar ratio of 5:4:1) were freeze-dried in the presence or absence of sugar and then rehydrated with a solution of meglumine antimoniate at a Sb/lipid mass ratio of 0.58:1.
[b] SD: standard deviation (n = 3–8).

sugar. Thus, sucrose at a sugar/lipid ratio of 3:1 resulted in significant reductions of the liposome diameter, from 1200 to 410 nm, and of the polydispersity index, from 0.7 to 0.3.

Fig. 1 shows the impact of sucrose on the kinetic of release of antimony from liposomes at 37 °C, after elimination of non-encapsulated drug and resuspension of liposomes in PBS. The kinetics of drug release were biphasic, with a first phase of fast drug release, followed by a second phase of sustained release. The amount and rate of drug release during the first phase were found to be significantly higher for the formulation prepared with sucrose, when compared to that prepared without sugar. Thus, the presence of sucrose enhanced antimony release from liposomes. This effect of sucrose may be explained by the increase in osmotic pressure difference between internal and external compartments, as a result of the co-encapsulation of sucrose and meglumine antimoniate.

3.2. Pharmacokinetics of antimony in the blood, liver, spleen and bone marrow of dogs following administration of liposomes with reduced size

The novel liposomal formulation of meglumine antimoniate was further evaluated in mongrel dogs naturally infected with *L. chagasi*. For this purpose, the liposomal drug was given intravenously as a bolus injection at 4.2 mg Sb/kg and 20 mg lipid/kg of body weight. Fig. 2 shows the pharmacokinetics of antimony in the blood. Data are consistent with a biexponential open model. The elimination of antimony from blood occurred within 3 h after administration, showing a first rapid phase followed by a second slower phase. From 3 to 96 h, the blood concentrations of antimony were below the method quantification limit (0.2 μg Sb/ml). Table 2 displays the pharmacokinetic parameters obtained from this experiment.

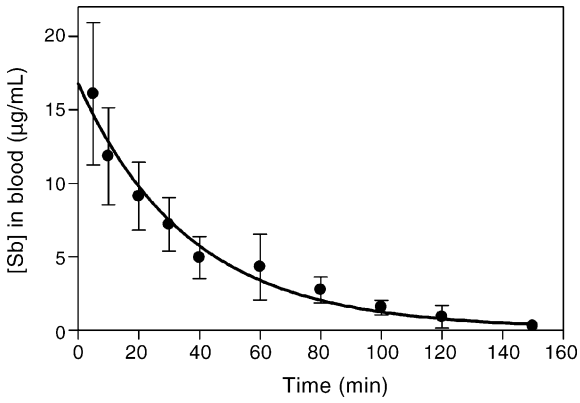

Fig. 2. Pharmacokinetics of antimony in the blood of dogs with VL, after intravenous bolus injection of meglumine antimoniate-containing liposomes prepared with sucrose, at 4.2 mg Sb/kg of body weight. Data are given as means ± standard deviation (n = 4–5 animals). Data were fitted to a biexponential decay. The model fitted the mean data well with a coefficient of determination (r^2) of 0.995.

Fig. 3 shows the distribution of antimony in the MPS organs which are known to harbor *Leishmania* parasites, at different times after injection of the liposomal drug. High levels of antimony, in the range of 25–60 μg Sb/g of wet organ, were found in the liver and spleen of animals. On the other hand, in the bone marrow, about 10-fold lower antimony levels, in the range of 1.2–5 μg Sb/g of wet tissue, were determined. Strikingly, no

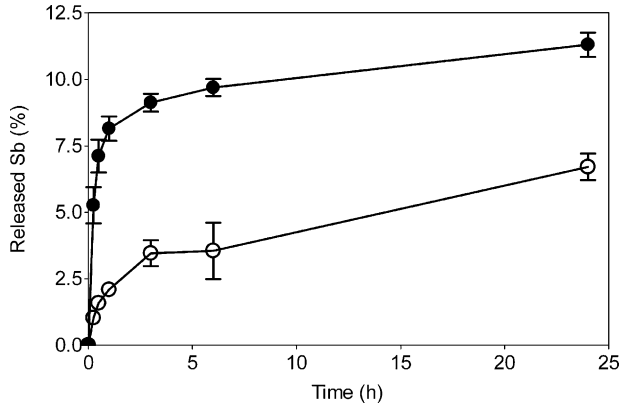

Fig. 1. Kinetics of release of antimony at 37 °C from meglumine antimoniate-containing liposomes prepared with (filled circles) or without (empty circles) sucrose and resuspended in drug-free PBS. Data are given as means ± standard deviation (n = 3).

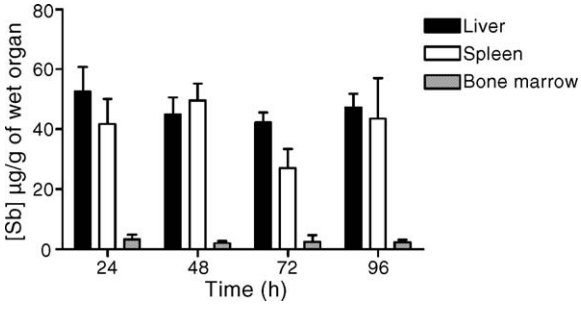

Fig. 3. Distribution of antimony in dogs with VL, after intravenous bolus injection of meglumine antimoniate-containing liposomes prepared in the presence of sucrose, at 4.2 mg Sb/kg of body weight. Data are given as means ± standard deviation (n = 3–5 animals).

Table 2
Pharmacokinetic parameters in dogs with VL[a], corresponding to analysis of blood concentration after intravenous injection of meglumine antimoniate-containing liposomes

Animal	Weight (kg)	$t_{1/2\alpha}$ (min^{-1})	$t_{1/2\beta}$ (min^{-1})	$AUC_{0-\infty}$ (g min/l)	V_{ss} (ml/kg)	CL (ml/min/kg)	$MRT_{0-\infty}$ (min)
1	15.8	11.9	47.1	0.83	230.6	5.1	45.4
2	13.5	0.57	34.5	1.78	34.0	2.4	14.4
3	26.2	0.59	46.6	2.99	27.8	1.4	19.8
4	11.7	31.8	72.8	0.63	306.7	6.7	39.9
5	13.0	3.2	27.7	0.54	270.4	7.8	34.9
Mean	16.0	10	46	1.35	174	4.7	31
S.D.	5.9	14	18	1.04	133	2.8	14

[a] Dogs ($n=5$) were given a single bolus intravenous injection of meglumine antimoniate-containing liposomes prepared with sucrose, at 4.2 mg Sb/kg of body weight. Abbreviations: $t_{1/2\alpha}$, disposition phase half-life; $t_{1/2\beta}$, terminal phase half-life; $AUC_{0-\infty}$, area under the blood concentration–time curve projected to infinity; V_{ss}, volume of distribution at steady state; CL, total body clearance; $MRT_{0-\infty}$ mean residence time projected to infinity.

significant variation of antimony levels as a function of time was observed in the three organs (one-way ANOVA, $P>0.05$), indicating a long persistence of antimony in these organs for at least 4 days.

3.3. Impact of drug encapsulation on antimony levels in MPS organs

Fig. 4 compares the levels of antimony achieved in the different MPS organs, 96 h after administration either of the novel liposomal formulation (4.2 mg Sb/kg) or of the free drug given at a therapeutic dose (100 mg Sb/kg). According to this data, the liposomal drug promoted a marked targeting of antimony to MPS tissues. Even at a 23-fold lower dose of antimony, the liposomal formulation resulted in antimony levels, two-fold, 63-fold and 68-fold higher in the bone marrow, liver and spleen, respectively, when compared to the free drug. It is also noteworthy that the antimony concentration after liposomal drug was significantly lower in the bone marrow than in the other MPS organs. This is in contrast with the free drug that did not show any significant difference in antimony levels between the three MPS organs (one-way ANOVA, $P>0.05$).

The proportions of total antimony dose recovered from the liver, spleen and bone marrow were also calculated (Table 3) to further evaluate the impact of drug encapsulation on in vivo antimony distribution. This data clearly confirms that treatment with the liposomal drug directed a much greater proportion of the dose to these tissues.

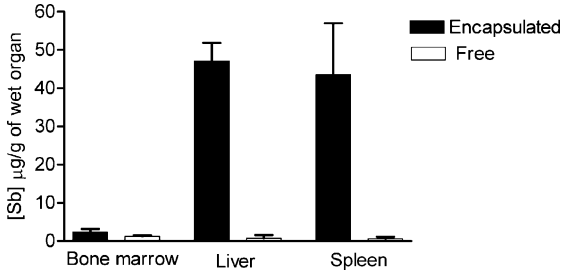

Fig. 4. Comparison of antimony levels at 96 h in different MPS organs of dogs with VL after administration of meglumine antimoniate-containing liposomes prepared with sucrose (4.2 mg Sb/kg) or free meglumine antimoniate given at a therapeutic dose (100 mg Sb/kg). Data are given as means ± standard deviation ($n=4$–5 animals).

Table 3
The proportion (%, mean ± S.D.) of the total antimony dose recovered from liver, spleen and bone marrow of dogs, 96 h after dosing with free or liposomal meglumine antimoniate[a]

	Percentage of injected dose	
	Free	Liposomal
Liver	0.021 ± 0.019	38.0 ± 6.5
Spleen	0.0035 ± 0.0029	7.0 ± 4.3
Bone marrow	0.027 ± 0.007	1.15 ± 0.32

[a] Animals were given a single intravenous bolus injection of either free (100 mg Sb/kg) or liposomal meglumine antimoniate prepared with sucrose (4.2 mg Sb/kg).

3.4. Influence of liposome size on antimony targeting to the bone marrow of infected dogs

Fig. 5 compares the antimony level achieved in the bone marrow of dogs 96 h after injection of the novel liposomal formulation at 4.2 mg Sb/kg to that after a liposomal formulation exhibiting higher vesicle size (prepared without sucrose) given at 5.7 mg Sb/kg.

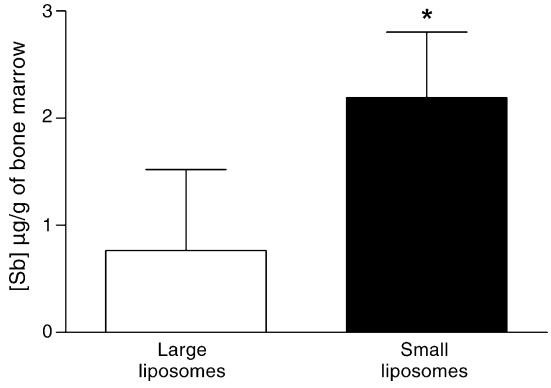

Fig. 5. Influence of liposome size on antimony level in the bone marrow of infected dogs, 96 h after intravenous bolus injection of meglumine antimoniate-containing liposomes. Small liposomes (prepared in the presence of sucrose, with a mean vesicle diameter of 410 nm) were given at 4.2 mg Sb/kg of body weight ($n=5$). Large liposomes (prepared in the absence of sucrose, with a mean vesicle diameter of 1200 nm) were given at 5.7 mg Sb/kg of body weight ($n=10$). Data are given as means ± standard deviation. $^{*}P=0.003$ for unpaired t-test.

A significantly (three-fold) higher antimony level was found in the bone marrow of infected dogs after small sized liposomes (mean diameter of 410 nm) than after large sized ones (mean diameter of 1200 nm). This data strongly suggests that the reduction of liposome size promoted a higher drug targeting to the bone marrow of dogs.

4. Discussion

Two advantageous encapsulation methods have recently been proposed by our group for meglumine antimoniate (Frézard et al., 2000, 2005; Demicheli and Frézard, 2005). The first method used the dehydration–rehydration procedure (Kirby and Gregoriadis, 1984), that consists of mixing a suspension of liposomes (SUVs) prepared in water with an aqueous drug solution and then freeze-drying the mixture. Rehydration of the resulting lyophilized powder with water and saline under controlled conditions of temperature and lipid concentration resulted in multilamellar vesicles with high drug encapsulation efficiency (>30%). The second method consisted of freeze-drying a suspension of SUVs prepared in water and then rehydrating the lyophilized powder with a solution of meglumine antimoniate (Frézard et al., 2000, 2005; Demicheli and Frézard, 2005). A significant technological advantage of these methods, when compared to conventional ones (Alving and Steck, 1980; Rao, 1986), is that the liposomal formulation may be stored as an intermediate lyophilized product: as a liposome–drug mixture (first method) or as pre-formed empty liposomes (second method). The final rehydration step may then be performed just before administration, using water (first method) or a meglumine antimoniate solution (second method). A significant advantage of the second method is that it does not expose the drug to freeze-drying, thereby reducing the risk of chemical alteration of the drug (Roberts et al., 1998). Finally, the resulting formulation also showed the expected high antileishmanial activity in hamsters infected with *L. chagasi* (Frézard et al., 2000). On the other hand, the large size of vesicles (mean diameter greater than 1 μm) in these formulations appears to be a problem for therapeutic use, because of the expected side effects following intravenous administration and, presumably, of the low drug targeting to the bone marrow of dogs (Schettini et al., 2003, 2005). In this context, the modification of the process of preparation of these formulations, based on the use of sucrose as cryoprotectant, represents an important achievement, since vesicles with significantly reduced size were obtained without affecting the drug encapsulation efficiency. Methods of drug encapsulation, relying on the rehydration of freeze-dried empty liposomes with a drug solution, have been described previously (Yachi et al., 1996; Frézard et al., 2000; Peer and Margalit, 2000; Stevens and Lee, 2003). However, the use of a cryoprotectant in this process in order to control the final size of vesicles, although already employed in active encapsulation methods (Stevens and Lee, 2003), had not yet been proposed for passive encapsulation. Therefore, the demonstration of the feasibility of this process is an important contribution of the present study to the field of liposome technology (Frézard et al., 2004). Even though the mean diameter of liposomes was significantly reduced from 1200 to 400 nm, we were unable to prepare by this process liposomes with a mean diameter of less than 200 nm, at least with this specific lipid composition.

The pharmacokinetics of antimony in the blood of dogs, following intravenous administration of the resulting liposomal formulation, was found to be biphasic and characterized by elimination half-lives of about 10 and 45 min. At least one of the two observed phases should correspond to the capture of liposomes by MPS organs, as more than 45% of injected antimony was encountered in the liver, spleen and bone marrow. Assuming that meglumine antimoniate remained essentially encapsulated after entering blood circulation, the biphasic profile of the blood pharmacokinetic curve may be interpreted by the existence of two distinct populations of phagocytic cells with either differing capture efficiencies or differing saturation levels. It is also noteworthy that the observed pharmacokinetic profile differs markedly from that of meglumine antimoniate alone in dogs, which exhibited a slower terminal phase with a half-life of 6–11 h (Valladares et al., 1996, 2001).

The long tissue persistence of antimony, following intravenous administration of the liposomal drug in dogs, constitutes an important finding of the present study. We observed no significant variation of antimony levels in the MPS organs over a period of at least 3 days after administration. The tissue persistence of antimony can be compared to that achieved with either the free drug (Valladares et al., 1996) or the drug encapsulated in liposomes of the same lipid composition but of larger size (Schettini et al., 2005). Valladares et al. (1996), in their study of the pharmacokinetics of free meglumine antimoniate in dogs, attributed the terminal phase of plasma drug elimination (with half-life of about 10 h) to the release of antimony from the tissues. On the other hand, Schettini et al. (2005) have reported a half-life of about 4 days for the elimination of antimony from the bone marrow of dogs with VL after administration of meglumine antimoniate-containing liposomes with a mean diameter in the micron range (Schettini et al., 2005). Accordingly, the rate of antimony elimination from the bone marrow of dogs would be slightly faster after large sized liposomes than after small sized ones. In order to account for the apparent difference in the rate of tissue elimination between the free and encapsulated drugs, two hypotheses can be suggested. The free and encapsulated drugs may exhibit different tissue and/or cellular distributions. Alternatively, the degradation of liposomes within the phagolysosomes of macrophages may be the rate-limiting step for the release of antimony from MPS organs. Since the present formulation is made from a high phase transition temperature phospholipid (DSPC) and a high proportion of CHOL, a slow rate of lysosomal degradation is indeed expected (Moghimi and Patel, 1998). A possible explanation for the influence of vesicle size on the retention of antimony by the bone marrow may be that the higher number of vesicles, expected in the formulation of smaller liposomes, resulted in a higher number of vesicles per macrophage and in a reduction of the effectiveness of these cells for liposome degradation.

The demonstration that small sized vesicles resulted in a three-fold higher antimony level in the bone marrow of dogs when compared to larger vesicles (given at a 20% higher dose

of antimony) represents an important contribution of this work. Three different studies led us to hypothesize that small sized liposomes may target more effectively the bone marrow of dogs. Small sized liposomes (mean diameter less than 100 nm) containing an antimonial drug were more effective than large sized vesicles in reducing the number of *Leishmania* parasites in the bone marrow of mice (Carter et al., 1989). Moreover, small liposomes (mean diameter less than 150 nm), made from equimolar amounts of CHOL and DSPC, showed a prolonged circulation time and a much higher accumulation in the bone marrow of rats, when compared to larger vesicles (Senior et al., 1985). Nevertheless, these results obtained in mice and rats may not apply to dogs, since the capture of lipoprotein particles by the bone marrow was found to be strongly species-dependent (Hussain et al., 1989). Finally, small non-ionic surfactant vesicles (mean diameter less than 100 nm) given at 0.68 mg Sb/kg (Collins et al., 1993) promoted antimony levels in the bone marrow of healthy dogs similar to those of larger conventional liposomes (mean diameter greater than 1000 nm) given at 3.8 mg Sb/kg (Schettini et al., 2003). The present study, however, is the first to demonstrate unambiguously, in infected dogs and for liposomes with the same lipid composition and a mean diameter in the range of 300–2000 nm, that smaller vesicles improved the drug targeting to the bone marrow.

Despite the numerous investigations on the factors affecting the in vivo fate of liposomes (Senior et al., 1985; Hwang, 1987; Woodle and Lasic, 1992; Moghimi and Patel, 1998), the mechanistic nature of the effect of liposome size remains unclear and no universal model has emerged so far. The influence of curvature in terms of changes to the lipid packing may apply to radii of less than 150 nm, but differences are not expected above this size (Woodle and Lasic, 1992). It has been proposed that liposomes of differing morphologies (size and lamellarity) and surface characteristics may attract different arrays of plasma proteins, called "opsonins", the content and conformation of which may account for the different pattern in the rate and site of vesicle clearance from the blood (Moghimi and Patel, 1998). Accordingly, a possible explanation to our findings is that, by reducing the size of liposomes, the total exposed surface area of liposomes is increased and the pattern of bound opsonins is altered. Alternatively, considering that the capture of liposomes by macrophages is a saturable process and should depend on the total surface area (Abra and Hunt, 1981) and that liposomes are taken up primarily by liver and spleen macrophages (Hwang, 1987), one can propose that the higher concentration of exposed lipid approached the level of saturation of these organs and that a higher proportion of liposomes remained available to the bone marrow. These interpretations are consistent with the fundamental nanotechnology concept that significantly modified properties of a system are observed, when the size of this system is reduced from the micron to the nanometer range, as a result of increased surface area.

In conclusion, a novel method is reported for the encapsulation of meglumine antimoniate in liposomes, that resulted in high drug encapsulation efficiency and reduced vesicle size. The resulting formulation showed an improved targeting to the bone marrow of dogs with VL and promoted a higher tissue retention of antimony, when compared to the free drug and to a liposomal formulation with larger vesicle size. Future studies will aim at evaluating the antileishmanial efficacy of this novel formulation in dogs naturally infected with *L. chagasi*.

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