FOLIA PARASITOLOGICA 41: 161–167, 1994

A comparative study of the utilization of ether- and ester-linked phospholipid-containing liposomes by J774.E1 macrophage cell-line infected with *Leishmania mexicana mexicana* amastigotes

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Key words: *Leishmania m. mexicana*, ester- , ether-linked phosphatydilcholine, ligands (native LDL, acetylated LDL), liposome uptake

Abstract. The macrophage cell-line J774.E1 and *Leishmania m. mexicana* infection was used to investigate the uptake of liposomes, which differed in their bulk phospholipid: ester- or ether-analogue of phosphatydilcholine (PC). The receptor-mediated uptake of both species of liposomes, containing native or acetylated LDL as ligands was also evaluated. Uninfected and infected J774.E1 cell-line accumulated more ester- and ether-liposomes alone than mixed type (50:50, ester/ether). The utilization was significantly enhanced when both types of liposomes contained native LDL. The highest uptake was recorded for liposomes bearing acetylated LDL by infected J774.E1 cells. Accumulation of ester- and ether-liposomes with the same ligand was not markedly affected by different chemical nature of PC. Finally, ether-liposomes alone possessed certain activity against *Leishmania m. mexicana* amastigotes. The results presented here demonstrated the usefulness of ether-liposomes with specific ligands in site-specific delivery of antileishmanial compounds *in vitro*.

Leishmaniasis is a widespread disease affecting some 20 million people, with approximately 400 thousand new cases each year (Behin and Louis 1984). The amastigote form of *Leishmania* is an obligate intramacrophage parasite, proliferating in phagolysosomes of phagocytic cells. It has been shown that liposomes containing antileishmanial drugs (e.g. Pentostam, Glucantime) greatly enhanced pharmacological efficacy of these drugs against the parasites *in vivo* (Alving et al. 1978, New and Chance 1980). Liposomes are gradually cleared from the circulation by macrophages in the reticuloendothelial system (RES). Macrophages also play an important role in the metabolism of lipids which are transported in plasma by lipoprotein vesicles. The involvement of mononuclear phagocytic cells in the clearance and degradation of chemically-modified, low-density lipoprotein (LDL) has been reported by many authors (Goldstein et al. 1979, Quinn et al. 1987, Nicolas et al. 1990). During the chronic stages of *Leishmania* infection, the receptor-mediated uptake and degradation of lipoproteins by the host's macrophages could be used by the parasite as a source of lipids for amastigotes (Hart 1987).

The group of phospholipids containing an ether- instead of an ester-bond between the alkyl chain and the glycerol backbone have many physiological functions in living organisms, including prokaryote cells (Mangold and Webber 1987). Lipids with an ether-bond are known to be highly resistant to metabolic conversion (Stein et al. 1986), and liposomes containing ether-lipids as their bulk phospholipids show a prolonged circulation time and increased resistance against lysosomal degradation (Derksen et al. 1988).

In the present work, an *in vitro* experimental-model system, the macrophage cell-line J774.E1 and *Leishmania mexicana mexicana* infection, was used to investigate the utilization of different kinds of liposome preparations by uninfected and infected macrophages. Comparison has been made between the uptake of the liposomes, which differ in their bulk phospholipid: ester- or ether-analogue of phosphatidylcholine and also the utilization of both types of liposomes, containing native or acetylated LDL, by the same cell-line. The basic physico-chemical characteristics of prepared liposomes were determined, as regards their possible role on cell-liposome interactions. Finally, antileishmanial activity of ether-liposomes alone on *Leishmania* infection in the peritoneal macrophages *in vitro* was also investigated.

MATERIALS AND METHODS

Liposome preparation. Multilamellar liposomes (MLV) were prepared by a proliposome method, after Perrett et al. (1991). For the liposome preparation the following were used: dipalmitoyl phosphatidylcholine (DPPC), dicetylphosphate (DCP), cholesterol (Chol), all of which were purchased from Sigma Chemical Company Ltd, U.K. The ether analogue of DPPC (1-O-octadecyl-2-O-methyl-rac-glycerol-3-phosphorcholine) was a gift from Medmark Pharma GmbH (Grunwald bei München, Germany). Aliquots of stock solutions in chloroform/methanol (3:1) of DPPC, Chol and DCP were mixed in molar ratio 42:12:6. The corresponding weights of these lipids were decreased 4 times in comparison with the original method. With the other liposomes, either 100% of the ether-analogue of DPPC or a 50:50 mixture of ester- and of ether-analogue of phosphatidylcholine (PC) replaced DPPC in the initial mixture of lipids. The molar ratio of all lipid substances was the same in each kind of liposome. As a hydrophilic radioactive marker, ^{14}C-sucrose (5 μCi/ 5μl) was added to the initial lipid suspension and unentrapped sucrose was removed by dialysis (pore size, 4.2 nm) in distilled water at 4°C for 24 h. Liposome preparations were then filtered through a 0.4-m size sterile polycarbonate membrane.

Liposomes with ligands, both native LDL (nat.LDL) and acetylated LDL (ac.LDL) were prepared, following the same protocol as outlined above. Nat.LDL was isolated from human serum of healthy donors and was a gift from Professor David Leake (London Hospital). Nat.LDL or ac.LDL was added to the initial lipid mixture in a quantity such that each liposome preparation contained 0.5–1.0 mg of apoprotein-B.

Acetylation of apo-B from LDL. The determined concentration of apo-B in nat.LDL was 13.4 mg/ml and its acetylation was carried out with acetic anhydride. The volume of acetic anhydride used for acetylation was in the ratio of 1mg apo-B : 10 μl acetic anhydride. Aliquots of acetic anhydride were added dropwise over a period of 1 h, stirring continuously in an ice-water bath under sterile conditions. The reaction mixture was stirred for a further hour and then transferred to the dialysis tube (4.2 nm pore). Dialysis was carried out in buffer (pH 7.4) containing NaCl, NaH_2PO_4, Na_2HPO_4 and EDTA.Na_2 in a molar ratio (mMol) of 154:16.7:21.1:100, in distilled water overnight at 4°C. After dialysis, the ac.LDL was transferred under sterile conditions into tubes and stored at 4°C.

Acetylation confirmation. For the confirmation of apo-B acetylation, electrophoresis on 1% agarose gel was performed. The gel was run at a constant 85 volts for 3 h and was then fixed in 100 ml methanol/ acetic acid solution (40%/10%) for 1 h. Staining of the gel was performed with Coomassie blue (0.025%) in distilled water under continuous stirring for 2 h, following which the gel was destained within 2 h using a 50% ethanol/10% acetic acid solution.

Physico-chemical characteristics of liposomes. For each liposome type used the total amount of lipid in the liposomes was determined by a modified method of Knight et al. (1972), using standard chemicals (Bio-la test, Lachema Brno, Czech Republic). The average recovery of lipids in liposomes was 83.7%. Entrappment of the radioactive marker, ^{14}C-sucrose in liposomes varied from 69.4% to 82.7%, and the final radioactivity for 1 μg of liposomal lipids in each sample was then calculated. Size analysis of liposomes was performed on an Autosizer Model 700 (Malvern Instruments Ltd., Malvern, U.K.), using laser light scattering (photon correlation spectroscopy) as a basis for measurement of particle size. The measurement of the size distribution of liposomes without ligands showed that the suspension of ether-liposomes was comprised mostly of smaller particles (40–109 nm in diameter), the fraction with a diameter of 83 nm being the most frequent (37%). The most common fraction (21.5%) of ester-liposomes had an average size of 109 nm. The "mixed" type of liposomes formed the less-homogeneous suspension. Compared with the two previous kinds of liposomes the larger particles were the most abundant (the most frequent fraction (26.4%) being 160 nm). In the suspension of ester-liposomes with ligands (nat.LDL, ac.LDL), the fraction with an average size of 109 nm in diameter was the most frequent (38.9%). Determination of the size distribution of ether-liposomes with ligands showed that they were less homogeneous than the ester-liposomes with ligands; two peaks were found at 109 nm (19.3%) and 160 nm (13.6%), respectively.

Protein content. Protein was assessed by the Bradford protein macroassay (Bradford 1976), using bovine serum albumin (Sigma, U.K.) as the protein standard. The absorbance was read at 595 nm in a Uvicon 810P Kontron spectrophotometer.

Radioactivity counting. The specific activity of ^{14}C-sucrose in the lysate of cells was measured using a liquid scintillation counter. After the particular incubation period, the medium from the wells was removed, the cell monolayers washed three times with 1 ml phosphate-buffered saline (PBS) (pH 7.2) and dissolved in 1 ml of 10 mM NaOH/0.1% (v/v) Triton X-100. Twenty μl of cell suspension were kept for protein determination, and the rest was used for counting in a Beckman LS-6800 scintillator counter. Radioactivity in the aliquots of liposome samples was also measured before and after dialysis.

Parasites. *Leishmania m. mexicana* (strain M379) was isolated originaly from a patient with the cutaneous form of the disease in Belize. The promastigotes of *Leishmania m. mexicana* were cultured continuously in monophasic semi-defined medium (SDM), supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) and 0.005% haemin, and maintained as described in Hart and Opperdoes (1984). For experimental purposes, the *Leishmania* promastigotes were harvested at the late log-phase of growth.

Infection of macrophages with *Leishmania*. The macrophage-like cell line, J774.E1 is maintained continuously at King's College London in RPMI-1640 (Flow, U.K.), supplemented with 20 mM L-glutamine (Flow, U.K.) and 20% (v/v) heat-inactivated FCS (Gibco, U.K.) at 35°C in 5% CO_2 and 85% humidity. The cells were maintained by subpassing once a week. Peritoneal exudate cells from mice were removed in RPMI-1640 medium, centrifuged at 1000 rpm/min for 10 min, and, resuspended in RPMI-1640 containing 20% FCS-HI, was added to the cells. For the uptake study, the adherent J774.E1 cells were detached by freshly-thawed trypsin/EDTA (Flow, U.K.) treatment on ice, spun at 1000 rpm for 10 min at 4°C, then re-suspended in 1 ml of medium and counted using a Burker haemocytometer. The final volume of

medium was calculated so as to obtain a dilution of $2x10^5$ cells/ml and 1 ml of cell suspension was added per well (24 Well Linbro plates) in quadruplicate. Peritoneal macrophages from mice BALB/c strain were counted, then plated at a density of $6x10^4$ macrophages/0.5 ml/well in 8-well Lab-tek slides and allowed to adhere for 4 h. Macrophages were infected with *Leishmania m. mexicana* promastigotes at a 1:10 ratio. After incubation overnight the medium with the nonadherent cells and untransformed promastigotes were removed, washed with RPMI only, and the appropriate amount of liposomes in RPMI (without FCS) added and incubated at 35°C.

For the evaluation of ether-liposomes toxicity on amastigotes, the mean number of amastigotes per 100 peritoneal macrophages at each liposome lipid concentration and control cells was measured. Antileishmanial activity (%) of ether-liposomes was expressed as follows:

$$\text{antileishmanial efficacy of liposomes (\%)} = \frac{\text{No. amastigotes (control)} - \text{No. amastigotes (exposed)}}{\text{No. amastigotes (control)}} \times 100$$

Statistical analysis. An analysis of variance was used to evaluate the differences in uptake of the different kinds of liposome, at 0.05 level of significance. In toxicity study of ether-liposomes the differences between groups were analysed using Student's *t*-test on the same level of significance.

RESULTS

In all of the experiments, the amount of liposomes taken up by the J774.E1 macrophage cell-line was calculated on the basis of radioactivity counts of ^{14}C-sucrose entrapped in liposomes. Uninfected macrophages of the J774.E1 cell-line were incubated with ester-, ether- and mixed-types of liposomes for up to 6 h, at a concentration of 50 μm liposomal lipids/$2x10^5$ cells/well. The amount of liposomes phagocytosed by the cells was determined after 2, 4 and 6 h of incubation and expressed as nanomols of liposomal lipids/μg cell protein. The uptake of liposomes by the non-infected J774.E1 cell-line is shown in Fig. 1A. Ester-liposomes were phagocytosed by the cells at the highest rate, ether-liposomes nonsignificantly less so, with the mixed types of liposomes (50:50 ether/ester) accumulating least of all ($P<0.05$). Initially within over first 2 h of incubation, the rate of liposome sequestration was greatest, after which liposome-cell interaction slowed down until, by the end of the incubation period (6 h), there was apparent saturation of the process. A very similar uptake kinetics was observed for all of the liposome preparations when J774.E1 cells infected with *Leishmania m. mexicana* amastigotes were used instead (Fig. 1B).

The effect of liposome concentration on the phagocytosis of liposomes by uninfected J774.E1 cells was measured. Liposome preparations were added to the incubation medium in an arithmetically increasing concentration of liposomal lipids/ml, and the portion taken up after 4 h of incubation determined. The highest rate of liposome uptake was found at a concentration of

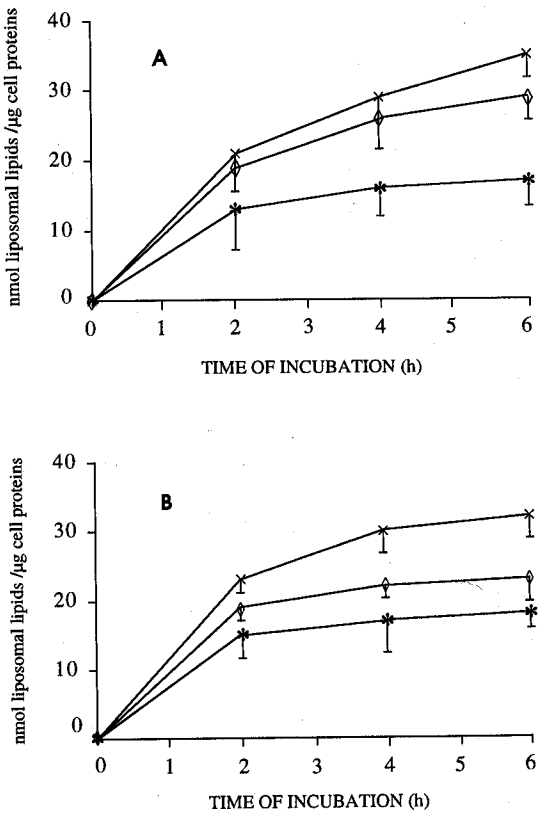

Fig. 1A–B. The time-dependent uptake of ester-, ether- and mixed (50:50 ester:ether) type of liposomes by the J774.E1 cell-line *in vitro*. **A** – uninfected cells, **B** – infected cells with of *Leishmania mexicana mexicana* amastigotes. Dose of liposomal lipids/$2x10^5$ cells/well = 50 μg. (—×— ester-liposomes, ····· ◊ ····· ether-liposomes, —✱— mixed type of liposomes).

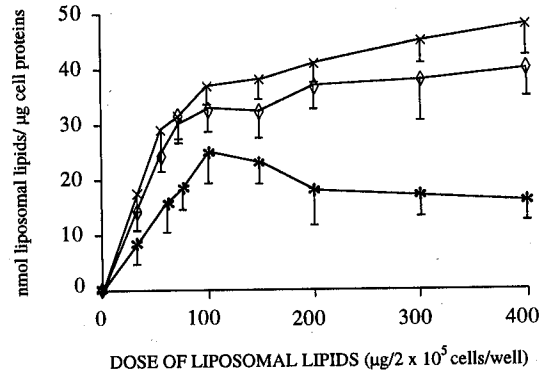

Fig. 2. Concentration-dependent uptake of ester-, ether and mixed type of liposomes by uninfected J774.E1 cell-line. (—×— ester-liposomes, ····· ◊ ····· ether-liposomes, —✱— mixed type of liposomes). Time of incubation = 4 hours.

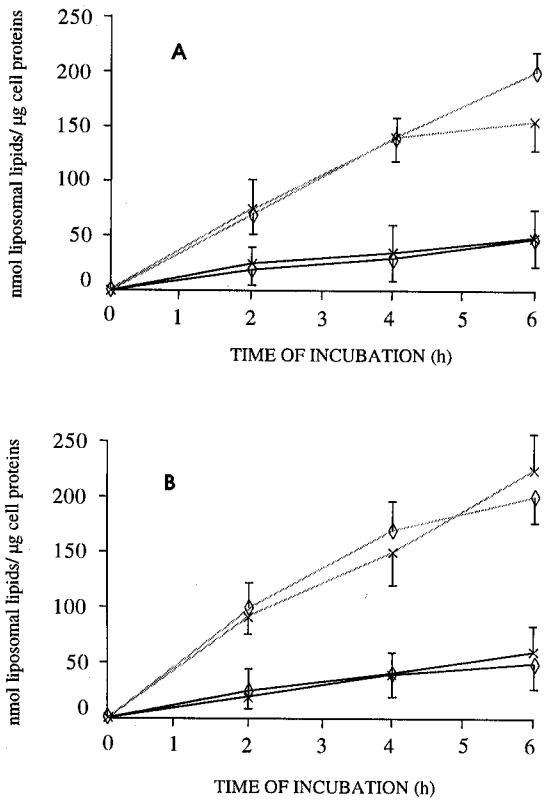

Fig. 3A–B. Time-dependent uptake of ester- and ether-liposomes without ligand and with native apo-B of LDL as ligand. **A** – uninfected cells, **B** – infected cells. Dose of liposomal lipids/2x10^5 cells/well = 50 g – liposomes alone; 120 g – liposomes with ligand. (—×— ester-liposomes, —◊— ether-liposomes × ester-nat.LDL liposomes, ◊ ether-nat.LDL liposomes).

200 µg liposomal lipids/ml (100 µg/2x10^5 cells/well) for ether- or ester-liposomes. Above this concentration the uptake leveled off. Using mixed type of liposomes, the uptake reached a peak concentration at 100 µg/ml, decreased with increasing concentration (Fig. 2).

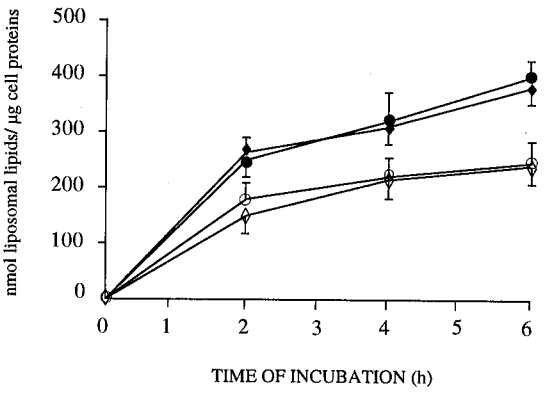

Fig. 4. The receptor-mediated endocytosis of ester- and ether-liposomes with acetylated apo-B of LDL as ligand by J774.E1 cell-line uninfected and infected with *Leishmania mexicana mexicana* amastigotes. Dose of liposomal lipids/2x10^5 cells/well = 120 g. (—○— ester-ac.LDL liposomes, —◊— ether-ac.LDL liposomes, unshaded – uninfected cells, shaded – infected cells).

A series of experiments was designed to compare the utilization of ester- and ether-liposomes, with and without ligands (nat.LDL, ac.LDL), by uninfected and infected J774.E1 cells *in vitro*. The apoprotein-B part of LDL served as the ligand. Each kind of liposome preparation was diluted so that the concentration per well (2x10^5 cells) was 50 µg of liposomal lipids. This concentration was calculated from the initial mixture of standard lipids used. However, in the case of liposomes with incorporated LDL, the concentration per well increased (120 µg), due to the lipid portion of LDL being added to the initial mixture of lipids. The resulting uptake of these liposomes is shown on Fig. 3A. A significantly ($P<0.05$) higher uptake of both ether- and ester-liposomes containing nat.LDL was found in comparison with liposomes alone. The differences between the particular kinds of liposomes, with or without ligand, were not significant. The kinetics of uptake for the same kinds of liposomes by cells infected with

Table 1. Activity of ether-liposomes against amastigotes of *Leishmania m. mexicana**.

Incubation time (h)	24 h		48 h		96 h	
Dose of added liposomal ether-lipids **	No. of amastigotes/cell ($\bar{x}$± SD)	Antileishmanial activity (%)	No. of amastigotes/cell ($\bar{x}$± SD)	Antileishmanial activity (%)	No. of amastigotes/cell ($\bar{x}$± SD)	Antileishmanial activity (%)
0	57.1 ± 9.3	–	69.2 ± 11	–	73.1 ± 10	–
1 µg/ml	54.0 ± 10	6.0	58.6 ± 6.1	15.0	60.0 ± 8.2	17.9
2 µg/ml	51.7 ± 8.1	9.4	48.3 ± 8.8$^+$	29.6	43.8 ± 7.8$^+$	40.0
4 µg/ml	53.7 ± 7.4	5.8	46.3 ± 9.3$^+$	34.0	37.4 ± 7.4$^+$	48.7
8 µg/ml	48.6 ± 9.6	14.8	36.9 ±10$^+$	46.5	27.2 ± 9.2$^+$	62.8

* Peritoneal macrophages (6 x 10^4/well) infected with amastigotes of *L. m. mexicana in vitro*

** Volume of ether-liposomes = 0.5 ml/well

$^+$ Significant difference between control (without liposomes) and liposome affected macrophages ($P<0.05$)

Leishmania m. mexicana are given in Fig. 3B, and show a pattern similar to that for the noninfected cells. Again, a markedly higher accumulation of liposomes containing nat.LDL (approximatelly 200 μg of nmol lipids/μg cell protein) in J774.E1 infected cells was found.

The exploitation of the acetylated LDL (ac.LDL) receptor-mediated pathway of liposomes with chemically modified (ether- and ester-bond) bulk phospholipid (DPPC) containing ac.LDL was investigated, comparing also their uptake by uninfected and infected J774.E1 cells. The presence of acetylated apo-B on the surface of liposomes resulted in their uptake by uninfected cells to a simillar extent as was determined for liposomes with nat.LDL. The *Leishmania* infection in macrophages enhanced ($P<0.05$) the accumulation of both ether- and ester- liposomes, in comparison with uninfected J774.E1 cells (Fig. 4). The kinetics of endocytosis of liposomes bearing native or acetylated LDL as ligands was similar, in both cases there was two-stage kinetics, rapid uptake over the first two hours and then slow uptake for the next four hours. The main difference was the range of liposome accumulation by infected cells, higher for ac.LDL liposomes. Finally, the possible antileishmanial effect of ether-lipids administered *via* liposomes on the viability of *Leishmania* amastigotes was studied. Liposomal lipids were added to the incubation medium from a concentration of 1 to 8 μg/ml, and comparison of the number of parasites in peritoneal macrophages treated by liposomes with numbers in unaffected control was used to calculate the efficacy (Table 1). Ether liposomes themselves had no toxic effects on normal macrophages (results not shown). At dose of 1 μg/ml of ether lipids amastigote proliferation was slowed down, above this concentration the reduction of the parasite number with prolonging incubation time was observed. The number of amastigotes was significantly lower against control ($P<0.05$) from concentration 2 μg/ml of liposomes in medium and this effect was pronounced after 48 and 96 hours of incubation. The highest antileishmanial effect was 62.5%, and this was recorded for infected macrophages incubated with 8 μg liposomal lipids/ml after 96 hours.

DISCUSSION

The present work has shown that the macrophage sarcoma-cell line J774.E1 sequestered at different rates ligands free liposomes containing ether- or ester-analogues of phosphatidylcholine (PC) as bulk phospholipids. It is possible that the observed differences in uptake may have been influenced by differences in the size distribution of the liposomes used and by the average size of the most frequent fractions of liposomes. The higher frequency of uptake of smaller particles in suspension of ether-liposomes was probably caused by the ether-bond in the molecule of the ether-analogue of PC, resulting in a more tight packing of molecules in the liposome bilayers (Agarwal et al. 1986). Macrophages are considered to be one of the most active phagocytic cells of the reticuloendothelial system (Roit et al. 1985), and the J774 cell-line possesses all of the properties of phagocytosing cells (Ralph and Nakoinz 1975).

In the present experiments, the uptake was highest in favour of ester-liposomes without ligands for both uninfected and infected cells, compared with the other kinds of liposome. The differences are likely to be of a physico-chemical nature as we suppose that chemical composition and physical composition of liposomes had a key role in kinetic of their phagocytosis. A similar conclusion for the uptake of phosphatidylcholine/cholesterol and sphingomyelin/cholesterol liposomes by rat hepatocytes was reported by Spanjer et al. (1986). Since the incorporation of native LDL into the liposome's structure placed apoprotein-B of LDL on the liposome surface, such liposomes can then be internalized by cells *via* the specific apo-B receptor (Brown et al. 1981). For both ether- and ester-liposomes with nat.LDL as the ligand, there was a significantly higher uptake by the J774 cell-line, compared with liposomes without ligand. This finding can be interpreted to indicate that in the binding of liposomes with nat.LDL by macrophages more binding sites are involved or that receptor-mediated internalisation of nat.LDL liposomes is a more effective process. The work of Margolis et al. (1982, 1984) and Galkina et al. (1992) have shown that the plasma membrane of cells contains sites that are capable of binding of liposomes, and that liposome-binding sites on cells participate in the binding of LDL. In the present study, such "special receptors" probably contributed to the uptake of liposomes with nat.LDL, and that apo-B receptors were also involved.

The presence of *Leishmania* amastigotes in J774.E1 cells did not increase markedly the uptake of comparable liposome species. In the work of Hart (1987), the accumulation of native LDL particles in infected J774.G8 macrophages was only slightly elevated over that evident in uninfected cells. When ether- or ester-liposomes contained acetylated apo-B of LDL as ligand, their uptake was enhanced dramatically in the case of infected J774.E1 cells and less by uninfected cells. The chemically-modified but not native LDL are taken up via specific "scavenger" receptors (Goldstein et al. 1979) which are highly expressed on J774 cells, compared with other cell types (Lee et al. 1992). Our results highlight the selective accumulation of ac.LDL-liposomes within infected macrophages. The uptake of ac.LDL particles by infected peritoneal macrophages

has been shown to be even more enhanced (Nicolas et al. 1990), reflecting perhaps physiological differences between the two different cell types (J774.E1 cell-sarcoma line and peritoneal macrophages). It is still not clear why the scavenger-receptor pathway was affected by the *Leishmania* infection and why the parasites preferred the acetylated form of apo-B.LDL.

In the present work we have also showed that ether-liposomes without incorporated antileishmanial drug possessed a certain activity against *Leishmania* amastigotes. Some ether-lipids are present in small quantities in *Leishmania* promastigotes, but the higher concentration of ether-lipids in the medium led to their accumulation. A similar antileishmanial effect was reported by Achterberg and Gercken (1987), and in the work of Croft et al. (1987) several ether-lipids were shown to be active against *Leishmania donovani* amastigotes *in vitro*. The same ether-analogue of dipalmitoyl phosphatydilcholine was able to activate the cytotoxic activity of peritoneal macrophages (Ngwenya et al. 1991) and such activation could also contribute to the antileishmanial activity of ether liposomes.

The results presented here demonstrate the usefulness of ether-liposomes with specific ligands in site-specific delivery of antileishnamial compounds *in vitro*. The chemical nature of such liposomes did not affect the kinetics of uptake when compared with liposomes prepared from natural phospholipids, and in *in vivo* studies it should contribute to the enhancement of antileishmanial therapy.

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Received 3 February 1994

Accepted 3 May 1994