Anti-Leishmanial Drug Delivery: Acetylated LDL as A Site-Specific Delivery Ligand

AKRAM SHAH* AND DAVID HART

Life Sciences Department, School of Life, Basic Medical and Health Sciences, King's College London, 150 Stamford, St. London SE1 9NN
E-mail: akramkokab@yahoo.com

Abstract.- The potential utility of acetylated LDL incorporated reverse-phase evaporation vesicles as J774.E1 macrophage specific delivery system was studied using Pentostam as anti-leishmanial drug and Leishmania mexicana as model macrophage disease. The investigations have shown that Apoprotein-B moiety of acetylated-LDL is incorporated into reverse-phase evaporation vesicles (acytalted-liposomes) allowing exploitation of the targeting properties of apoprotein-B ligand. Incorporation of apo-B into liposome carriers significantly enhances their uptake by Leishmania infected macrophages via the LDL and acetylated LDL receptors. The leishmanicidal action of Pentostam entrapped in acetylated liposomes was greater than native LDL containing liposomes and significantly higher than untargeted liposomes. Indeed targeted liposomes with acetylated LDL ligand have highly beneficial effect on the anti-leishmanial action of entrapped drugs and could contribute to a reduction in toxicity and increase in therapeutic index of currently prescribed anti-leishmanial drugs.

Key Words: Acetylated LDL, receptor-mediated endocytosis, acetylated-LDL incorporated liposome, Pentostam, Site-specific drug delivery, J774.E1 macrophages, Leishmania mexicana mexicana

INTRODUCTION

Leishmaniasis is a cosmopolitan disease resulting in severe morbidity and mortality. It is caused by the intra-macrophage Leishmania spp. parasitic protozoan that are transmitted via the bite of an infected female phlebotomine sand fly (Herwaldt, 1999). The leishmaniasis is endemic in 88 countries, 72 of which are developing countries, across 5 continents; Africa, Asia, Europe, North America and South America, with an estimated 350 million people at risk and 12 million affected at one time. Leishmania life cycle involves an insect vector (i.e., different species of sand-fly) and a vertebrate host. Infection may be classified into 3 clinical syndromes: cutaneous, mucocutaneous, and visceral leishmaniasis (Desjeux, 2001). Leishmania pathogenicity is attributable to survival and proliferation of the amastigote stage within macrophages of the host. These amastigotes survive the anti-microbial action and multiply within the phagolysosome (Swanson and Fernandez, 2002), thus presenting permeability problem for anti-leishmanial drugs, which must be translocated through at least two membrane systems before being accumulated in this sub-cellular organelle.

The current situation for the treatment of leishmaniasis is not good. All available antileishmanial drugs are non-discriminatory between host and parasite / parasite-infected cells. They, therefore, have, at best, a moderate chemotherapeutic index, which means that they are only slightly less toxic to the host than they are to the parasite. Sodium stibogluconate (Pentostam) is the most commonly prescribed treatment for leishmaniasis yet has extensive side effects and many infected areas are displaying increasing resistance to this drug (Sundar and Rai, 2002). Pentavalent antimonials are still the first-line drugs for the treatment of visceral leishmaniasis, but there is a new liposomal amphotericin B formulation that seems promising as an alternative therapy (Minodier et al., 2003). The drugs such as Glucantime, Amphotericin B, AmBiosome and aminosidine are also used as alternative therapies (Berman, 1997). There is increased concern over rising resistance in Leishmania donovani against standard antimonials and emergence of Leishmania-HIV co-infection (Alvar et al., 1997). An ether-lipid analogue hexadecylphosphocholine (Miltefosine) originally developed as anti-leukemia drug (Berdel et al.,

* Present address: Parasitology Section, Department of Zoology, University of Peshawar NWFP Pakistan 25120.
1987) as well as anti-neoplastic drug (Ungar et al., 1989) has proved to be less toxic and highly effective in Phase-III clinical trials for oral treatment of Kala-azar in India (Sundar et al., 1998). Similarly, oral treatment with 100mg Miltefosine per day for 4 weeks was effective in treating 95% of the Kala-azar cases in India (Jha et al., 1999). In addition to clinical trials, ether-lipids analogues have also demonstrated potent in vitro and in vivo anti-leishmanial activity (Achterberg and Gercken, 1987; Croft et al., 1987, 1993, 1996; Hart et al., 1995, 1998; Croft, 1997; Unger et al., 1998; Lux et al., 2000; Escobar et al., 2001). We were the first in King's College London to demonstrate antileishmanial action of ether-liposomes made by proliposome method (Shah, 1993; Shah and Hart, 1993; Hrckova et al., 1994).

Liposome has gained increased attention as systemic drug delivery vehicles following recent regulatory approvals of several vesicle-formulated drugs (Gregoriadis, 1995). These products have demonstrated improved therapeutic indices over their corresponding conventional drugs by avoiding sensitive tissues and/or increasing delivery to specific targets in vivo. The use of liposomes as delivery vesicles has been shown to improve the anti-leishmanial action of these drugs (Alving et al., 1978a,b; Guru et al., 1989; Gregoriadis, 1995; Agarawal and Gupta, 2000). To successfully access target tissues, liposomes must be able to overcome several factors limiting their biodistribution and uptake. These limitations include short circulation half-lives, slow extravasation into target tissues, and penetration into sites of action. Several delivery systems, including lipopeties, ‘‘ligands’’, incorporated into the liposome have provided measurable improvements for membrane surface. In site-specific drug delivery the success of liposomes as delivery vesicles could be rationalized to deliver drug to infected cells via the incorporation of site-specific ligands in to the liposomal membrane and selectively accumulated via receptor-mediated endocytosis processes, which are exclusive to, or elevated in, Leishmania-infected cells.

Human plasma low-density lipoproteins (LDLs) are water-soluble nanoparticles, which may be regarded as natural counterparts of liposome (Hammel et al., 2003). The receptor-mediated endocytosis of modified LDL has been shown to be elevated in Leishmania-infected macrophages (Hart, 1987; Shah, 1993) and is therefore a prime target for rational drug delivery.

In this study, we investigated the use of acetylated-LDL incorporated liposomes as a putative site-specific drug delivery system for intracellular delivery of liposomal drug, Pentostam. We have developed an in vitro model using cultured J774.E1 macrophage-like cells infected with Leishmania mexicana mexicana M379 to screen for anti-leishmanial drug delivery.

**MATERIALS AND METHODS**

**In vitro culture of J774.E1 cells**

J774.E1 a selected clone was maintained in RPMI 1640 growth medium (Invitrogen, Ltd. UK) supplemented with 10% heat-inactivated fetal calf serum (FCS-HI) (Invitrogen, Ltd. UK). These cells were then plated in 24 well tissue culture plates (Beckton Dickinson, UK) at a density of 1x10^5 cells/ml and were cultured in 5% CO2 humidified chamber at 37°C. After 16 hours the non-adherent cells were removed and adherent monolayer was used for experiments. J774.E1 cells were maintained by sub-passaging once a week.

**In vitro culture and maintenance of Leishmania parasites**

In their mammalian hosts, Leishmania parasites exist primarily as amastigotes within phagolysosomes of macrophages. Extra- and intracellular promastigotes occur only during a few hours after infection and extra cellular amastigotes appearing between disruption of one host cells and uptake by the next.

Promastigotes of Leishmania were continuously cultured in semi-defined medium (SDM) generally at 23-27 °C in sealed 25cm² tissue culture flasks (Beckton Dickinson, UK). It was supplemented with 10% FCS-HI. Promastigote of Leishmania were harvested at late log phase of growth. Suppassages of Leishmania promastigotes were limited to less than 12, as after this the infectivity is less reliable (unpublished observations).
Infection kinetics of J774.E1 cells with Leishmania parasites

Assessment of effects on the intracellular survival of amastigotes is more complex. Primary macrophage cultures or monocytic cell lines are parasitized in vitro with promastigotes of Leishmania cultures or freshly isolated amastigotes. The former should be cultured at 37°C at least over night to allow infection and intracellular transformation to amastigotes.

To examine the anti-leishmanial action of Pentostam containing liposome, native LDL liposome and acetylated LDL liposomes, J774.E1 macrophages were washed twice with PBS. These cells were then infected with promastigotes of Leishmania mexicana mexicana at a ratio of 10 parasites to 1 macrophage in 24well plates or 8-chambered lab-tek slides for 16 hours at 37°C in 5% CO2 and 85% humidity. These cells were then washed three times to remove non-phagocytosed promastigotes. Fixing these cells in Methanol and staining with Geimsa’s stain determined percentage of infected cells. After counting 100 cells from each chamber results were expressed as mean amastigotes/cell.

Preparation of reverse-phase evaporation vesicles (REVs)

Liposomes were prepared by the Reverse Phase Evaporation method of Szoka and Paphadjopoulus (1978) as follows. Dipalmitylphosphatylcholine (DPPC) 15mg, cholesterol 7.5mg and dicetyl phosphate 2.5mg were dissolved in 2.5 ml chloroform. Sterile de-ionized distilled water (0.75ml) containing 200µCi/ml of U-14C sucrose (Amersham) was then added. This was sonicated at 50°C for 5-15 minutes to produce an emulsion. To some preparations 0.5 mg of apo-B from 125I-native and 125I-acetylated-LDL and Pentostam was added to the initial aqueous phase. Chloroform was removed under vacuum to leave a gel which collapsed to leave a concentric liposome preparation which was then made up to 10ml with sterile de-ionized double distilled 0.22µm filtered water and then filtered through 0.22µm polycarbonate filter. Un-entrapped U-14C sucrose was removed by exhaustive dialysis in sterile 0.9% saline buffered to pH 7.4 at 4°C with 3 volume changes of 24 hours each. The physico-chemical characterization of liposomes was done for lipid content, entrapment of radioisotope, apo-B and pentostam, size determination, lamellarity and electron microscopy before uptake studies (for details see Shah and Hart, 1993).

Liposome uptake assay

For liposome uptake studies 2x10^5 J774.E1 cells (both infected and uninfected) were plated in 24 wells Linbro plates overnight, washed 3 times with PBS and 25-30µM liposomal lipid was added. These cells were then incubated for 2, 4 and 6 hours at 37°C in 5%CO2 and 85% humidity. After interaction of the cells for the indicated time the cells were washed 3 times with PBS and were dissolved in 1ml of 0.2MNaOH+0.1% TritonX100. About 50µl of this lysate was removed for protein macro-assay and the rest was placed in scintillation vials for counting the activity of U-14C sucrose using liquid scintillation counter. Uptake of liposomes was expressed as nano-moles of lipid/µg of cell protein.

Measurement of cell protein content

To measure protein content of macrophages, monolayer were washed twice with PBS. The cells were then lysed using 0.1% Triton-X100 with 0.2M NaOH. The protein content was then measured by using Bio-Rad protein assay, using bovine serum albumin (BSA) as a standard.

RESULTS

Uptake of liposome, acetylated-LDL-liposome and native-LDL-liposome by J774.E1 cells

Uptake of three different liposome preparations as a function of time into uninfected J774.E1 is shown in Figure 1. It can clearly be seen that liposomes with acetylated-LDL incorporated significantly increases their uptake by J774.E1 cells. Liposomes uptake increased with time and showed no sign of saturation for up to 6 hours of incubation. Uptake was lowest for liposomes. This suggests that the apoprotein-B from modified LDL may be a powerful ligand for the development of anti-leishmanial drug delivery systems. It is assumed that liposomes in the presence of acetylated-LDL
interact with the receptors for modified LDL on the J774.E1 macrophage-like cells.

Endocytosis of liposome, native-LDL-liposome and acetylated-LDL-liposome by Leishmania maxicana mexicana infected J774.E1 cells

Figure 2 shows the time course of uptake of three preparations of liposome by Leishmania infected J774.E1. Uptake increased with time and no saturation seen at up to 6 hours. When Leishmania infected J774.E1 were incubated with acetylated-LDL-liposome the uptake was 77% more than the uninfected J774.E1 cells. Therefore infected cells take more acetylated-liposome as compared to the un-infected J774.E1 cells.

Intracellular leishmanicidal action

The Figure 3 shows the comparative antileishmanial activity of free Pentostam and Pentostam encapsulated in liposomes, native-LDL-liposomes and acetylated-LDL-liposomes. It is evident from Figure 3 that during the 72-hour incubation period, acetylated-LDL-liposomes with Pentostam 62% of the intracellular amastigotes were killed. In contrast, an equivalent amount of liposomes encapsulated Pentostam and native-LDL-liposomes with Pentostam eliminated 30% and 40% amastigotes respectively. It can clearly be seen that leishmanicidal action of Pentostam is greatly enhanced when incorporated in acetylated-LDL targeted liposomes and more than 60% increase in anti-leishmanial activity is demonstrated. It can be seen from the Fig. 3 that the use of liposomes improved anti-leishmanial drug delivery, and that acetylated-LDL-proved to be the most effective drug targeting ligand for liposomes, which correlated with the highest uptake rate shown in Figure 2.

Concentration dependent uptake of liposomes and acetylated-liposomes by J774.E1 cells

Incubation of liposomes and acetylated-liposomes increasing concentration at 37°C resulted saturation in the cellular radioactivity (Fig. 4). It is evident from the figure that receptor-mediated endocytosis of acetylated-liposomes is linear up to 50µg concentration, while in liposomes high-efficiency binding was achieved at 25µg concentration and at 100 µg concentration both liposomes and acetylated-liposomes uptake reached steady-state plateau. In the case of acetylated-liposomes, half-maximal value was achieved at 25µg while in liposomes it was at 12.5µg. These results showed that for both liposomes and acetylated-liposomes binding site in J774.E1 cells showed high-affinity and saturability and that it was possible that untargeted negatively charged vesicles
were also taken up through endocytosis. Since untargeted control liposomes are also endocytosed by J774.E1 cells, we conclude that the uptake of acetylated-liposomes by J774.E1 cells is the net result of at least two mechanisms operating simultaneously. Firstly, the most significant part of the uptake of acetylated-liposomes by J774.E1 cell is inhibited by acetylated-LDL (data not shown) indicating involvement of acetylated-LDL receptor. Secondly, endocytosis of un-targeted liposomes because of their net negative charge.

Temperature dependent uptake of Ac.LDL-targetted and untargeted vesicles by J774.E1 cells.

The surface binding of both liposomes and acetylated-liposomes was measured by incubating J774.E1 with increasing time-points at 4°C. Both liposomes (Fig. 5) and acetylated-liposomes (Fig.6) show no significant difference in between them for their binding at 4°C with J774.E1 plasma membrane. At the same phospholipid concentration in the same cell population at 37°C both untargeted and acetylated-LDL-targeted vesicles showed 3-4 fold higher cellular content of radioactivity without saturation. These results also explain the possibility of discrete receptor molecule involvement in the endocytosis of liposomes. At an incubation temperature of 37°C a substantially larger number of liposomes associate with the J774.E1 cells, indicating internalisation and perhaps also increased binding to the cells.

DISCUSSION

Increase in uptake of acetylated-LDL-liposomes by J774.E1 cells after infection with Leishmania mexicana mexicana M379 showed that acetylated-LDL-liposome is an excellent model for anti-leishmanial drug delivery. The ability of drug carriers such as acetylated-LDL-liposome to be taken up by the cells of the reticulo-endothelial system makes them ideal carrier for the selective transport of drugs to target tissue in disease where phagocytic cells are involved. This natural trait has been clearly demonstrated by taking advantage in treatment in the case of experimental visceral leishmaniasis; where liposomes loaded with antimonial drugs have greatly increased efficacy in comparison with the un-encapsulated compounds. Liposomes have demonstrated improved therapeutic indices over their corresponding conventional drugs by avoiding sensitive tissues and/or increasing delivery to specific targets in vivo and has been shown to improve the anti-leishmanial action of these drugs (Alving et al., 1978ab; Guru et al., 1989; Gregoriadis, 1995; Foressen and Willis, 1998; Agarwal and Gupta, 2000; Proulx et al., 2001).
One of the most interesting potential applications of ligand bearing liposomes is the specific delivery of drugs to the cells. There are two problems, which must be overcome before use of such liposomes in the therapy of *Leishmania*-infected macrophages can be considered. The first is that the liposomes must be able to reach only the infected cell population. The second is that the liposomes must release their content at the intracellular site of action (Chauderi et al., 1989; Agarawal and Gupta, 2000), which is phagolysosome in the case of *Leishmania*.

Macrophages isolated from various organs in several animal species have exhibited high affinity binding sites for acetylated-LDL. Such chemically modified protein ligands not only bind to macrophages through these binding sites but also are internalised by the process of endocytosis and degraded in lysosomes (Chauderi et al., 1989). In the present study of targeted delivery, the LDL “receptor” system was selected because (1) J774.E1 cells possess these binding sites on their surfaces; (2) “Receptor” system mediates rapid internalisation and degradation of the protein ligands recognized by the binding sites; (3) The “receptor” system is probably recycled with little or no down regulation. It was therefore thought that the amastigotes of *Leishmania* causing the J774.E1 to increase the number of acetylated-LDL-receptors and/or recycling rate of finite number of receptors. The *Leishmania* amastigotes rapidly multiply and therefore have a high demand for ergosterol or it is possible that the *Leishmania* has the ability to modify the receptor-mediated uptake of acetylated LDL to its advantage as a major source of lipid for the amastigote survival. Previous studies showed elevation of receptor-mediated endocytosis of modified LDL in *Leishmania*-infected macrophages (Hart, 1987; Chauderi et al., 1989; Shah, 1993) and also may be regarded as a natural counterpart of liposomes (Hammel et al., 2003).

To achieve site-specific drug delivery to *Leishmania* infected J774.E1 we encapsulated the Pentostam in liposomes coupled with acetylated-LDL and it was proved to be an efficient way to kill more than 60% amastigotes of *Leishmania mexicana mexicana* within J774.E1. The results of our study demonstrate that it is possible to transport the contents of liposomes inside cells via the receptors for modified LDL. Since these particles may serve as containers both for hydrophobic and hydrophilic substances. They also demonstrate that liposomes can be a useful investigative tool in study of receptor-mediated processes.

These findings indicate that encapsulation of Pentostam in acetylated-LDL liposomes significantly enhances the drug efficacy against the
Leishmania mexicana mexicana infection in J774.E1 cells. Studies with Leishmania donovani infection in hamsters have shown that encapsulation of Pentostam in tuftsin bearing liposomes has 85-92% inhibition of infection with 250-500mg antimony/kg on 28th day post treatment as compared to 20-37% inhibition of infection with the same dose on 28th day post treatment with only liposomes (Guru et al., 1989). Furthermore, our results have shown that liposomes with acetylated apo-B incorporated into the membrane are taken up as Chauderi phagolysosomes. The efficacy of 20(S)-camptothecin (CPT), free and incorporated into sterically stabilized liposomes, have significantly reduced the parasite loads in the murine macrophage like cell line and leprosy, monocytic leukaemia and tuberculosis are the diseases with macrophage as primary target where effective delivery of drug through this approach is possible.

ACKNOWLEDGEMENTS

Financial support of Ministry of Science and Technology and HEC, Govt. of Pakistan to first author is gratefully acknowledged.

REFERENCES


CROFT, S.L., NEAL, R. A., PENDERGAST, W. AND CHAN,


(Received 6 December 2003)