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

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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 mexicana* as model macrophage disease. The investigations have shown that Apoprotein-B moiety of acetylated-LDL is incorporated into reverse-phase evaporation vesicles (acetylated-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 dug 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* pathogenecity 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.*,

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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 lipoeties, ‘‘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 $25cm^2$ 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-^{14}C 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 ^{125}I-native and ^{125}I-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-^{14}C 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%CO_2 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-^{14}C 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 J77.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-leishamanial 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 machrophage-like cells.

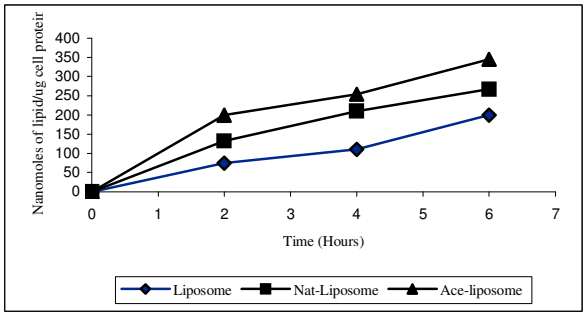

Fig. 1. Uptake of liposome, native-LDL-liposome and acetylated-LDL-liposome by un-infected J774.E1 cells. Incubation was at 37°C. The liposomes containing aqueous (U-^{14}C) Sucrose were incubated at a concentration of 30μM phospholipid. The data show mean of the three independent experiments.

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 lipsomes, which correlated with the highest uptake rate shown in Figure 2.

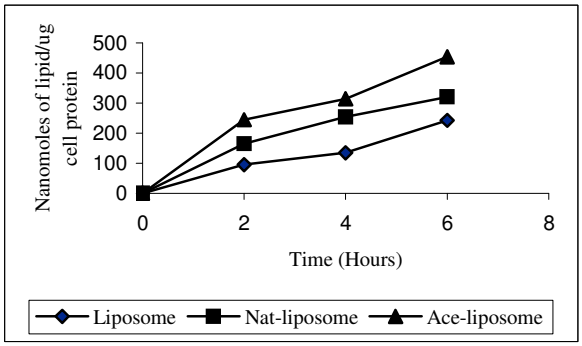

Fig. 2. Accumulation of liposome, native-LDL-liposome and acetylated-LDL-liposome by *Leishmania maxicana mexicana* infected J774.E1 cells. Each monolayer received 1 ml of RPMI 1640+0%FCS-HI with liposomes concentration of 30μM phospholipid. After the indicated time period, the monolayers were washed and the amount of phospholipid bound to the cells was determined as described in Materials and Methods. The data show mean of the three independent experiments.

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.Ei 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 a 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.

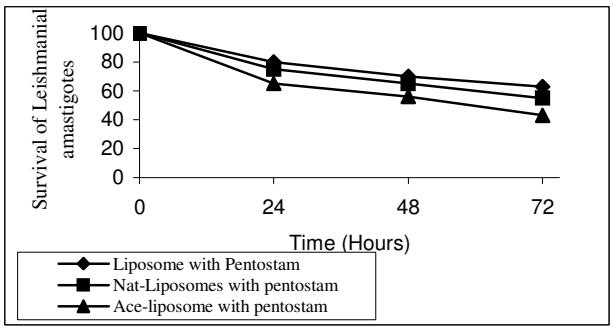

Fig. 3. Comparison of Anti-leishmanial activity of liposome, nat–LDL-liposome and acetylated – LDL - liposome encapsulated Pentostam in J774.E1 cells infected with *Leishmania maxicana maxicana* as described in Materials and Methods. Each infected monolayer received 0.5 ml of medium containing 3 µg of Pentostam either free or encapsulated in liposomes. They were incubated at 37°C for 24, 48 and 72 hours.

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.

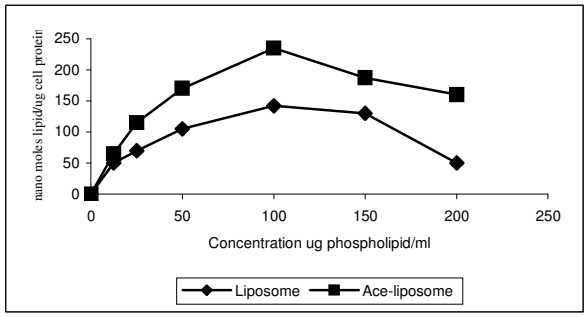

Fig. 4. Accumulation of liposomes and acetylated-liposomes by J774.E1 cells. Each well received increasing concentration of liposomes or acetylated-liposomes. J774.E1 cells were incubated for 4 hours at 37°C and uptake was measured. Each point is the mean for two independent determinations.

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; Agarawal and Gupta, 2000; Proulx *et al.*, 2001).

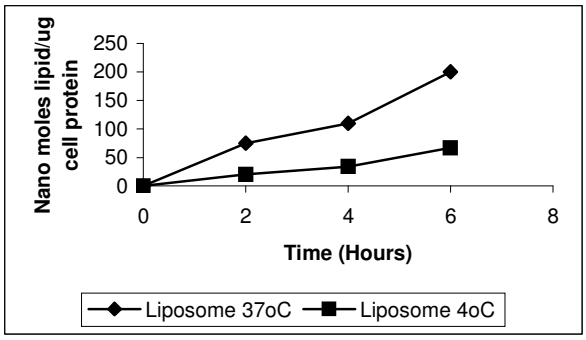

Fig. 5. Binding of liposomes by J774.E1 cells at 4°C. Each well of the 24 well plates with $2x10^5$ J774.E1 cells received ice-cold medium containing 30μM of phospholipid and was incubated at 4°C for the indicated time points. For 37°C same procedure was adopted except the temperature. The data represents average of three representative experiments.

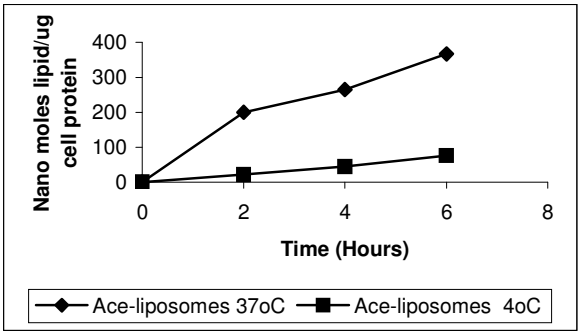

Fig. 6. Binding of acetylated-liposomes by J774.E1 cells at 4°C. Each well of the 24 well plates with $2x10^5$ J774.E1 cells received ice-cold medium containing 30μM of phospholipid and was incubated at 4°C for the indicated time points. For 37°C same procedure was adopted except the temperature. The data represents average of three representative experiments.

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 posses 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 liposmes have 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 Hart (1987) has shown for acetylated LDL. This modified LDL was shown to be taken up by receptor mediated processes and was accumulated in at higher rates in both infected and uninfected J774-G8 as compared to native LDL. Degradation of both acetylated and native forms of LDL was somewhat reduced in infected J774-G8. Colloidal gold labelled acetylated LDL was used to follow the sub-cellular routing with in infected macrophages and the gold was shown to be accumulated in the phagolysosomes.

Chauderi *et al.* (1989) have shown that methotrexate conjugated with malelated BSA was taken up and degraded by the cultured hamster peritoneal macrophages through polyanion binding sites for acetylated LDL and eliminated intracellular amastigotes of *Leishmania donovani* three times more efficiently than free methotrexate. Similar selective killing of Leishmania *mexicana amazonensis* was also observed by delivering drug through scavenger receptor in hamster peritoneal macrophages (Mukhopadhay *et al*., 1989).

It has been shown by Ivanov *et al*. (1985) that malondialdehyde-treated LDL addition to the incubation medium resulted in 15-20-fold increase in uptake of carboxyflourescein labelled liposomes to J774 cells. Hsu and Juliano (1982) tested IgG antigen complex on liposome surface results in 102 fold enhancement of liposome uptake by the macrophages. The efficacy of 20(S)-camptothecin (CPT), free and incorporated into sterically stabilized liposomes, have significantly reduced the *Leishmania donovani* parasite loads in the murine livers by 43 and 55%, respectively, compared with the loads for untreated controls (Proulx *et al.*, 2001).

These studies serve to expand our knowledge of liposome-macrophage system interactions and the liposomes can be a useful investigative tool in studying a non-specific and receptor mediated endocytotic processes.

From all these studies we can conclude that it is possible to incorporate acetylated-LDL into the liposomes to achieve selective killing of intracellular parasites residing in J774.E1 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.

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