

Characterization of Ace-Liposome Receptor-mediated Endocytic Pathways in *Leishmania* Infected J774 Cells: Effect of Lysosomotropic Agents and Inhibitors of Oxidative Phosphorylation*

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Abstract.- Previously, we have demonstrated that incorporation of apoprotein-B into liposome and acetylated LDL containing liposome carriers significantly enhance their uptake by *Leishmania* infected J774 macrophages via LDL and acetylated LDL receptors. To further enhance the potential of our drug delivery system, in the present investigation we have embarked upon characterization of the acetylated LDL containing liposome receptor-mediated endocytic pathways. The J774 model system was used to study the effect of a series of inhibitors and modulators of endocytosis including lysosomotropic agents and inhibitors of energy metabolism. Uptake of liposomes and acetylated LDL Liposomes were amplified significantly in the presence of lysosomotropic agents and considerably inhibited when cells were treated with azide, a metabolic inhibitor.

Keywords: Lysosomotropic agents, ace-liposomes, oxidative phosphorylation, J774 cells, lysosome, receptor-mediated endocytosis.

INTRODUCTION

The use of drug delivery systems as nanocarriers for chemotherapeutic agents can improve the pharmacological properties of drugs by altering drug pharmacokinetics and biodistribution (Aliving *et al.*, 1978a,b; Hrckova *et al.*, 1994). Among the many drug delivery systems available, both micelles and Liposomes have gained the most attention in recent years due to their clinical success (Cukierman and Khan, 2010), which ultimately are deposited in lysosomes of J774 cells. Lysosomes are a major subcellular site for the degradation of a large variety of endogenous and exogenous macromolecules (Heuser, 1989). The lysosomal proton pump is used as an ion transporter. Translocating ATPase's pump protons into lysosomes by utilizing the energy from ATP hydrolysis and produce the acidic intralysosomal environment. In drug delivery systems for leishmaniasis that employ active and passive macrophage targeting strategies, polymer-drug conjugates have been used (Guru *et al.*, 1989; Mukhopadhyay *et al.*, 1989; Gregoriadis, 1995;

Croft, 1997; Proulx *et al.*, 2001; Escobar *et al.*, 2001; Hammel *et al.*, 2003; Nicoletti *et al.*, 2009). Macrophage lysosomal pH ranges from 4.7-4.8 and it is affected (*i.e.*, the pH increases) when cells are exposed to weak bases, such as potassium ionophores. Inhibitors of glycolysis (*e.g.*, 2-deoxyglucose) and oxidative phosphorylation *e.g.*, azide or cyanide also effect lysosomal pH. Transmembrane proteins destined to endosomes are selectively accumulated in clathrin-coated pits at the plasma membrane and rapidly internalized in clathrin-coated vesicles before their delivery to lysosomes (Sorkin, 2004).

Clearly, understanding and quantitating the effect of certain drugs on cells require as accurate an estimate as possible of the kinetics of drug entry into the cells, rather than total uptake (Nir and Nieva, 2003). A series of lysosomotropic compounds (*e.g.*, chloroquine, monensin and ammonium chloride) have been identified as weak bases whose unprotonated forms can translocate by diffusion across biological membranes while their protonated forms cannot. This generates a concentration gradient which drives the lysosomotropic affect. Indeed in *Plasmodium* infected RBC(s) accumulation of quinine is 600-fold higher than extracellular concentrations. The acidic compartment will therefore have a very high concentration of these agents and this may certainly aid in the mechanism of chloroquine action in anti-

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malarial chemotherapy. Chloroquine (CQ) has several pharmacokinetic and pharmacological advantages over all the other antimalarial drugs, which accounts for its excellent performance over eight decades of malaria therapy, despite the CQ-resistance developed by *Plasmodium* strains. The mode of action of CQ is based on its accumulation in the food vacuole of the parasites (lysosomotropic character). This interferes with the polymerization of the toxic heme monomers leading to parasite death by heme poisoning (Santos-Magalhaes and Mosqueira, 2010).

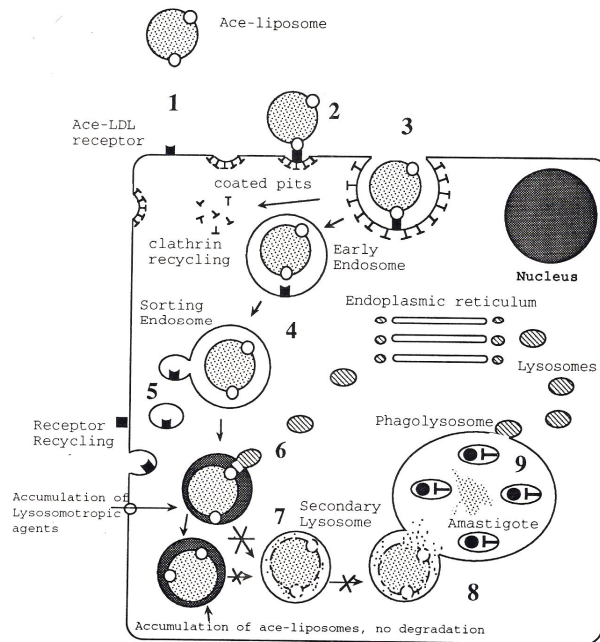


Fig. 1. Schematic representation for inhibition of receptor-mediated endocytosis of ace-liposomes by *Leishmania* infected J774 cells. 1. Ace-LDL receptor. 2. Migration of receptor to coated pit and binding with ace-liposome ligand. 3. Invagination of coated pit to form early endosome. 4. Dissociation of receptor-ligand complex due to endosome acidification. 5. Recycling of receptor to cell surface. 6. Endosome-lysosome fusion to form secondary lysosome. 7. Accumulation of lysosomotropic agents in lysosomes and inhibition of degradation.

The pH of the acidic compartments would thus increase as a result of accumulation of lysosomotropic agents and therefore inhibition of the hydrolase of lysosomes and proton shuttling

across the membrane takes place (Ginsburg, 1990). Monensin ($C_{36}H_{62}O_{11}$), a carboxylic ionophore and major factor in antibiotic complex separated from *Streptomyces cinnamomensis*, for example has been found to inhibit plasma membrane recycling by raising the pH of acidic compartments and disrupting the proton gradient at a concentration of $50\mu M$. Lysosomal degradative enzymes had no detectable effect at pH 7.0 and 6.5 whereas proteolysis increases 1.5 times at pH 5.5-6.0 (Vorgas *et al.*, 1990), this rise in pH may therefore affect the degradative enzymes. In normal conditions the lysosomes are clustered around the centre in macrophages but they were moved to very edge of the cells, lowering the pH of the cytoplasm to 6.8 by applying acetate. Lysosomal clustering around the centre resumed promptly, after applying a weak base or by removing the acetate, that is, the rebound alkalinization of the cytoplasm (Heuser, 1989).

In this study the uptake and degradation of liposomes and ace-liposomes by J774 cells in the presence and absence of ammonium chloride (NH_4Cl), sodium azide (NaN_3), chloroquine and monensin has provided interesting information on the endocytosis of both liposomes and ace-liposomes in J774 cells. Figure 1 summarizes the inhibition of probable subcellular routing of receptor-mediated endocytosis of ace-liposome by lysosomotropic agents.

MATERIALS AND METHODS

In vitro culture of J774 cells

J774 were maintained in RPMI 1640 growth medium (Invitrogen, Ltd. UK) supplemented with 10% heat-inactivated fetal calf serum (FCS-HI). These cells were then plated in 24 well tissue culture plates (Becton Dickinson, UK) at a density of 1×10^5 cells /ml and were cultured in 5% CO_2 humidified chamber at $37^\circ C$. After 16 hours the non-adherent cells were removed and adherent monolayer was used for experiments. J774 cells were maintained by sub-passaging once a week.

In vitro culture and maintenance of *Leishmania* parasites

Promastigotes of *Leishmania mexicana*

mexicana were continuously cultured in semi-defined medium (SDM) generally at 23-27°C in sealed 25cm² tissue culture flasks (Becton 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 cells with Leishmania parasites

To examine the uptake of , native LDL liposome and acetylated LDL liposomes, J774 macrophages were washed twice with PBS. These cells were then infected with promastigotes of *Leishmania mexicana mexicana* at a ratio of 10 parasites: 1 macrophage in 24well plates or 8-chambered lab-tek slides for 16 -48 hours at 34°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 (REV)

Liposomes were prepared by the Reverse Phase Evaporation method of Szoka and Paphadjopoulos (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 UK) was then added. This was sonicated at 50°C for 5-15 minutes to produce an emulsion. To some preparations 0.5-1mg of apo-B from ¹²⁵I-native and ¹²⁵I-acetylated-LDL 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, size determination, lamellarity and electron microscopy before uptake studies (Shah and Hart, 1993).

Liposome uptake assay

For liposome uptake studies 2x10⁵ J774 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.

RESULTS AND DISCUSSION

Time-dependent effect of intralysosomal perturbators and inhibitor of terminal respiration on the endocytosis of ace-liposomes

In Figures 2 and 3 the time-dependent effect on the endocytosis of ace-liposome by *Leishmania* infected and uninfected J774 cells of lysosomotropic agents (Chloroquine and monensin), the weak base (NH₄Cl) and neutral salt (NaCl) as well as cytochrome oxidase inhibitor (sodium azide) is shown. Uptake was inhibited by a approximately 70% after treatment of the cells with sodium azide and NH₄Cl at 6-8 hour incubation, while significant enhancement of uptake was observed by treatment of the J774 cells by chloroquine and monensin. There was no significant effect on the endocytosis when cells were treated with the neutral sodium salt NaCl.

Monensin and chloroquine are potent lysosomal inhibitors (Morselli *et al.*, 2009), and in the presence of these inhibitors, endocytosis of ace-liposomes was increased by up to 70% of the control values. There was no significant difference

in the enhancement of ace-liposomes endocytosis by these lysosomal inhibitors by infected and uninfected J774 cells. Only slightly higher values were found in the infected J774 cells. This indicates the possibility that lysosomal fusion with the parasitophorous vacuole was not taking place or was slowed down or maturation of the primary lysosome to secondary lysosome was not taking due to higher intralysosomal pH.

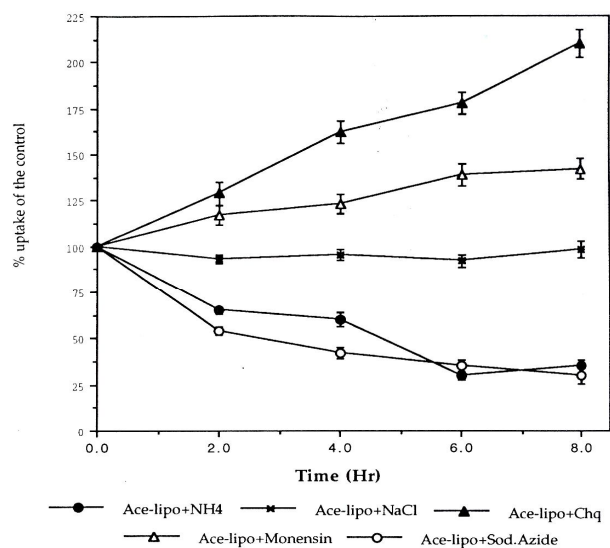


Fig. 2. Time dependent effect of ammonium chloride (NH₄), sodium chloride (5mM), monensin, sodium azide (2.5mM) and chloroquine (Chq) 50 μ M on the endocytosis of ace-liposomes by un-infected J774 cells. Points are the mean \pm SE of 9 determinations from three experiments.

Our results are supported by studies conducted by Rathore and Ghosh (2008), where monensin and NH₄Cl significantly enhanced the cytotoxicity of ricin entrapped in negatively charged liposomes composed of soya phosphatidylcholine. Similarly, Barratt *et al.* (1986) have found a 20% increase (per hour) in the endocytosis of ¹²⁵I-BSA containing liposomes in the presence of 40 μ M chloroquine with marked reductions in the exocytosis of the products and inhibition of degradation.

However anti-leishmanial action of methotrexate-coupled maleylated-BSA was suppressed more than 60% in the presence of

monensin and chloroquine (Chauderi *et al.*, 1989), suggesting that intact lysosomal function is necessary for effective anti-leishmanial activity.

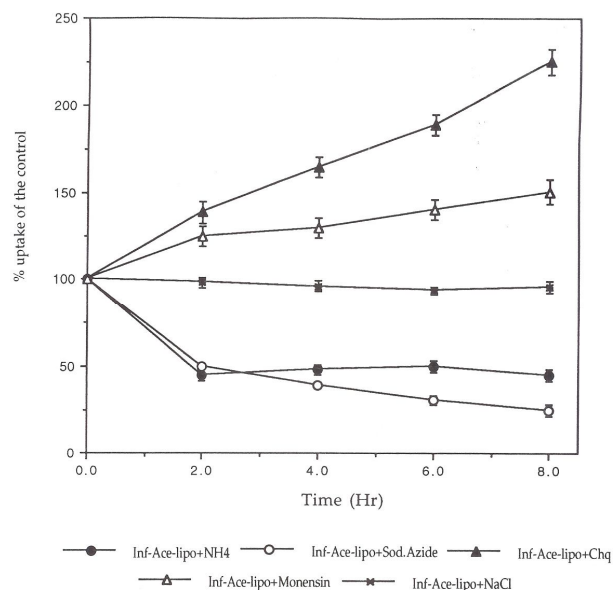


Fig. 3. Time dependent effect of ammonium chloride (NH₄), sodium chloride (5mM), monensin, sodium azide (2.5mM) and chloroquine (Chq) 50 μ M on the endocytosis of ace-liposomes by *Leishmania* infected J774 cells. Points are the mean \pm SE of 9 determinations from three experiments.

Treatment of J774 cells with monensin also enhanced the uptake by up to 65%, this may be the result of inhibition of degradation and continued accumulation of ace-liposomes in the lysosomes. Monensin has a similar effect to chloroquine, that of disrupting the proton gradient and increasing the pH of the acidic lysosomal compartment resulting in inhibition of degradation and plasma membrane recycling (Kiss and Rohlich, 1989).

Treatment of J774 cells with sodium azide, an inhibitor of terminal respiration, significantly reduced endocytosis of ace-liposomes, suggesting that endocytosis is dependent on energy produced by oxidative phosphorylation. Straubinger *et al.* (1983) found similar results with negatively charged liposomes which were bound to the cell surface and were not endocytosed. These results indicate that ace-liposomes may also be bound and not endocytosed in cells treated with sodium azide.

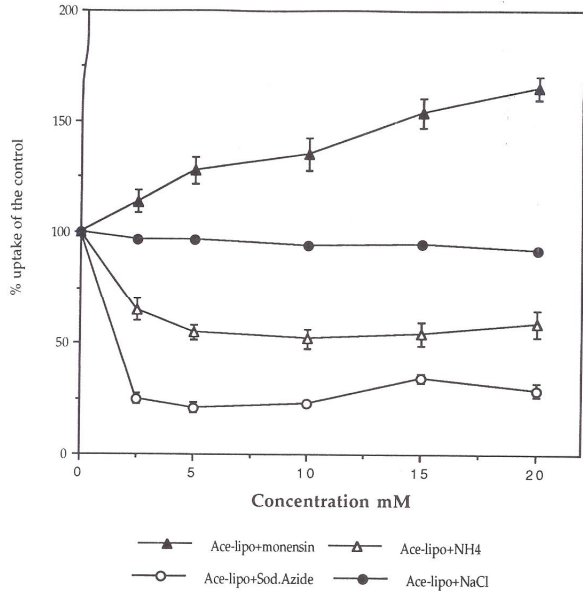


Fig. 4. Concentration dependent effect of lysosomotropic agents and sodium azide on the uptake of ace-liposome by J774 cells. Points are the mean ± SE of 8 determination from two independent experiments.

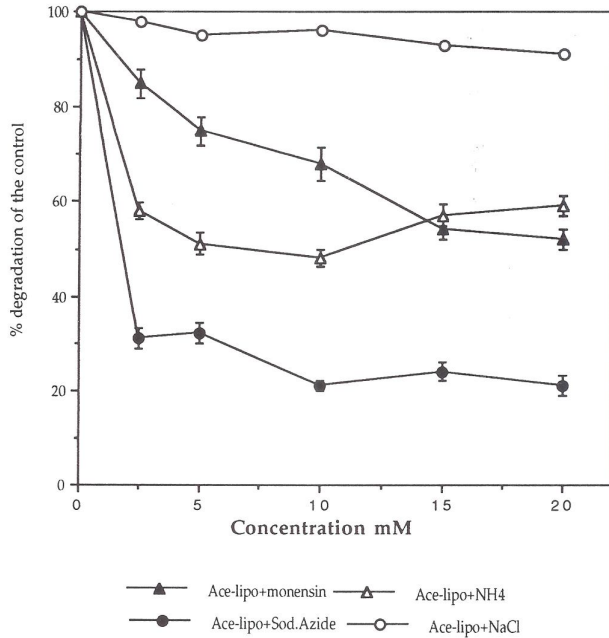


Fig. 5. Concentration dependent effect of lysosomotropic agents and sodium azide on the degradation of ace-liposome by J774 cells. Incubation was for 3 hours at 35°C. Points are the Mean ± SE of 12 determination from three independent experiments.

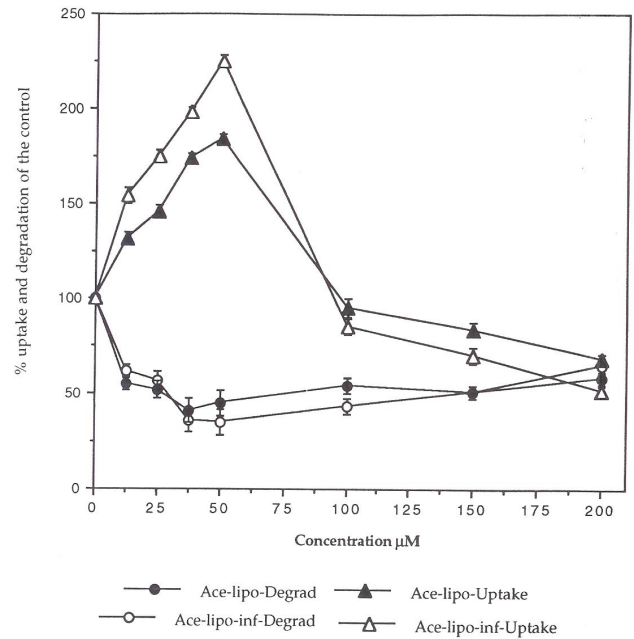


Fig. 6. Concentration dependent effect of chloroquine on the endocytosis and degradation of ace-liposome by *Leishmania* infected and uninfected J774 cells.

The weak base NH_4Cl also inhibited endocytosis of ace-liposomes in J774 cells by more than 60%. NH_4Cl , as reported by D'Arcy-Hart and Young (1978) is an inhibitor of phagosome-lysosome fusion and lysosomal movement, which induces phagosome-endosome fusion. It was found to significantly reduce the uptake of ace-liposomes in J774 cells. In the cells treated with NH_4Cl it is possible therefore that it accumulates in the endosome raising the pH so that the dissociation of ace-liposome ligand from the receptor is not taking place. This would result in inhibition of receptor recycling. The number of available ace-liposome receptors would decrease and consequently uptake of the vesicles would be dramatically reduced. Another possibility is that NH_4Cl inhibits

Salutatory movements of lysosomes. It is possible that endosome-lysosome fusion is suddenly slowed down by NH_4Cl and consequently uptake is decreased. It is possible that endosomal pH is higher in cells treated with NH_4Cl so that the pH dependence of ace-liposomal passage to cytoplasm is delayed and would therefore accumulate in

endosomes (Straubinger *et al.*, 1983).

It can be concluded therefore, that both lysosomotropic agents as well as inhibitors of energy metabolism (oxidative phosphorylation) are found to be effective in perturbation of acetylcholinergic receptor-mediated pathways.

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