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NEOGLYCOPROTEIN CONJUGATED LIPOSOMES AS MACROPHAGE SPECIFIC DRUG CARRIER IN THE THERAPY OF LEISHMANIASIS

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SUMMARY : The potential utility of neoglycoprotein conjugated multilamellar liposomes as macrophage specific drug delivery system was studied using hamycin as the model drug and visceral leishmaniasis as the model macrophage disease. Hamycin, a polyene antibiotic, was found to have a growth inhibitory effect on cultured Leishmania donovani promastigotes at a concentration of 0.05 μg/ml. Hamycin entrapped in neoglycoprotein conjugated liposome (neohamysome) eliminated intracellular amastigotes of L. donovani in peritoneal macrophages 10 and 1.5 times more efficiently than did the free and liposome entrapped drug (hamysome), respectively. Moreover, neohamysome possibly could completely eliminate splenic intracellular parasites in a 45 day BALB/c mouse model of visceral leishmaniasis at a dose of 1.5 mg/Kg/day given for 4 consecutive days. Hamysome at a similar dose had 80% parasite suppressive effect whereas free drug could not be administered more than the dosage of 0.5 mg/Kg/day due to mortality problem. Neohamysome and hamysome were generally less toxic than the free drug as judged by erythrocyte lysis and several clinical parameters of liver toxicity. These results suggest a possible use of neoglycoprotein conjugated liposomes in macrophage-associated diseases.

One of the most recent trends in pharmaceutics is to deliver active drug moieties specifically to their site of action. Carbohydrate receptor mediated targeting of cytotoxic drugs to specific cell types is an useful approach for drug delivery as mannose receptor on macrophages (1), mannose-6-phosphate receptor on fibroblasts (2) and galactose receptor on hepatocytes (3) are well documented. The exclusive presence of mannose receptors on macrophages has been exploited by us in developing the neoglycoprotein, mannosyl serum albumin as an efficient macrophage-directed drug carrier (4). Cytotoxic drugs like methotrexate and doxorubicin were found to be highly effective against visceral leishmaniasis, a macrophage-associated parasitic disease, when conjugated to the neoglycoproteins (5,6). The polyene antibiotic, amphotericin B is used extensively for most systemic fungal infections including antimony resistant leishmaniasis. Very recently hamycin, another polyene

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antibiotic, now in extensive use in the treatment of candidiasis and otomycosis, has been found to be remarkably effective in killing Leishmania parasites (7). But the use of these drugs suffer from the limitations of acute and chronic toxicity. However, it has now been established that encapsulation of amphotericin B in liposomes can reduce the toxic effects of the drug without decreasing its efficacy (8). Liposome-encapsulated hamycin when compared to the free drug, also has greatly reduced in vivo and in vitro toxicity (9). The natural homing of liposomes by macrophages has been exploited to activate the tumoricidal properties of macrophages, by liposome-associated immunomodulators (10,11), or for the treatment of diseases linked to macrophage-resident microorganisms (12) and parasites including Leishmania (13). However, an useful approach for promoting the uptake of liposomal content by macrophages is to incorporate ligands capable of interacting with macrophage surface receptors. Taking into account the exclusive presence of mannose receptors on macrophages and the successful delivery of methotrexate and doxorubicin in the treatment of leishmaniasis by conjugating the drug to mannosyl serum albumin (5,6), we decided to prepare liposomes bearing this neoglycoprotein on their surface as an alternate means of delivering hamycin. We herewith report the efficacy of neoglycoprotein-liposome encapsulated hamycin in the treatment of both in vitro and in vivo models of visceral leishmaniasis.

MATERIALS AND METHODS

Parasites and macrophages : Leishmania donovani AG83 (MHOM/IN/1983/AG83) was isolated from an Indian patient with kala-azar (14,15). Strain AG83 was maintained in BALB/c mice by intravenous passage every 6 weeks. For in vitro studies, parasites were grown in liquid medium of M199 (GIBCO Laboratories, grand Island, NY) supplemented with 10% fetal bovine serum (FBS). Macrophages were collected by peritoneal lavage from mice (BALB/c, 20-25 g) given intraperitoneal injections of 0.5 ml of thioglycollate broth 5 days before harvest and were cultured as described earlier (16). The composition of the macrophage culture medium (α-10) as described earlier (16) is α-minimal essential medium (α-MEM) (GIBCO Laboratories, Grand Island, NY) plus $NaHCO_3$ (2.2 g/l), 10% (v/v) FBS, 100 units of penicillin/ml and 100 µg of streptomycin/ml.

Preparation of hamycin containing liposome (hamysome) : Multilamellar liposomes were prepared with egg lecithin, cholesterol, dicetylphosphate and a bovine brain type III ganglioside (all from Sigma Chemical Co., MO, USA) in a molar ratio of 35:45:5:15 according to the method described earlier (17). Hamycin dissolved in ethanol was mixed with the lipids in chloroform-methanol (2:1, v/v) at a drug to lipid ratio of 1:10 as described in (9). The organic solvents were evaporated under vacuum using a rotary evaporator. The thin dry film consisting of lipid mixtures was dispersed in 0.025M sodium phosphate buffer, pH 7.2 containing 0.15M NaCl. Unentrapped material was separated by ultracentrifugation (Beckman, model L868) at 105,000g for 90 min (three times). An aliquot of final liposome suspension was dissolved in ethanol and used to determine the amount of hamycin entrapped in liposomes by measuring absorbance at 380 nM according to (9).

Covalent coupling of neoglycoprotein to hamysome (neohamysome) : The neoglycoprotein, mannosyl human serum albumin (Man-HSA) was prepared according to the method described earlier (18). Liposome suspensions consisting of the above mentioned lipid composition and hamycin were prepared in 0.02M borate buffer, pH 8.4 containing 0.14M NaCl. The conjugation of neoglycoprotein to liposome was carried out by reductive amination using sodium cyanoborohydride according to Yamazaki et al. (19). Liposome suspension after conjugation was dialyzed against 0.025M phosphate buffer, pH 7.2 containing 0.15M NaCl. Neohamysome thus prepared was found to contain 0.14g Man-HSA (determined by modified Lowry method (20)] per g of of lipid [determined by first measuring the cholesterol content (21) and then calculating the amount of total lipid on the basis of molar ratio].

Effect of hamycin on L. donovani promastigotes : Hamycin was dissolved in dimethyl sulfoxide and diluted with saline to required concentrations. Growth inhibition studies in liquid media on L. donovani strain AG83 promastigotes were done as described recently for doxorubicin (22).

Treatment of parasite infected macrophages with free hamycin, hamysome and neohamysome : L. donovani promastigotes were used to infect cultures of adherent macrophages at a ratio of 10 parasites per macrophage. Infection was allowed to proceed for 4h, and the unphagocytized parasites were removed by washing with medium. After 24h in α-10 medium, infected cells were placed in α-10 medium containing hamycin, hamysome and neohamysome for various times. Drugs were then removed by washing and cells were placed in α-10 medium for an additional 20h. Cells were then fixed in methanol and stained with Giemsa stain. The number of amastigotes in 200 macrophages in drug treated and control cultures was determined.

Drug treatment of infected mice : Leishmanicidal potency of hamycin, hamysome and neohamysome was tested in BALB/c mice (~20g body weight) infected intravenously with Leishmania parasites ($2x10^6$ parasites/mouse). Various dosages of test materials in 0.2 ml of volume were injected through the tail vein for 4 consecutive days 15 days after inoculation of parasites. Forty five days after the start of infection, the animals were sacrificed, multiple spleen impression smears were prepared and stained with Giemsa stain (23). The number of amastigotes per spleen cell nucleus was then determined in drug-treated and saline-treated animals.

Toxicity studies : Lysis of erythrocytes by various concentrations of free and liposomal hamycin was quantitated by measuring the hemoglobin release according to Mehta et al (9). Blood urea nitrogen, serum transaminases (SGOT and SGPT), cholesterol and alkaline phosphatase were analysed in the serum of animals one day after drug treatment (0.25 mg/Kg/day for 4 days for free hamycin and 1.5 mg/Kg/day for 4 days for hamysome and neohamysome) according to Chaney and Marbach (24), Henry (25), allain et al (26) and Walter and Schult (27) respectively.

RESULTS

Liposome encapsulated hamycin

1.21±0.21 µg hamycin was encapsulated per µmol of total lipid for both hamysome and neohamysome. The percent encapsulation (amount of hamycin in liposomes as a percent of initial amount of hamycin) was 73.5±11.2%. The $T_{\frac{1}{2}}$ of permeation of hamycin from liposomes was 32 days at 4°C in PBS, 4 days at 37°C in PBS and 5 days at 37°C in presence of 20% FBS.

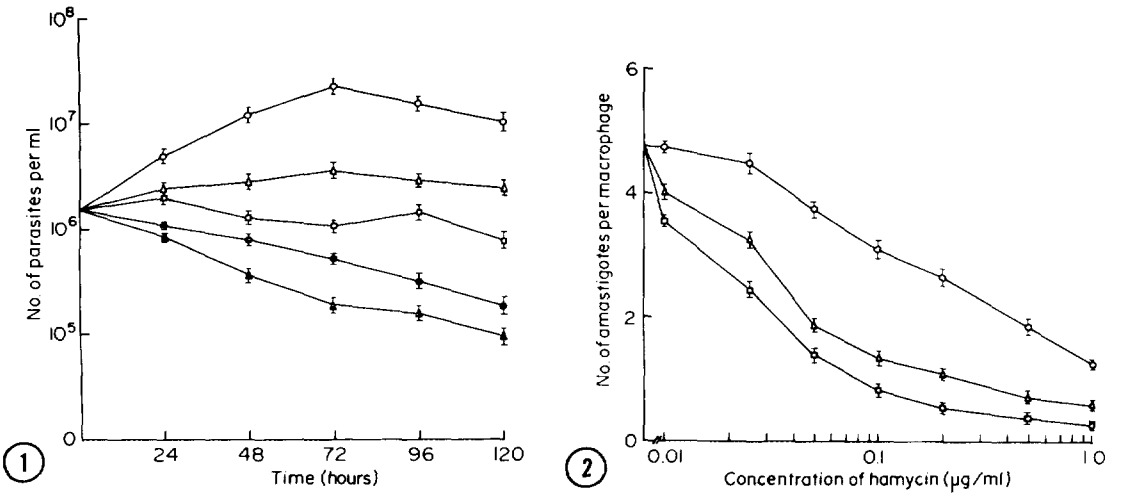

Fig.1. Effect of hamycin on L. donovani promastigotes. Hamycin concentration (µg/ml);○,0; △,0.02; □, 0.05;●, 0.1;▲, 0.2.

Fig.2. Effects of various drug forms on growth of L. donovani amastigotes in mouse peritoneal macrophages. L. donovani infected cells were treated with various concentrations of hamycin given as free drug (○), hamysome (△) and neohamysome (□). Data represent mean ± SD of three experiments.

Effect of hamycin on L. donovani promastigotes

Hamycin was found to have profound growth inhibitory effect on L. donovani promastigotes (**Fig.1**). Promastigotes could not sustain growth at concentrations greater than 50 ng/ml. To determine whether the drug was killing the organism or inhibiting growth, samples were taken, placed in fresh medium without hamycin and examined microscopically for growth. Hamycin appeared to be a leishmanicidal agent as it caused a decrease in the number of viable organism.

Treatment of L. donovani infected macrophages with hamycin, hamysome and neohamysome

The suppression of multiplication of macrophage-contained amastigotes by free hamycin, hamysome and neohamysome in representative experiments is shown in **Fig.2**. Infected macrophages were treated with various drug forms for 3h at 37°C in α-10 medium. Neohamysome was the most effective of all drug forms with a 50% inhibitory concentraation of .026 µg/ml as compared to .038 µg/ml for hamysome and 0.26 µg/ml for free hamycin. The antileishmanial effect of hamysome and neohamysome was not due to the drug that may have leaked from the liposomes during the time of treatment. Treatment for 3h with cholesterol free liposomes, from which most of the drug would be liberated did not suppress parasite multiplication. Suppression of Leishmania was not associated with phagocytosis of liposome alone, since liposomes without hamycin were not suppressive (data not shown).

Antileishmanial activity of various drug forms against infected mice model

The efficacy of hamycin, hamysome and neohamysome for the treatment of visceral leishmaniasis was tested in a mouse model. The BALB/c mice were infected intravenously with L. donovani strain AG83 as described in Methods. The infection was allowed to proceed for 45 days, when spleen weight increased from 0.103±0.013g to 0.914±0.106g. Various drug dosages ranging from 0.1 mg/Kg/day to 1.5 mg/Kg/day were administered intravenously daily for 4 consecutive days, 15 days after infection. Free hamycin could not be given more than 0.5 mg/Kg/day as it caused mortality problem. Almost 20% mortality was observed at 45 days when free hamycin was given at a dose of 0.75 mg/Kg/day. In contrast, a dose of 1.5 mg/Kg/day given in the form of hamysome and neohamysome resulted in 100% survival. All animals were sacrificed 45 days after parasite inoculation and the degree of antileishmanial potency of various drug forms were assessed in terms of spleen weight and splenic amastigote burden. As shown in **Fig.3** neohamysome therapy at 1.5 mg/Kg/day for 4 days resulted in possibly complete elimination of spleen parasite burden 45 days after infection with an accompanying reduction in spleen weight to nearly normal level. Culturing of splenic specimens in transformation medium at 22°c for 4 days did not show the presence of parasites. Hamysome at the same dose caused an 80% suppression of spleen parasite burden. Free hamycin at the maximum possible dose of 0.5 mg/Kg/day caused very little (~8%) suppression whereas neohamysome and hamysome at the same dose caused 70% and 55% suppression respectively.

Toxic effects of various drug forms

Free hamycin was found to have a lytic effect on erythrocytes in a concentration-dependent manner with 50% lysis effected at a dose of 1.5 µg/ml.

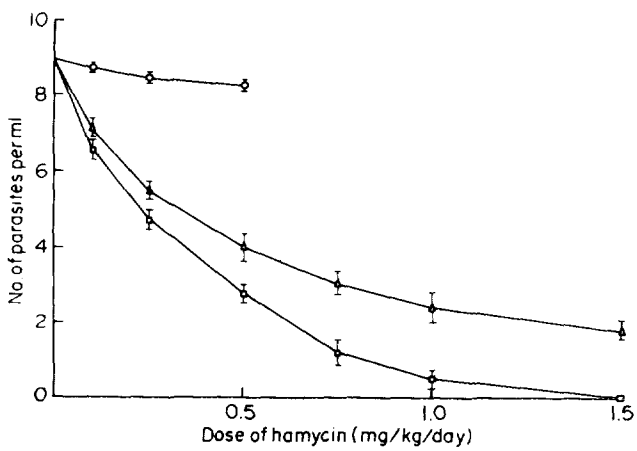

Fig.3. Suppression of spleen parasites by hamycin (O), hamysome (Δ) and neohamysome (□). Drugs at indicated doses were administered daily for 4 consecutive days 15 days after infection. All data are mean ± SD of 6 mice.

Table 1. Serum levels of various clinical parameters after treatment with different drug forms of hamycin

Parameters	Normal mice	Infected mice	Infected mice administered with		
			Free drug (0.25 mg/ Kg/day)	Hamysome (1.5 mg/ Kg/day)	Neohamysome (1.5 mg/ Kg/day)
SGOT (Units/l)	39.2±4.3	48.1±5.92	21.1±1.8	55.1±6.4	52.5±5.3
SGPT (Units/l)	21.4±2.8	27.2±3.3	82.8±7.6	24.1±3.2	24.3±2.8
Alkaline phosphatase (Units/l)	48.7±3.9	58.6±4.7	137.5±11.2	57.9±4.2	58.2±4.9
Blood urea nitrogen (mg/dl)	18.9±2.3	23.3±2.8	81.4±6.1	18.3±1.6	19.2±2.2
Cholesterol (mg/dl)	119.4±9.5	125.6±10.4	58.4±6.1	123.8±11.2	124.5±10.8

Unit of SGOT and SGPT : μmoles of pyruvate formed per min. Unit of alkaline phosphatase: μmoles of p-nitrophenol formed per min. All the parameters were measured in the serum of animals one day after drug treatment at indicated doses for four consecutive days. Data represent mean ± SD of three experiments.

Hamysome and neohamysome, however, showed very little lysis (2.2%) at a similar dose. Even at a high concentration of 10 μg/ml of hamycin, hamysome and neohamysome caused only 8% lysis as compared to 100% lysis when given in the free form. The presence of neoglycoprotein on liposomes did not alter the lytic effects of hamycin containing liposomes. Some of the clinical chemistry parameters of liver dysfunction like serum cholesterol, serum transaminases, alkaline phosphatase and blood urea nitrogen level were found to be significantly altered in case of animals treated with free hamycin at a dose of 0.25 mg/Kg/day for 4 days indicating thereby some sort of hepatotoxicity (**Table 1**). However, hamycin given in the form of hamysome and neohamysome did not result any hepatotoxicity even at a much higher dose of 1.5 mg/Kg/day for 4 days as reflected by the unaltered levels of clinical parameters compared to control values.

DISCUSSION

The present study describes a new carrier system for macrophage-specific drug trgeting in the therapy of experimental visceral leishmaniasis. A toxic antifungal polyene antibiotic, hamycin was encapsulated in neoglycoprotein-conjugated liposomes (neohamysome). Ample evidences were provided in our earlier studies (**5,6,18**) for macrophage-specific navigatory potential to

the neoglycoprotein, mannosyl human serum albumin. The superior efficacy of the encapsulated drug in eliminating intracellular amastigotes of L. donovani both in vitro macrophage model and in vivo mouse model of leishmaniasis demonstrates the effectiveness of this approach. The potential utility of neoglycoprotein-conjugated liposomes as a new type of drug targeting device exploiting cellular functions of carbohydrate-binding proteins has been suggested by Gabius et al (19).

In the present study, neohamysomes were consistently more effective than hamysomes and free hamycin in causing parasite suppression in infected macrophages. Moreover, hamycin given in the form of neohamysome could completely eliminate the spleen parasite burden in infected animals at a dose of 1.5 mg/Kg/day for 4 consecutive days. Non encapsulated hamycin injected by the same route and schedule was toxic at a much lower dose as revealed by various clinical parameters. In the erythrocyte toxicity test hamysome and neohamysome showed no damage in concentrations of 10 μg/ml, almost 10 times the concentration at which free hamycin caused lysis. Liposome encapsulated drugs had significantly reduced toxicity as well as had improved the killing of intracellular parasites. The mechanism by which neohamysome improved the therapeutic index of hamycin in the treatment of leishmaniasis is presumed to be a facilitated delivery of the drug to macrophages of the liver and spleen through mannose receptors. However, prolonged presence of hamycin in liver and spleen resulting from entrapment of drug within liposomes may also account for the improved therapeutic activity. The combined effect of neoglycoprotein and liposomes indeed resulted in an increased availability of the drug for intracellular parasites. Macrophage mannose receptors have also been exploited for improved therapy against experimental aspergillosis by using amphotericin B encapsulated in liposomes having α-mannoside on their surface (28). The results obtained in this study seems encouraging for the use of neoglycoprotein conjugated liposomes in the therapy of other macrophage-associated infections.

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