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SUGAR RECEPTOR MEDIATED DRUG DELIVERY TO MACROPHAGES IN THE THERAPY OF EXPERIMENTAL VISCERAL LEISHMANIASIS

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SUMMARY : Methotrexate (MTX) conjugate of a neoglycoprotein, mannosyl bovine serum albumin, containing an average of 30 moles of MTX per mole of neoglycoprotein was taken up efficiently by murine peritoneal macrophages through cell surface mannosyl receptors. The conjugate strongly inhibits the growth of Leishmania donovani inside macrophages, with 50% inhibitory dose of 0.11 μg/ml MTX, which makes it 100 times more active than free MTX (50% inhibitory dose of 12.1 μg/ml). MTX conjugated to BSA or other non-specific neoglycoproteins like galactose-BSA and glucose-BSA have leishmanicidal effects comparable to free MTX. Moreover, in a murine model of experimental visceral leishmaniasis, the drug conjugate reduced the spleen parasite burden by more than 85% in a 30 day model whereas the same concentration of free drug caused little effect. The results demonstrate that neoglycoproteins may be useful as carriers for receptor mediated drug delivery to treat macrophage associated diseases.

Leishmania parasites are obligate intracellular parasites that replicate exclusively within the phagolysosome of their host's macrophages (1). Leishmaniasis, the disease caused by these parasites, occurs in large areas of the tropics and subtropics. Most of the drugs used for the therapy of this disease like antimonials, amphotericin B and pentamidine have serious side effects that limit their clinical applications. One novel approach to Leishmania chemotherapy is the encapsulation of clinical agents within macrophage directed carriers like liposomes (2,3), niosomes (4) and erythrocyte ghosts (5). Although some very encouraging results have been obtained in animal model, these carriers suffer from the limitations of complexity of their composition and limited shelf-life. We describe an alternative approach for selective delivery of drugs to macrophages in which a cytotoxic drug, methotrexate (MTX) was conjugated to a neoglycoprotein mannosyl bovine serum albumin, recognized by the mannosyl

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receptors present exclusively on the surface of macrophages. Mannosyl receptor is one of the most well characterized cell surface receptors in terms of number of receptors per cell, their affinity for the ligand and turnover (**6,7**). In this report we show the superior efficacy of the conjugated drug compared to free drug in both *in vitro* macrophage model and *in vivo* murine model of visceral leishmaniasis.

MATERIALS AND METHODS

Parasites : Leishmania donovani strain UR6 (MHOM/IN/1978/UR6) and strain AG83 (MHOM/IN/1983/AG83) were isolated from Indian patients with Kala-azar (**8,9**). UR6 was maintained in modified Ray's medium (**8**) and AG83 was maintained in BALB/c mice by intravenous passage every 6 weeks.

Macrophage culture : Macrophages were collected by peritoneal lavage from mice (BALB/c, 20-25g) given intraperitoneal injections of 0.5 ml 4% thioglycollate broth 5 days before harvest and cultured as described earlier for rats (**10**). Composition of macrophage culture medium (α-10) as described earlier (**10**) is MEMα medium (GIBCO 430-1900) plus 2.2 g/l $NaHCO_3$, 10% fetal bovine serum, 100 IU of penicilin and 100 µg of streptomycin per ml medium.

Neoglycoprotein-MTX conjugate : Neoglycoproteins like mannose bovine serum albumin (BSA), galactose-BSA and glucose-BSA were prepared as described earlier (**11**) and found to contain 25,28 and 23 moles of sugar residues respectively per mole of protein. MTX was coupled to the neoglycoproteins through 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC). One µmole of neoglycoprotein was added to a stirring solution of 100 µmole of MTX in a volume of 6 ml 0.05M $NaHCO_3$, pH 6.0. A solution of EDC in water (1 ml) was added dropwise over a period of 30 min at room temperature and the pH was maintained at 6.0 with 0.5 M HCl. Unreacted MTX was separated from the conjugate by eluting through a Sephadex G-50 column with 0.05 M $NaHCO_3$, pH 6.0. Conjugates were found to contain an average of 30 moles of MTX per mole of neoglycoprotein as determined by absorbancy at 340 nm.

Binding assay with the conjugate : Mannose-BSA and mannose-BSA-MTX were iodinated by the chloramine T method (**12**) to a specific activity of 3-5 $x10^6$ cpm/µg. Binding studies on macrophages were performed according to (**3**).

Effect of free and conjugated MTX on infected macrophages : Promastigotes were used to infect cultures of adherent macrophages at a ratio of 10 parasites per macrophage. Infection was permitted to proceed for 4 h and the non-phagocytized parasites were removed by washing with medium. After 24 h in α-10, infected cells were placed in α-10 containing drug or drug conjugate for 3 h. Drugs were then removed by washing, cells were replaced in α-10 and returned for 20 h to the CO_2 incubator. Cells were then fixed in methanol and treated with giemsa stain and the number of amastigotes in 100-200 macrophages in drug treated and control cultures were determined. The mean percent survival of Leishmania in drug treated cultures was calculated on the basis of considering no. of Leishmania in untreated cultures as 100%. The dose of drug corresponding to survival of 50% of organisms compared to controls, was determined by non-linear regression (**13**).

Treatment of L. donovani infected mouse with free or conjugated drug : BALB/c mice of about 15-20g were infected intravenously with AG83 strain ($2x10^6$ cells per mice). After 15 days post inoculation of parasites, test drugs (both free and conjugated) in different dosage levels were injected by tail vein of 0.2 ml volume for four consecutive days. Thirty days after the start of infection the surviving animals were sacrificed and multiple spleen impression smears prepared and stained using giemsa stain. Parasite burden of spleen were assessed by Stauber method (**14**). The number of parasites per host cell nucleus in the drug treated experimental groups were expressed as a percentage of the number of parasites per host cell nucleus in saline treated controls.

RESULTS

Binding of mannose-BSA and mannose-BSA-MTX by macrophages

Binding experiments were carried out on macrophages using both mannose-BSA and mannose-BSA-MTX as ligands (**Fig 1**). Binding was determined at 4°C where uptake is essentially zero. A scatchard plot of the binding data indicated the presence of single class of binding sites for the ligands with high affinity. The K_d values for mannose-BSA and mannose -BSA-MTX were identical indicating thereby that the conjugation did not affect the affinity of mannose-BSA for its cell surface receptor.

Killing of Leishmania within macrophages by MTX-conjugates

The suppression of multiplication of L. donovani amastigotes within macrophages by MTX conjugated to various neoglycoproteins were compared to free MTX and MTX-BSA (**Fig 2**). Results show that mannose-BSA-MTX

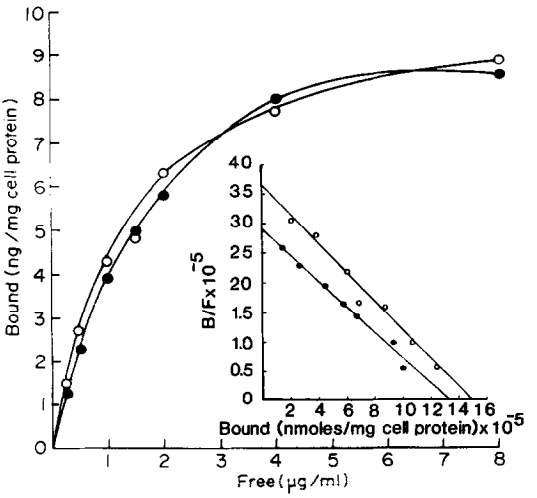

Fig.1 Binding of mannose-BSA and mannose-BSA-MTX by macrophages. Binding was determined on ice (4°C) with $3.0\text{-}5.0x10^5$ cells and ^{125}I-mannose-BSA and ^{125}I-mannose-MTX-BSA ($3.5x10^6$ cpm/ug) as ligands according to Stahl et al (3). Non-specific binding (binding in the presence of mannan, 2 mg/ml) was subtracted from the total binding. A scatchard plot (inset) was constructed from the specific binding data and K_d was calculated to be $4.12x10^{-9}$M for mannose-BSA and $4.54x10^{-9}$ M for mannose-BSA-MTX. Mannose-BSA (O); mannose-BSA-MTX (●).

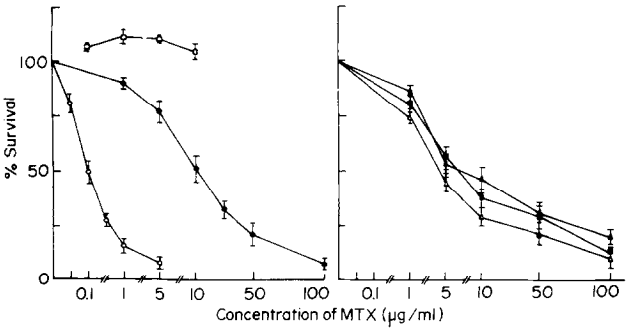

Fig.2 Effects of MTX and its conjugates on the growth of L. donovani within mouse peritoneal macrophages. L. donovani infected cells were treated with various concentrations of MTX given as free drug (●), mannose-BSA-conjugate (O), galactose-BSA-conjugate (△), glucose-BSA conjugate (▲) and BSA-conjugate (■). Unconjugated mannose-BSA was also tested for antileishmanial effect at protein concentrations corresponding to those of conjugates containing 0.1 to 10 ug/ml MTX (□). Data represent mean (±SD) of three determinations.

was the most effective of all drug forms with 50% inhibitory concentration of 0.11 µg/ml MTX, compared to 12.1 µg/ml and 7.6 µg/ml for free and BSA-conjugated MTX respectively. The effect of mannose-BSA-MTX is not due to the antileishmanial activity of mannose-BSA because the drug free mannose-BSA did not cause any significant suppression of multiplication at protein concentrations corresponding to those of conjugates containing 0.1 µg/ml to 10 µg/ml MTX. The effect of conjugates with other neoglycoproteins like galactose-BSA and glucose-BSA are comparable to that of BSA-MTX conjugate.

Efficacy of MTX and MTX-conjugate in the therapy of visceral leishmaniasis in mice

BALB/c mice were infected intravenously with L. donovani strain AG83 as described in Methods. The spleen weight increased from 16.5±2.3mg in normal animals to 150.2±19.8 mg in infected animals 30 days after infection. Fifteen days after post inoculation of parasites, the animals were given intravenous injections of free MTX and conjugated MTX daily for four consecutive days. Various drug dosages were injected at MTX equivalent concentrations ranging from 3.12 µg/kg/day to 625 µg/kg/day. As shown in Fig.3 conjugated MTX therapy at 62.5 µg/kg/day for 4 days resulted in more than 85% suppression of spleen parasite burden 30 days after infection. Free MTX at the same concentration caused ~ 10% reduction in spleen parasite burden. At higher doses, however, free MTX had significant effects. For ex, at a dose of 625 µg/kg/day free MTX, 55% suppression of parasite burden was observed. In the case of conjugated MTX, increasing the dose from 62.5 µg/kg/day to 312 µg/kg/day resulted in little increase in suppression of parasite burden from 85% to 95%. Spleen size reduced to nearly normal size in the case of conjugated MTX therapy.

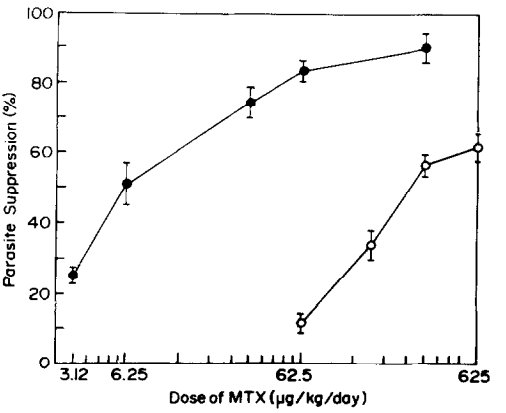

Fig.3 Suppression of Leishmanial infection in mice by MTX and mannose-BSA-MTX. Mice had been infected for 15 days prior to treatment and the indicated doses were administered intravenously daily for four consecutive days. Animals were sacrificed after a total infection period of 30 days. In the control equivalent volumes of normal saline were given instead of drugs. Data represent mean (±SD) of five animals. Animals were administered with free MTX (O) and conjugated MTX (●).

DISCUSSION

Although a number of work has been carried out on the encapsulated drug therapy for leishmaniasis using liposomes (2,3) and erythrocyte ghosts (5), studies on receptor mediated targeting of antileishmanial drugs exploiting specific macrophage receptors are very limited. Only recently scavenger receptor mediated drug delivery has been reported in the therapy of experimental cutaneous leishmaniasis (15). In the present work, MTX-neoglycoprotein (mannosyl BSA) conjugate has been designed to achieve selectivity towards macrophages. The impetus for the preparation of neoglycoprotein-drug conjugate was the selective presence of well characterized mannosyl receptors on macrophage surface. The results obtained in this study show that MTX conjugate has a considerably higher leishmanicidal activity than the free drug at the same concentration. The amplification factor (A.F), accounting for the improvement of drug action by a targeting process can be defined as the ratio of the concentration of free drug to that of the targeted drug, measured at the same antimicrobial activity (16). The A.F. obtained in our targeting experiment is about 10^2.

The evidence for proper subcellular delivery of MTX conjugate was obtained by its strong leishmanicidal effect on both infected macrophages and infected animals. An intracellular effect of carrier-coupled drug is of course the best demonstration of their internalization. The internalization of the drug conjugate probably occurs by endocytosis via the mannosyl receptor, since a strong antileishmanial effect was obtained with mannose-BSA-MTX but not with other non-specific conjugates like BSA-MTX, glucose-

BSA-MTX or galactose-BSA-MTX (**Fig 2**). Neither the dosages of MTX nor any of the neoglycoproteins tested proved to be toxic for macrophages as evidenced by viability (trypan blue) and the release of lactate dehydrogenase from the cells (data not shown). All of the chemotherapeutic effects shown in Fig 3 were due to MTX only as there was no effect when infected macrophages were treated with mannose-BSA (**Fig 2**). The neoglycoprotein served solely as targeting and drug carrying device.

The idea of targeting substances of biological importance to macrophages using mannose is not entirely new. Introduction of α-mannoside onto the liposomal surface resulted in the preferential targeting of these liposomes to Kupffer cells of liver (**17**) and mannosyl liposomes have been used to load macrophages with glucocerebroside in developing a cell culture model for Gaucher disease (**18**). Moreover, Roche et al (**19**) have activated macrophages by means of muramyl dipeptide covalently bound to mannose- or mannose-phosphate-terminated glycoproteins. The present study indicates that sugar receptor mediated targeting of neoglycoprotein conjugated cytotoxic drugs to specific cell types is feasible. This could be a useful approach for treatment of diseases involving particular cell types, known to have specific sugar receptors on their surface. Also as modified drugs, the conjugates are novel pharmacological agents which may become important in the elucidation of basic rules of site directed chemotherapy against certain other macrophage diseases.

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