Journal of Antimicrobial Chemotherapy (1993) **31**, 151–159

Pharmacokinetics and biodistribution of methotrexate conjugated to mannosyl human serum albumin

Rupnarayan Sett, Himadri Sekhar Sarkar and Pijush Kanti Das*

Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Calcutta-700 032, India

The superior efficacy of mannosylated neoglycoprotein-conjugated methotrexate, compared with free drug, in eliminating the parasite burden in both the in-vitro macrophage model and in-vivo mouse model of visceral leishmaniasis has been demonstrated previously. In the present study it was found that: (i) methotrexate conjugated to mannosyl human serum albumin (Man-HSA) was taken up rapidly by the liver and spleen, whereas the free drug was taken up by the kidney; (ii) uptake of the conjugate was ten-fold more efficient in liver macrophages (Kupffer cells) than in hepatocytes; (iii) most of the drug conjugate reached the lysosomes of Kupffer cells; and (iv) the active drug was released in the lysosomes of macrophages to act on *Leishmania* parasites.

Introduction

The stability of glycoproteins and glycoconjugates in mammalian plasma is known to be determined by the nature of exposed or terminal sugar residues associated with the carbohydrate chains. Mannose-specific receptors are present exclusively on the surface of differentiated macrophages, which are responsible for the internalization of mannose-terminated glycoproteins infused i.v. (Gordon & Makena, 1989). Mannose receptors are well-characterized in terms of their number/cell, their affinity for the ligand, and turnover (Stahl *et al.*, 1980; Stahl & Gordon, 1982; Wileman, Boshans & Stahl, 1985). *Leishmania donovani*, the causative agent of visceral leishmaniasis, is known to be harboured and to multiply in the lysosomes of macrophages from spleen, liver and bone-marrow in man (Chang & Fong, 1983). We have developed a macrophage-specific drug delivery system exploiting mannose receptors, with leishmaniasis as the model macrophage disease. Methotrexate (MTX) was selected as the model drug, with the neoglycoprotein, mannosyl human serum albumin (Man-HSA), as the carrier. Methotrexate is an anti-tumour drug, but also has a profound antileishmanial effect (Scott, Coombs & Sanderson, 1987; Chakraborty, Bhaduri & Das, 1990). The exclusive expression of mannose receptors on the surface of macrophages, along with the parasitic location of *Leishmania* amastigotes in macrophage lysosomes, formed the basis of drug targeting. The leishmanicidal efficacy of the drug conjugate was found to be almost 100-fold greater than that of the free drug on both the in-vitro macrophage culture model and an in-vivo BALB/c mouse model of visceral leishmaniasis (Chakraborty *et al.*, 1990). The present study reports biodistribution studies designed

*Corresponding author.

0305–7453/93/010151+09 $08.00/0

to ascertain the physiological concentration, as well as the fate, of the drug in tissues after conjugation with the carrier.

Materials and methods

Preparation of Man-HSA-(^{3}H)-MTX conjugate

The neoglycoprotein, Man-HSA was prepared in a multi-step reaction by a modification of the procedure described by Lemieux, Bundle & Baker (1975). In short, 8-methoxycarbonyloctylmannose was first synthesized by condensing acetobromomannose with 8-ethoxycarbonyloctanol, followed by treatment with sodium methoxide in dry methanol. The ester intermediate was coupled after hydrazinolysis with HSA by the azide procedure at -50°C. The final product was dialysed and lyophilized. The various intermediates of mannose derivatives were characterized by spectroscopic and analytical methods. The number of mannose residues was determined by the phenolsulphuric acid method (Dubois *et al.*, 1956), and was found to be 45 mol mannose/mol HSA. (^{3}H)-MTX was coupled to Man-HSA through 1-ethyl-3(3-dimethylaminopropyl-carbodiimide hydrochloride (EDC) as described by Chakraborty *et al.* (1990). Almost 30 mols MTX were coupled/mol HSA.

Tissue distribution studies

BALB/c mice weighing *c.* 20–25 g were used throughout the experiment. Each mouse received a single dose iv (through the tail vein) of 0·2 mL conjugate (0·34 mg protein) containing 1×10^5 cpm (^{3}H)-MTX. As a control, free (^{3}H)-MTX and (^{3}H)-MTX-HSA were also injected. After time intervals of 5, 10 15, 30, 60 and 90 min, respectively, groups of three mice were killed and the livers, kidneys, spleens and lungs were removed. Each sample of tissue was washed with 0·9% saline and blotted with filter paper. Concomitantly, 0·1 mL of blood was collected from the heart. Samples were prepared for scintillation counting according to the method of Kimelberg (1976). Tissue samples were solubilized, with 1 mL of Protosol (New England Nuclear, Boston, MA, USA) for each 100 mg wet weight of tissue, and heated at 60°C for 3 h in a water bath. After cooling, each sample was decolourized with 0·5 mL 30% H_2O_2. Blood samples (0·1 mL) were solubilized in 1 mL Protosol for 1 h at room temperature, after which 0·5 mL isopropanol and 0·5 mL 30% H_2O_2 were added. All samples treated with Protosol were neutralized with one-tenth the volume of 5 N HCl. All the samples were dried at 45°C on filter paper discs (2·5 cm, GF/C; Whatman, Maidstone, UK). Dried discs were immersed in 10 mL of scintillation fluid (Cocktail T: Spectrochem, Bombay, India) and counted in a L5500 TD Liquid Scintillation Counter (Beckman Instrument, Fullerton, CA, USA).

Sub-cellular fractionation of liver

After appropriate treatment, the mice were killed and their livers removed. The livers were washed with 0·9% saline, and then blotted with filter paper. Each whole liver was then homogenized in 0·32 M sucrose (4 mL/g tissue), and subsequently fractionated by differential centrifugation into nuclear, mitochondrial-lysosomal, microsomal and soluble fractions, as described by Gregoriadis & Sourkes (1967). Samples were prepared with Protosol for scintillation counting as described earlier.

Isolation of parenchymal and non-parenchymal cells

Liver cell suspensions were obtained by perfusion of liver *in situ* according to the modified method of Berry & Friend (1969) and Munthe-Kaas & Seglen (1974), as described by Murray (1987). Calcium-free Krebs-Henseleit buffer containing 0·025 M HEPES was used as the perfusate. The concentration of collagenase was 0·03%. Perfusion was discontinued after 25 min and the liver was cut into small pieces in 50 mL of oxygenated calcium-containing Krebs-Henseleit buffer. The mixture was swirled gently for 2 min with a magnetic stirrer. The cell suspension was passed through a 0·03 mm nylon mesh and centrifuged at 50 **g** for 2 min. Parenchymal cells were sedimented and washed three times with the same buffer. The supernatant, containing the nonparenchymal cells, was purified further by underlayering 23 mL of the supernatant with 13 mL of metrizamide solution (16 g metrizamide, 0·24 g HEPES, 0·05 g KCl, 0·018 g $CaCl_2 \cdot H_2O$, 5·5 mL 0·1 N NaOH, adjusted to 100 mL with water, pH 7·6). Tubes were centrifuged for 45 min at 3000 **g**, after which the nonparenchymal cells recovered from the metrizamide interphase were washed with calcium-containing Krebs-Henseleit buffer, spun at 500 **g** for 3 min, resuspended in the same buffer, and counted. In the competition experiments, yeast mannan (1 mg/mL) and Man-HSA (2 mg/mL) were injected 5 min before Man-HSA-(^{3}H)-MTX. Samples for radioactive counting were prepared with Protosol as described earlier.

Sub-cellular fractionation of isolated liver cells

The cell suspensions were homogenized at 4°C in a Teflon Glass Homogeniser (Thomas Scientific, Philadelphia, PA, USA), followed by sub-cellular fractionation as described by Dasgupta & Bachhawat (1985). Radioactivity in each fraction was determined as described earlier, and was expressed as a percentage of the total radioactivity present in the homogenate before sub-cellular fractionation.

Degradation of drug conjugate by lysosomal enzymes

An extract of mouse liver lysosomes was prepared by methods similar to those of Das & Bishayee (1980). The drug conjugate was incubated in phosphate buffered saline (pH 7·5) and citrate-phosphate buffer (0·05 M, pH 5·0) at 37°C in the presence of lysosomal extract or trypsin as control. The proportion of Man-HSA-(^{3}H)-MTX to lysosomal extract or trypsin was taken as 1 : 10 or 1 : 1 respectively. Protein concentrations were determined by the method of Lowry *et al.* (1951). ^{3}H counts in the pellet and supernatant were measured after precipitation with an equal volume of 20% TCA.

Results

Plasma clearance and tissue distribution pattern of methotrexate and its conjugates

Figure 1 compares the ^{3}H counts in plasma after administration iv of comparable amounts of free MTX, Man-HSA-(^{3}H)-MTX, and HSA-(^{3}H)-MTX. Plasma clearance was much faster for both free MTX and Man-HSA-MTX compared with HSA-MTX. The $T_{\frac{1}{2}}$ was 4 min for free MTX, and 10 min for Man-HSA-MTX, whereas for HSA-MTX it was 25 min. This rapid clearance of Man-HSA-MTX resulted perhaps from rapid uptake by the liver and spleen, as evidenced by tissue distribution studies

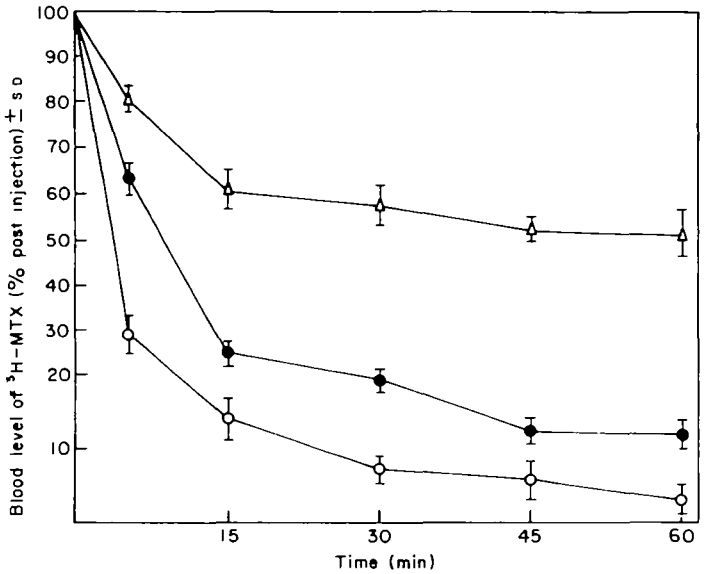

Figure 1. Blood levels of ^{3}H in mice following administration iv of. (^{3}H)-MTX (○); Man-HSA-(^{3}H)-MTX (●), and HSA-(^{3}H)-MTX (△). Mean ± standard deviation of three mice/group

(Table I). After 15 min, 52% of the administered Man-HSA-MTX was recovered (relative radioactivity/100 mg tissue) in the liver, and 13% in the spleen. In contrast, the rapid clearance of free MTX resulted probably from its substantial accumulation in the kidney (36%). The uptake rates of the various forms of (^{3}H)-MTX by the liver are shown in Figure 2. For all the drug forms, the maximum uptake of (^{3}H)-MTX was 15 min after administration. However, in each case, there was a subsequent gradual decrease in the amount of (^{3}H)-MTX in the liver. The tissue levels of (^{3}H)-MTX reflect the difference between uptake and possible clearance and loss. The possible role (if any) of the protein carrier on the clearance of drug from the liver has not been investigated.

Sub-cellular distribution of the various forms of (^{3}H)-MTX in the liver

At the time point of highest uptake of MTX-neoglycoprotein conjugate, sub-cellular fractionation of liver by differential centrifugation revealed that most of the mannosylated drug conjugate had reached the lysosome-rich fraction; almost 55% of the total count, compared with 20% and 41% for free MTX and HSA-MTX, respectively

Table I. Tissue distribution of various forms of (^{3}H)-MTX, measured 15 min after administration iv. Values shown are means of four independent observations

	% Injected dose/organ		
Organ	(^{3}H)-MTX	HSA-(^{3}H)-MTX	Man-HSA-(^{3}H)-MTX
Liver	10·1 ± 1·8	18·2 ± 2·5	51 4 ± 7·8
Spleen	6·2 ± 1·1	9·3 ± 1·6	13·3 ± 2·7
Kidney	36·4 ± 4·2	8·1 ± 0·6	4·1 ± 0·5
Lung	0·5 ± 0·1	0·4 ± 0·1	0·4 ± 0·1

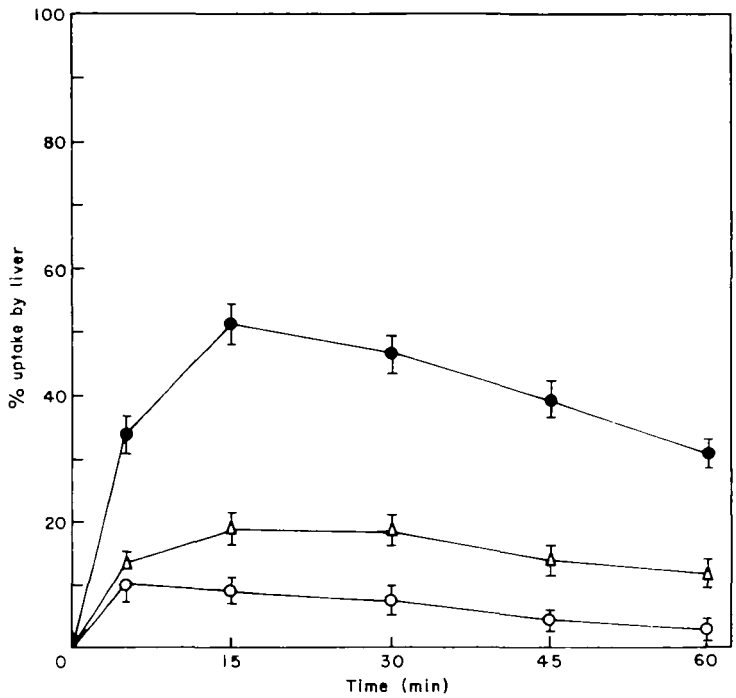

Figure 2. Uptake rates of various forms of (^{3}H)-MTX by mouse liver (^{3}H)-MTX (○), Man-HSA-(^{3}H)-MTX (●), and HSA-(^{3}H)-MTX (△). Mean ± standard deviation of three mice/group.

(Table II). Uptake of HSA-MTX by the lysosome-rich fraction was greater than uptake of free MTX, probably because of the high concentration of phagocytic cells in this tissue. The further enhancement observed with mannose substitution indicated the special role of mannose receptors in the process of receptor-mediated endocytosis. The greater accumulation of free MTX in the soluble supernatant fraction indicated its presence in extracellular fluid and/or passive transport to cytosol.

Table II. Sub-cellular distribution of (^{3}H)-MTX in the liver, measured 15 min after administration iv. Values shown are means of four independent observations

	(^{3}H)-MTX		HSA-(^{3}H)-MTX		Man-HSA-(^{3}H)-MTX	
Fraction	cpm × 10^{-3}	% of total	cpm × 10^{-3}	% of total	cpm × 10^{-3}	% of total
Original homogenate	8·1 ± 1·1	100	17·5 ± 2·1	100	49·8 ± 4·2	100
Cell debris-nuclear	2·2 ± 0·5	27·2	4·5 ± 0·7	25·7	10·3 ± 1·5	20·7
Mitochondrial lysosome	1·6 ± 0·2	19·8	7·1 ± 1·1	40·6	27·2 ± 3·3	54·6
Microsome	0·6 ± 0·1	7·4	2·1 ± 0·4	12·0	3·6 ± 0·7	7·2
Soluble supernatant fraction	3·2 ± 0·5	39·5	3·1 ± 0·4	17·7	5·8 ± 0·8	11·6

Table III. Uptake of various forms of (^{3}H)-MTX by mouse liver cells. Values shown are means of four independent observations made 15 min after administration

Experiment	Radioactivity infused (cpm × 10^{-5})	Radioactivity (cpm/mg cell protein ± S.D.)	
		Hepatocytes	Non-parenchymal
(^{3}H)-MTX	5·0	327 ± 33	214 ± 38
HSA-(^{3}H)-MTX	5·0	475 ± 57	783 ± 81
Man-HSA-(^{3}H)-MTX	5·0	276 ± 32	2562 ± 363
Man-HSA-(^{3}H)-MTX + Man-HSA (2 mg/mL)	5·0	524 ± 71	887 ± 102
Man-HSA-(^{3}H)-MTX + Mannan (1 mg/mL)	5·0	465 ± 56	1120 ± 118

Uptake of methotrexate and its conjugates by mouse liver cells

Parenchymal and non-parenchymal mouse liver cells were separated after i.v. administration of (^{3}H)-MTX, or its conjugates, and the counts of (^{3}H)-MTX were measured. It was found that non-parenchymal cells were ten-fold more efficient than hepatocytes in the assimilation of Man-HSA-(^{3}H)-MTX, as evidenced by the comparison of radioactivity/mg cell protein (Table III). The uptake was also greater for HSA-(^{3}H)-MTX, and almost double in non-parenchymal cells compared with hepatocytes. This probably resulted from non-specific engulfment of HSA-(^{3}H)-MTX by liver macrophages. In contrast, the distribution pattern of free (^{3}H)-MTX indicated non-specific passive transport to various liver cells; uptake was higher in hepatocytes than in non-parenchymal cells, possibly because of the variation in their size, shape and disposition. Enhanced uptake of Man-HSA-MTX was found to be inhibited competitively by mannan and Man-HSA. This indicated a possible involvement of hepatic mannose receptors in the recognition of Man-HSA-MTX by the Kupffer cells which, in turn, may be responsible for enhanced uptake of this drug form by the liver.

Sub-cellular distribution in isolated liver cell types

In general, the content of neoglycoprotein-conjugated drug was higher in lysosome-rich fractions of both hepatocytes and non-parenchymal cells than in other fractions such as

Table IV. Relative sub-cellular distribution of the various forms of MTX in mouse liver cells.* Values shown are means of four independent observations made 15 min after administration

Experiment	Parenchymal cells		Non-parenchymal cells	
	Mitochondrial/ lysosome fraction	Plasma and nuclear membrane fraction	Mitochondrial/ lysosome fraction	Plasma and nuclear membrane fraction
(^{3}H)-MTX	23·6 ± 3·6	58·6 ± 4·2	20·3 ± 3·4	62·2 ± 5·3
HSA-(^{3}H)-MTX	32·8 ± 4·4	39·4 ± 5·2	30·7 ± 5·4	42·5 ± 5·0
Man-HSA-(^{3}H)-MTX	43·5 ± 6·2	35·4 ± 4·3	53·5 ± 5·1	24·7 ± 3·1

*Percentage of total count in isolated cell fractions

Table V. Degradation of Man-HSA-MTX in the presence of lysosomal enzymes. Values shown are means of four independent observations made 15 min after administration

	(^{3}H)-MTX count (% of total)			
	pH 7·5		pH 5·0	
Experiment	TCA-soluble	TCA-insoluble	TCA-soluble	TCA-insoluble
No enzyme	1·1 ± 0·1	98·6 ± 0·4	0·6 ± 0·1	99·1 ± 0·5
Trypsin	52·6 ± 4·2	44·7 ± 3·8	31·7 ± 1·9	66·2 ± 3·9
Lysosomal extract	16·4 ± 0·9	82·3 ± 2·8	58·8 ± 3·2	38·6 ± 2·6

the plasma membrane and nuclear membrane fraction (Table IV). Again, this indicated possible entry into cells through an endocytotic process. In contrast, most free MTX was associated with the plasma membrane and nuclear membrane fraction.

Degradation of Man-HSA-MTX by lysosomal enzymes

To ascertain whether the active drug was released from the neoglycoprotein conjugate, Man-HSA-MTX was incubated in the presence of lysosomal extract from mouse liver at pH 5·0 and 7·5 (Table V). Trypsin was used as a control. Trypsin was able to catalyse degradation of the conjugate at both pH values, although the rate of degradation was much faster at pH 7·5 (52·6% TCA soluble radioactivity, compared with 31·7% at pH 5·0). Almost 60% of the bound drug was released from the neoglycoprotein carrier when incubated with lysosomal extract at pH 5·0. Only 16% of the drug was released when incubated at pH 7·5. This indicated that much of the delivered drug is freed from its carrier in the phagolysosomes of macrophages.

Discussion

The challenge in therapeutics is to develop drugs that can be delivered selectively to the target site. Since imparting target selectivity on to the drug itself is a difficult and uncertain proposition, much attention is being devoted to the development of drug delivery systems that can carry otherwise non-selective drugs to the appropriate infected site. Of all the drug carrier systems presently available, liposomes seem to be the most versatile (Ostro, 1987; Leserman *et al.*, 1990). However, one of the major limitations of liposomal delivery system is their instability and limited shelf-life. Chemical conjugation of drugs to carriers, such as antibodies raised against target cells or their membranes, have been investigated extensively (Pastan, Willingham & FitzGerald, 1986; Worrell *et al.*, 1986). Such conjugated drugs, although highly successful in killing target cells *in vitro*, have limited potency *in vivo*. As an alternative, neoglycoproteins have been investigated as a potential drug carrier system. Methotrexate conjugated to mannosylated bovine serum albumin (Man-BSA) was found to be highly-efficient in suppressing the parasite burden in both the in-vitro macrophage model and in-vivo mouse model of visceral leishmaniasis (Chakraborty *et al.*, 1990). This targeting efficiency is not surprising considering the fact that some lysosomal enzymes and serum glycoproteins are cleared rapidly and specifically from the circulation by a receptor-mediated endocytotic process (Stahl *et al.*, 1976), and

receptors are present on matured macrophages which are specific for α-mannose terminated glycoproteins.

In the present study, it was found that there was a striking pharmacokinetic difference between the drug and its conjugate. Methotrexate conjugated to HSA with a mannose residue is cleared rapidly from blood, and displays all the characteristics of receptor-mediated endocytosis. The prompt disappearance of Man-HSA-MTX from the circulation, increased uptake by the liver and spleen, the relatively greater assimilation by Kupffer cells, and the inhibition of uptake by both Man-HSA and mannan, suggest that the drug conjugate is taken-up specifically by liver Kupffer cells as a direct consequence of the specific interaction of exposed mannosyl residues with the mannose-binding protein described by Stahl (1990). The drug conjugate reaches the liver macrophages in large quantities, particularly in the lysosomes. The drug is then cleaved from the conjugate and is released in the lysosome, i.e. the precise location where *Leishmania* amastigotes multiply. The entire pharmacokinetic and biodistribution studies argue clearly in favour of constructing a drug delivery system based on a neoglycoprotein that can carry and release an active drug at the appropriate diseased site through a receptor-mediated endocytotic process.

Acknowledgements

This work was supported by grant IND/87/018/A/0199 from the United Nations Development Programme, by the Council of Scientific and Industrial Research, and by the Department of Biotechnology, India.

References

Berry, M. N & Friend, D. S. (1969). High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *Journal of Cell Biology* **43,** 506–20.

Chakraborty, P., Bhaduri, A. N. & Das, P. K. (1990). Sugar receptor mediated drug delivery to macrophages in the therapy of experimental visceral leishmaniasis *Biochemical and Biophysical Research Communications* **166,** 404–10.

Chang, K.-P. & Fong, D. (1983). Cell biology of host-parasite membrane interactions in leishmaniasis. In *Cytopathology of Parasitic Disease (Ciba Foundation Symposium, 99)*, pp. 113–37 Pitman Books, London.

Das, P. K. & Bishayee, S. (1980). Differential localisation of phosphorylated and non-phosphorylated forms of arylsulfatase A in lysosomes. *FEBS Letters* **111,** 43–6.

Dasgupta, P. & Bachhawat, B. K. (1985). Receptor-mediated uptake of asialoganglioside liposomes: sub-cellular distribution of the liposomal marker in isolated liver cell types. *Biochemistry International* **10,** 327–36.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Annals of Chemistry* **28,** 350–6.

Gordon, S. & Makena, T. (1989). Receptors for mannosyl structures on mononuclear phagocytes. In *Mononuclear Phagocytes* (Zembala, M. & Asherson, G. L., Eds), pp. 141–50. Academic Press, San Diego, CA.

Gregoriadis, G. & Sourkes, T. L. (1967). Intracellular distribution of copper in the liver of the rat. *Canadian Journal of Biochemistry* **45,** 1841–51.

Kimelberg, H. K. (1976). Differential distribution of liposome-entrapped (^{3}H) methotrexate and labelled lipids after intravenous injection in a primate. *Biochimica et Biophysica Acta* **448,** 531–50.

Lemieux, R. U . Bundle, D. R. & Baker, D. A. (1975). The properties of a "synthetic" antigen related to the human blood-group Lewis a. *Journal of the American Chemical Society* **97,** 4076 83.

Leserman, L., Machy, P., Leonetti, J.-P., Milhaud, P. G., Degols, G. & Lebleu, B. (1990). Targeted lipsosomes and intracellular delivery of macromolecules. In *Horizons in Membrane Biotechnology* (Nicolau, C. & Chapman, D., Eds), pp. 95–102. Wiley-Liss, New York.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193,** 265–75.

Munthe-Kaas, A. C. & Seglen, P. O. (1974). The use of metrizamide as a gradient medium for isopycnic separation of rat liver cells. *FEBS Letters* **43,** 252–6.

Murray, G. J. (1987). Lectin-specific targeting of lysosomal enzymes to reticuloendothelial cells. *Methods in Enzymology* **149,** 25–42.

Ostro, M. J. (1987). Liposomes. *Scientific American* **256,** 102–11.

Pastan, I., Willingham, M. C. & FitzGerald, D. J. P. (1986). Immunotoxins. *Cell* **47,** 641–8.

Scott, D. A., Coombs, G. H. & Sanderson, B. E. (1987). Effects of methotrexate and other antifolıates on the growth and dihydrofolate reductase activity of *Leishmania* promastigotes. *Biochemical Pharmacology* **36,** 2043–5.

Stahl, P. & Gordon, S. (1982). Expression of a mannosyl-fucosyl receptor for endocytosis of cultured primary macrophages and their hybrids. *Journal of Cell Biology* **93,** 49–56.

Stahl, P., Schlesinger, P. H., Rodman, J. S. & Doebber, T. (1976). Recognition of lysosomal glycosıdases *in vivo* inhibited by modified glycoproteins. *Nature* **264,** 86–8.

Stahl, P., Schlesinger, P. H., Sigardson, E., Rodman, J. S. & Lee, Y. C. (1980) Receptor-mediated pinocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. *Cell* **19,** 207–15.

Stahl, P. D. (1990). The macrophage mannose receptor: current status. *American Journal of Respiratory Cell and Molecular Biology* **2,** 317–8

Wileman, T., Boshans, R. & Stahl, P. (1985). Uptake and transport of mannosylated ligands by alveolar macrophages. *Journal of Biological Chemistry* **260,** 7387–93

Worrell, N. R., Cumber, A. J., Parnell, G. D. Ross, W. C. J. & Forrester, J. A. (1986). Fate of an antibody-ricin A chain conjugate administered to normal rats *Biochemical Pharmacology* **35,** 417–23.

(*Received 8 April 1992; accepted 12 August 1992*)