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SELECTIVE DELIVERY OF DRUGS TO MACROPHAGES THROUGH A HIGHLY SPECIFIC RECEPTOR

AN EFFICIENT CHEMOTHERAPEUTIC APPROACH AGAINST LEISHMANIASIS

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Abstract—Methotrexate (Mtx) conjugated with maleylated bovine serum albumin (Mtx-MBSA) was taken up and degraded by cultured hamster peritoneal macrophages through the polyanion binding site for acetylated low density lipoprotein. Mtx-MBSA also eliminated intracellular amastigotes of *Leishmania donovani* in cultured hamster peritoneal macrophages about three times more efficiently than free Mtx. The antileishmanial effect of Mtx-MBSA on parasitized macrophages was blocked by MBSA, lysosomal inhibitors (chloroquine and monensin), and metabolic antagonists of Mtx (folic and folinic acids). The primary sites of accumulation of radioactivity of injected ^{125}I-labeled Mtx-MBSA were the macrophage rich tissues, viz. liver and spleen. These results suggest that Mtx-MBSA would ensure rapid and effective killing of intracellular parasites harbored by macrophages. Furthermore, these results also indicate the feasibility of a general approach for rapid intracellular delivery of desired agents to macrophages for various purposes.

Leishmania donovani, the etiological agent for visceral leishmaniasis or *Kala-azar* in humans, reside and proliferate solely in the phagolysosomes of the macrophages of the infected hosts [1]. All currently used antileishmanial agents, such as antimony compounds, diamidines and amphotericin, often lead to severe toxic side reactions [2–5]. This probably happens because these drugs are taken up not only by the host macrophages which harbor *L. donovani* but also by other cell types. Attempts have been made in the past to develop a specific targeting rationale, so that the parasite could be specifically eliminated without affecting other cell types of the host. Encapsulation of the drugs within liposomes is one of the very promising methods of developing antileishmanial agents to reticuloendothelial systems [6–8].

Our aim was to design a drug-delivery system based on the exquisite specificity and high efficiency of the process of receptor-mediated endocytosis which would selectively deliver the drugs only to the host macrophages. To achieve this, we conjugated a model antileishmanial drug, methotrexate (Mtx‡) [9], with maleylated bovine serum albumin (MBSA), the macromolecular ligand which is recognized by specific binding sites located only on macrophages [10, 11]. Experimental evidence presented in this paper indicates that such drug conjugates are likely to ensure maximum delivery of the drugs to macrophages at low plasma concentration, resulting in rapid elimination of the intracellular parasites.

EXPERIMENTAL METHODS

Materials. Special biochemicals like methotrexate, bovine serum albumin (BSA), chloroquine, monensin, folic acid and folinic acid were purchased from the Sigma Chemical Co. (St Louis, MO). Tissue culture supplies were from Gibco (U.S.A.). Other reagents used were of analytical grade.

Parasite. The promastigotes of *L. donovani* (UR6) were maintained on modified blood agar slopes [12, 13] at 25°. For experimental purposes they were harvested from 3-day-old agar slopes by scraping into phosphate-buffered (20 mM, pH 7.5) saline (0.15 M) (PBS).

Macrophages. Peritoneal cells were harvested from either male or female young (70–75 *g*) hamsters (*Mesocricetus auratus*) that had received an intraperitoneal injection of 2 ml of Brewer's thioglycollate medium (DIFCO) 5 days previously, as described [14]. The peritoneal fluid from five to ten hamsters ($6–10 \times 10^6$ cells per hamster) was pooled, and the cells were collected by centrifugation (400 *g*, 10 min, 4°). Cells were suspended in medium A [RPMI 1640 medium containing 20% heat-inactivated fetal bovine serum (HIFBS), 25 mM Hepes, 100 I.U. penicillin/ml and 100 μg streptomycin sulfate/ml] at a final concentration of 2×10^6 cells/ml. Aliquots (1 ml) of this cell suspension were dispensed into

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‡ Abbreviations: Mtx, methotrexate; BSA, bovine serum albumin; MBSA, maleylated bovine serum albumin; PBS, phosphate-buffered (20 mM, pH 7.5) saline (0.15 M); HBSS, Hanks' balanced salt solution; HIFBS, heat-inactivated fetal bovine serum; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; and TCA, trichloroacetic acid.

plastic Petri dishes (35 × 10 mm) and incubated at 37° for 2 hr. Each dish was washed three times with PBS to remove non-adherent cells. The adherent macrophages were incubated for another 24 hr with fresh medium A at 37°, washed once with PBS, and used for experiments. Each dish of adherent macrophages contained about 20% of the total number of cells originally plated (20–25 mg cell protein).

Leishmania-*macrophage tissue culture model*. The macrophage monolayers, prepared as above on glass cover-slips (18 mm), were incubated at 37° with *L. donovani* promastigotes, in medium A with a parasite to macrophage ratio of 10:1. After 2 hr of incubation, the infected macrophages were washed with PBS to remove the unabsorbed parasites. The test compound was added to the medium and, after incubation of the parasite-laden macrophages at 37° for 20 hr, the number of the amastigotes per 100–200 macrophages was counted microscopically.

Maleylation of BSA. BSA was maleylated by the reaction of the protein with maleic anhydride at constantly maintained alkaline pH (8.0) as described [15] and extensively dialyzed against 10 L PBS. Electrophoretic mobility of the chemically modified protein was tested by agarose gel (0.7%) electrophoresis at pH 8.4 [16]. The relative electrophoretic mobilities ($R_f \times 100$) of BSA and MBSA were 23 and 72, respectively.

Coupling of Mtx to MBSA. MBSA (30 mg, in 0.15 M NaCl, pH 4.5) was mixed thoroughly with Mtx (100 mg) and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (100 mg) in a total volume of 3 ml. The pH of the reaction mixture was adjusted to 7.5 with sodium bicarbonate. The reaction mixture was stirred for 2 hr at 30° and incubated for 20 hr at 4°. The conjugate was purified from excess reactants and other small molecules by Sephadex G-50 gel filtration. The Mtx content of various fractions was assayed by measuring their absorbance at 380 nm. MBSA has a negligible absorbance at 380 nm. Coupling of Mtx to MBSA did not alter appreciably the electrophoretic mobility of the native protein as evidenced by agarose gel electrophoresis at pH 8.4 [16]. Protein was determined by the biuret method [17]. Conjugates with molar ratios of Mtx:MBSA of 36 and 10 were used in these studies.

Radioiodination of Mtx-MBSA. Mtx-MBSA was labeled with Na[^{125}I] by the iodine monochloride catalyzed reaction, as described [18]. More than 98% of the radioactivity was acid precipitable, and the specific radioactivity was ~550 cpm/ng protein.

Assay of uptake and degradation of [^{125}I]*Mtx-MBSA by macrophages*. Each Petri dish containing a cultured monolayer of hamster peritoneal macrophages received 1 ml of the culture medium A (without HIFBS but with 1 mg/ml BSA) containing different concentrations of [^{125}I]Mtx-MBSA. After incubation for 5 hr at 37°, the amount of labeled material in the cells and the amount of ^{125}I-labeled trichloroacetic acid (TCA)-soluble (noniodide) material in the medium were determined in duplicate dishes, as described [19].

Infusions of [^{125}I]*Mtx-MBSA*. Female hamsters (130–150 g) were anesthetized by intraperitoneal injection of sodium phenobarbital. The femoral vein and the carotid artery were cannulated for infusion and blood sampling respectively. Each hamster received about 50 μg of [^{125}I]Mtx-MBSA (5.5×10^5 cpm) in a single intravenous infusion of 250 μl. The animals were killed 10 or 20 min after the introduction of radioactivity, and different tissues were collected. Tissues were washed with Hanks' balanced salt solution (HBSS) and weighed, and the radioactivity was measured in a well-type gamma counter. In the competition experiments, 1 mg MBSA was injected, in a total volume of 250 μl, intravenously 2 min before the introduction of [^{125}I]Mtx-MBSA.

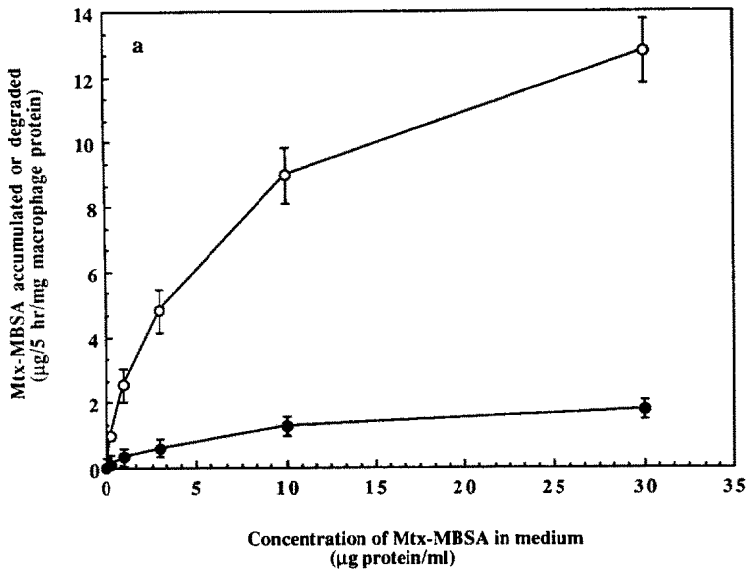
Isolation of hepatocytes and non-parenchymal cells from hamster liver. Liver cell suspension was obtained by the perfusion technique [20]. The organ was perfused *in situ* with Ca^{2+}-free HBSS for 15 min, followed by HBSS containing 0.05% collagenase for 10 min. The liver was cut into small pieces and transferred to 50 ml HBSS. The mixture was gently stirred for 2 min with a magnetic stirrer. The suspension was filtered three times through three layers of cheesecloth, and the cells were allowed to settle in a conical centrifuge tube for 15 min at 4°. Sedimented hepatocytes were washed three times with HBSS and counted in a hemocytometer. The cells in the supernatant fraction (mainly composed of nonparenchymal cells), were also washed with HBSS and counted. The nonparenchymal cell preparation contained about 13% hepatocytes, whereas contamination of the hepatocyte preparation by nonparenchymal cells was negligible.

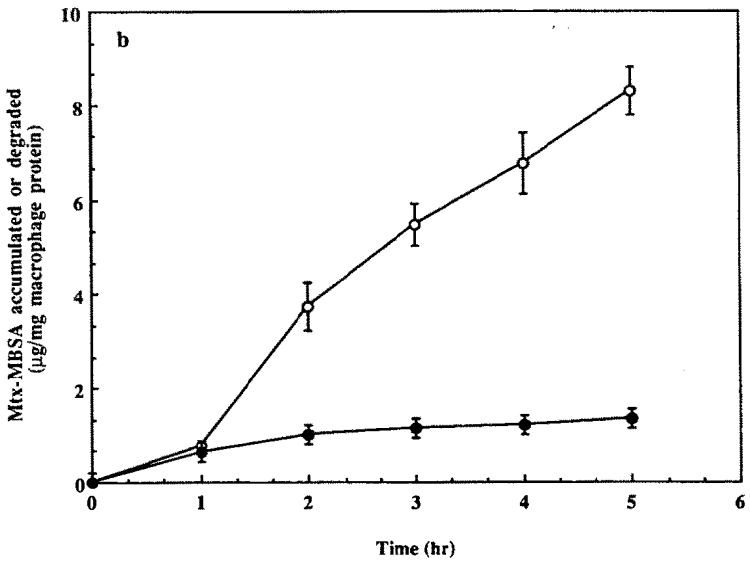
RESULTS

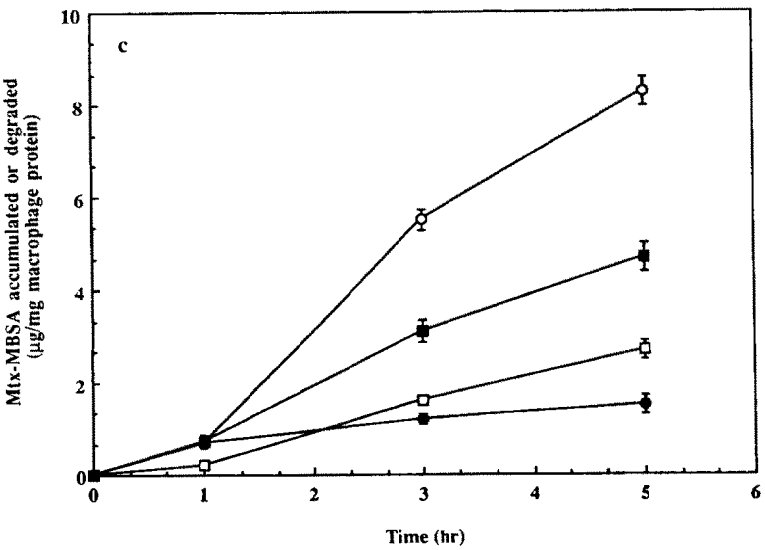
Uptake and degradation of Mtx-MBSA by macrophages. When freshly isolated hamster peritoneal macrophages were incubated with increasing concentrations of [^{125}I]Mtx-MBSA at 37°, cellular radioactivity increased in a saturable fashion (Fig. 1a). During the 5-hr incubation, a part of the added [^{125}I]Mtx-MBSA was degraded to TCA-soluble material (Fig. 1a). As the concentration of [^{125}I]Mtx-MBSA increased, the rate of degradation increased in parallel with the saturable component of the cellular uptake process. In both cases, half-maximal values were achieved at an [^{125}I]Mtx-MBSA concentration of about 5 μg protein/ml.

When macrophages were incubated with [^{125}I]Mtx-MBSA at 37° for various lengths of time, cellular radioactivity reached a steady-state (plateau) after about 1.5 hr (Fig. 1b). However, acid-soluble radioactivity continued to appear in the medium at a linear rate, reflecting the simultaneous uptake and

Fig. 1. Accumulation and degradation of [^{125}I]Mtx-MBSA by hamster peritoneal macrophages at 37°. (a) Concentration curves for accumulation (●) and degradation (○). (b) Time course of accumulation (●) and degradation (○). (c) Effect of chloroquine (3 μM) on the rate of accumulation [(●) control; (■) with chloroquine] and degradation [(○) control; (□) with chloroquine] of [^{125}I]Mtx-MBSA (10 μg protein/ml). The molar ratio of Mtx to MBSA in the drug conjugate used was 36. Results are means ±SD of three independent determinations.


a
Mtx-MBSA accumulated or degraded
(μg/5 hr/mg macrophage protein)
Concentration of Mtx-MBSA in medium
(μg protein/ml)


b
Mtx-MBSA accumulated or degraded
(μg/mg macrophage protein)
Time (hr)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Mtx-MBSA accumulated or degraded
(μg/mg macrophage protein)
Time (hr)

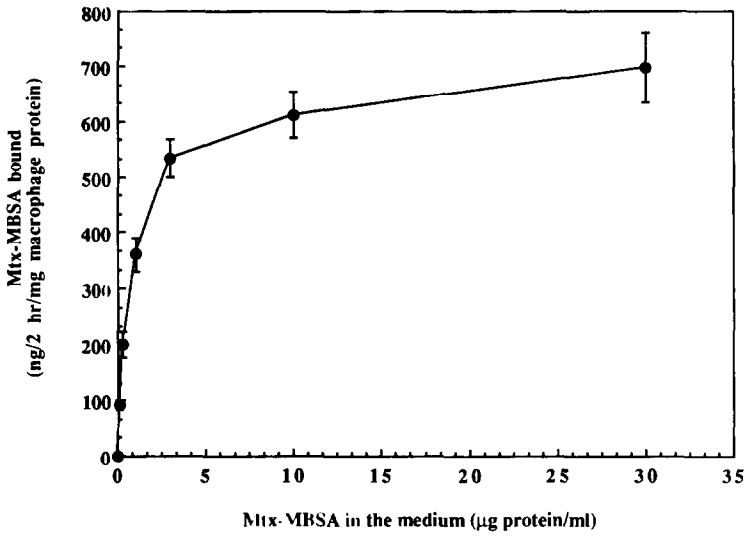

Fig. 2. Binding of [^{125}I]Mtx-MBSA by hamster peritoneal macrophages at 4° for 2 hr. The molar ratio of Mtx to MBSA in the drug conjugate used was 36. Results are means ±SD of three independent determinations.

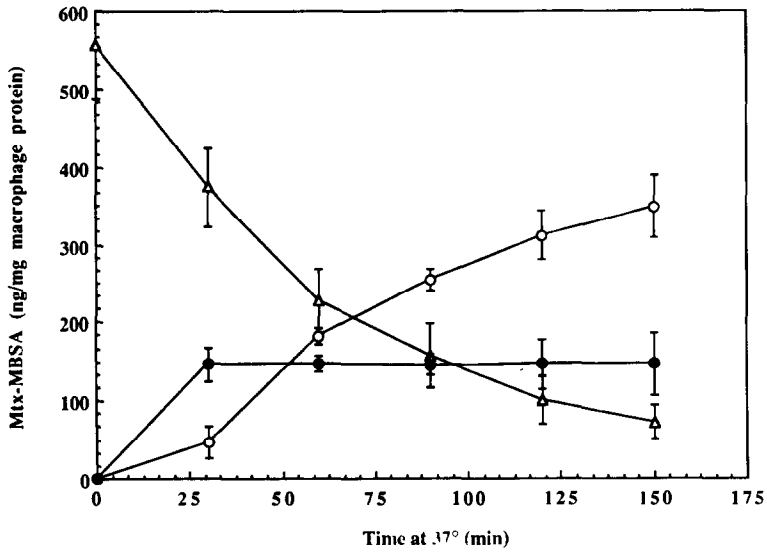

Fig. 3. Degradation at 37° of [^{125}I]Mtx-MBSA previously bound by cultured hamster peritoneal macrophages. Cultured macrophages were incubated with [^{125}I]Mtx-MBSA (10 μg protein/ml) for 2 hr at 4°. Cells were washed and reincubated with fresh medium at 37°. At different time points, the radioactivity bound to the cells (△) or released from the cells as TCA (6%) soluble (○) and TCA insoluble (●) molecules in the culture supernatant fraction was assessed. The molar ratio of Mtx to MBSA in the drug conjugate used was 36. Results are means ±SD of three independent determinations.

degradation of [^{125}I]Mtx-MBSA (Fig. 1b). After 5 hr, approximately twelve times as much [^{125}I]Mtx-MBSA had degraded, compared to the amount contained within the cells during the steady state. In the presence of the lysosomal inhibitor, chloroquine, degradation of [^{125}I]Mtx-MBSA was inhibited, and its cellular content increased linearly for 5 hr (Fig. 1c).

To measure the surface binding of [^{125}I]Mtx-MBSA, we incubated the macrophages with increasing concentrations of the ligand at 4°. [^{125}I]Mtx-MBSA bound to the cells with saturation kinetics (Fig. 2). Maximal binding was achieved within 2 hr at about 7 μg/ml. The half-maximum concentration for [^{125}I]Mtx-MBSA binding to macrophages at 4° (about 1 μg/ml) was lower than the half-maximum concentration for cellular uptake at 37° (about 5 μg/ml).

In another experiment, cells that had bound [^{125}I]Mtx-MBSA at 4° were subsequently warmed to 37°. The cell-bound radioactivity declined rapidly. About 70% of the decline was due to the release of

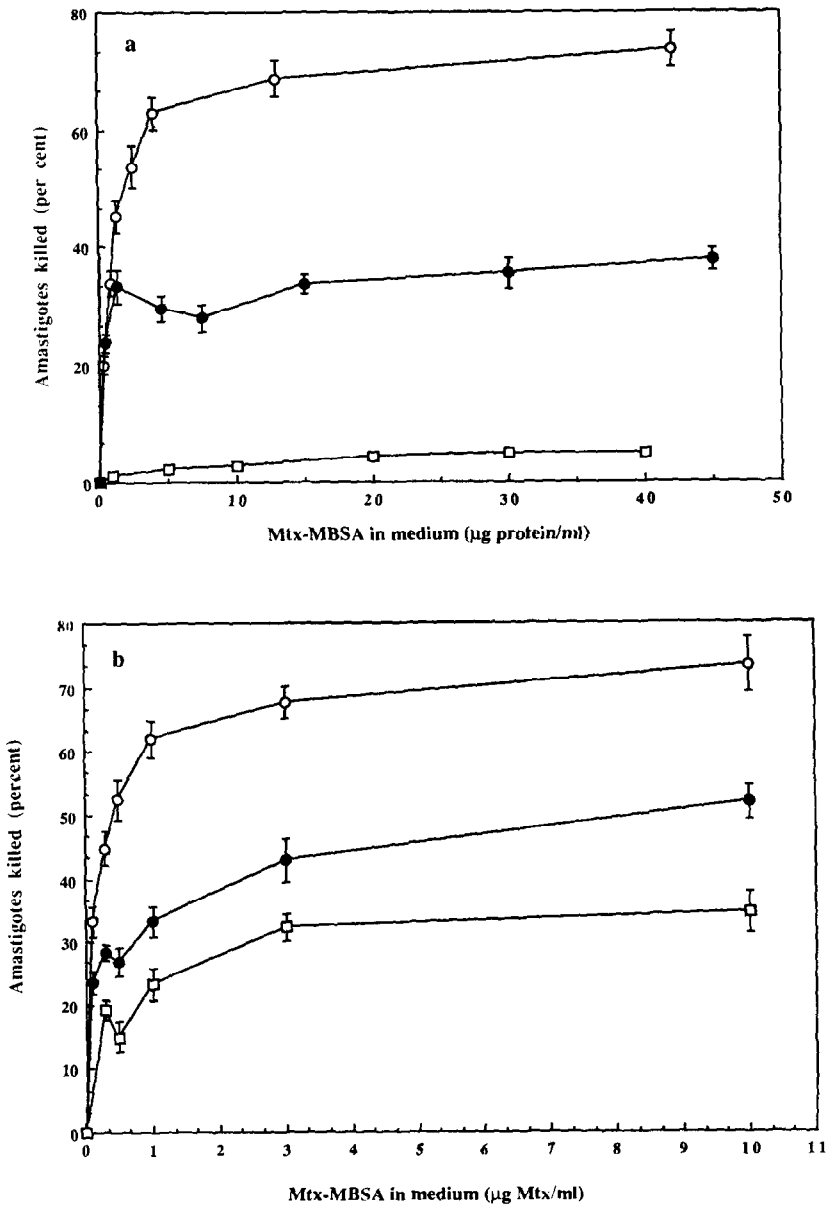

Fig. 4. Leishmanicidal effect of different preparations of Mtx-MBSA in cultured hamster peritoneal macrophages. Two preparations of Mtx-MBSA with molar ratios of Mtx to MBSA of 36 (A) and 10 (B) were tested. (a) Compared with respect to equivalent protein concentration: (○) with A; (●) with B; and (□) with free MBSA. (b) Compared with respect to equivalent Mtx concentration: (○) with A; (●) with B; and (□) with free Mtx. Results are means ±SD of three independent determinations.

acid-soluble radioactivity from the cells, a reaction that reached completion within 150 min. The 30% of the cell-bound [^{125}I]Mtx-MBSA that was not degraded dissociated from the cell surface soon after warming and appeared in the medium as acid-precipitable radioactivity (Fig. 3). Degradation of [^{125}I]Mtx-MBSA was effectively prevented by both unlabeled Mtx-MBSA and free MBSA; half-maximum inhibition occurred at about 25–30 μg of the competitors per ml of the medium.

Leishmanicidal action of Mtx-MBSA in macrophages. The parasite-laden macrophages were incubated with increasing concentrations of Mtx-MBSA at 37° for 3 hr, washed, and incubated in drug-free medium for up to 20 hr. The leishmanicidal activity of the drug conjugate showed saturation kinetics (Fig. 4, a and b). The concentrations of the drug conjugate for half-maximum killing were 0.40 and 0.75 μg protein/ml of the conjugates with molar ratios of Mtx : MBSA of 36 and 10 respectively. During a 3-hr exposure period, 60% of the intracellular amastigotes was eliminated at a concentration of 3 μg/ml Mtx when presented as Mtx-MBSA. In contrast, an equivalent amount of Mtx under the same conditions eliminated only 25% of the amastigotes. At concentrations lower than 0.1 μg/ml Mtx equivalent, the conjugate was

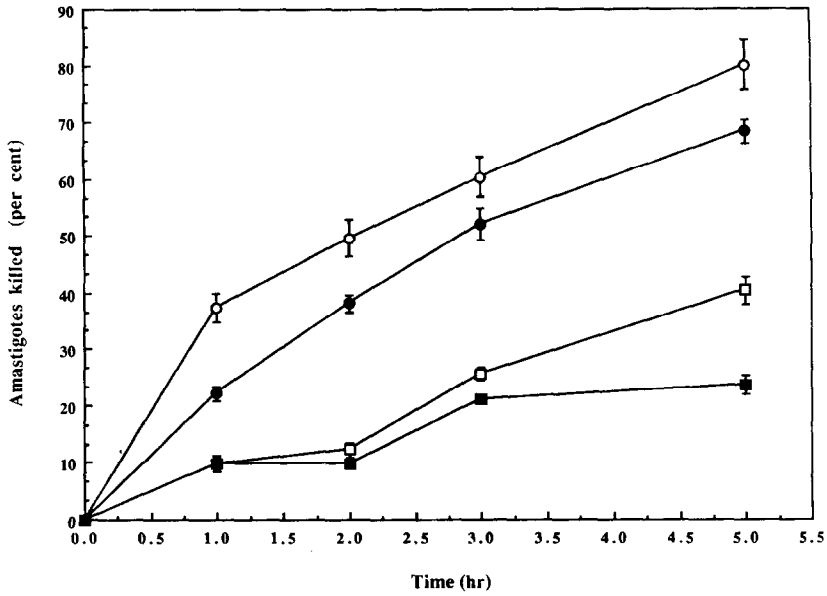

Fig. 5. Time course of killing of *Leishmania donovani* amastigotes by Mtx-MBSA or by free Mtx in cultured hamster peritoneal macrophages. Methotrexate, either as free drug [(■) 1 μg/ml; (□) 3 μg/ml] or drug conjugate (molar ratio of Mtx to MBSA in the drug conjugate used was 36) [(●) 1 μg Mtx equivalent/ml; (○) 3 μg Mtx equivalent/ml] was incubated with the parasite-infected macrophages for the specified time period at 37°. Cells were then washed and incubated for another 20 hr at 37°, after which the macrophages were stained and amastigotes inside the cells were counted microscopically. Results are means ±SD of three independent determinations.

nearly 100-fold more effective than free Mtx. Furthermore, when parasitized macrophages were incubated with Mtx-MBSA at 37° for various lengths of time, the *L. donovani* amastigotes decreased at a linear rate (Fig. 5). About 20% of the amastigotes were eliminated per hour of exposure of the parasitized macrophages to the drug conjugate. On the other hand, only 7.5% of the amastigotes were killed per hour by exposure of the cells to an equivalent amount of Mtx under identical conditions.

The leishmanicidal effect of the drug conjugate was suppressed when an excessive amount of MBSA was present in the medium (Table 1). Furthermore, lysosomotropic agents like chloroquine and monensin inhibited the antileishmanial activity of Mtx-MBSA (Table 1). There was no measurable effect of these lysosomotropic agents on the antileishmanial activity of free Mtx under the experimental conditions described (not shown). The killing effect of Mtx-MBSA was reversed by metabolic antagonists of Mtx, e.g. folic and folinic acids (Table 1).

Blood clearance and tissue distribution of [^{125}I]*Mtx-MBSA in the hamster.* More than 50% of the [^{125}I]Mtx-MBSA infused intravenously was removed from the

Table 1. Inhibition of the antileishmanial activity of Mtx-MBSA by MBSA, lysosomotropic agents and methotrexate analogs

Additions	Amastigote survival* (%)	
	Without Mtx-MBSA	With Mtx-MBSA
None†	100.0	37.5 ± 8.0
MBSA		
(100 μg/ml)	96.5 ± 3.2	64.3 ± 6.6
(500 μg/ml)	92.0 ± 2.6	78.4 ± 4.2
Monensin (3 μM)	74.8 ± 4.3	77.3 ± 6.7
Chloroquine (3 μM)	64.7 ± 7.3	55.0 ± 6.0
Folinic acid		
(10 μg/ml)	100.3 ± 3.7	79.3 ± 4.3
(100 μg/ml)	94.4 ± 2.5	85.8 ± 4.6
Folic acid		
(10 μg/ml)	72.3 ± 3.5	59.9 ± 3.7
(100 μg/ml)	84.4 ± 1.3	44.3 ± 7.2

* Results are means ±SD of three independent determinations. Control coverslips contained about 200–650 amastigotes per 100 macrophages.

† See text for the details of the assay conditions.

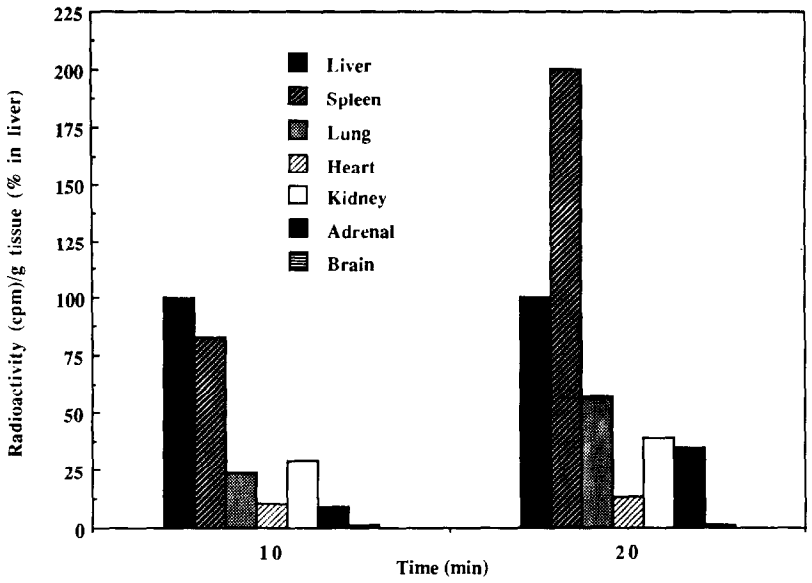

Fig. 6. Tissue distribution of intravenously injected [^{125}I]Mtx-MBSA in hamster. Results are averages of two independent determinations. See text for experimental details.

circulation of hamsters within 7 min. About 85% of the infused [^{125}I]Mtx-MBSA was cleared from the blood within 20 min. The radioactivity was found to be concentrated in the tissues containing large numbers of macrophages such as liver and spleen (Fig. 6). Of the total radioactivity taken up by the liver, about 75% was accumulated in the nonparenchymal cells (Table 2).

DISCUSSION

Macrophages isolated from a variety of organs in several animal species have exhibited high affinity binding sites for certain polyanionic macromolecules such as acetylated low density lipoprotein, maleylated BSA and fucoidin. Such chemically modified protein ligands not only bind to macrophages through these binding sites but also are internalized by the process of absorptive endocytosis and degraded in the lysosomes [10, 11]. In the present study of targeted delivery, the above polyanion "receptor" system was selected because (i) macrophages are the only cell type that harbors these binding sites; (ii) the number of the binding sites on the cell surface is high (20,000–40,000); (iii) the "receptor" system mediates rapid internalization and degradation of the protein ligands recognized by the binding sites; and (iv) the "receptor" system has no known down regulation and is probably recycled [10, 11].

To achieve specific drug delivery to leishmania-infected macrophages, we coupled methotrexate, a potent antifolate drug which has considerable anti-leishmanial activity [9], with the macromolecular ligand MBSA shown to be specific for macrophages. Because of the cytotoxic effect of Mtx at the therapeutic dose, it is currently not used in chemotherapy of leishmaniasis. The approach described in this paper is likely to overcome this problem.

We found that the cultured hamster peritoneal macrophages also have the polyanion binding sites, and such cells can endocytose and degrade [^{125}I]Mtx-MBSA in the lysosomes rapidly and efficiently through these binding sites. The rapid (about 8 μg/5 hr/mg protein) uptake and degradation of [^{125}I]Mtx-MBSA by hamster peritoneal macrophages was inhibited by MBSA, suggesting that the drug conjugate was taken up by the cell by the polyanion binding sites on macrophages. This observation was

Table 2. Uptake of [^{125}I]Mtx-MBSA by hamster liver cells

	Radioactivity (cpm/mg cell protein) in:	
Hamsters injected with:	Hepatocytes	Nonparenchymal cells
[^{125}I]Mtx-MBSA (5 μg/ml blood)	392	1225
[^{125}I]Mtx-MBSA (5 μg/ml blood) + MBSA (100 μg/ml blood)	200	550

See text for experimental details.

further strengthened by the fact that Mtx-MBSA did not exert its antileishmanial effect in the presence of free MBSA. Our finding also demonstrates that Mtx-MBSA conjugate was more efficient (nearly 100-fold at concentrations below 0.1 μg/ml Mtx equivalent and about 3-fold at higher concentrations) than free Mtx in eliminating *L. donovani* amastigotes from the infected cultured hamster peritoneal macrophages. Free MBSA had very little, if any, antileishmanial activity at low concentrations.

Prior incubation of macrophages with lysosomal inhibitors like chloroquine and monensin blocked the antileishmanial action of Mtx-MBSA, suggesting that intact lysosomal function is necessary for the drug conjugate to exert its antileishmanial effect. As expected, the antileishmanial action of Mtx-MBSA was also blocked when metabolic antagonists of the drug, e.g. folic or folinic acids, were present in the test medium. These observations suggest that the antileishmanial efficacy of Mtx-MBSA in the macrophage tissue culture model is probably due to intracellular liberation of Mtx or its biologically active derivatives by lysosomal degradation of Mtx-MBSA.

Finally, we demonstrated that in intact hamsters the radiolabeled Mtx-MBSA is rapidly cleared from circulation and is concentrated in tissues such as liver and spleen which are rich in macrophages.

Thus, we have shown that it is possible to deliver apparently cytotoxic drugs to macrophages through the polyanion "receptor" system. Further studies with other antileishmanial agents and *in vivo* experiments, currently underway, are necessary to evaluate the potential of this mode of specific antileishmanial chemotherapy. This approach to drug targeting may be useful in combating not only leishmaniasis but also other infections associated with macrophages, e.g. tuberculosis, typhoid and leprosy.

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