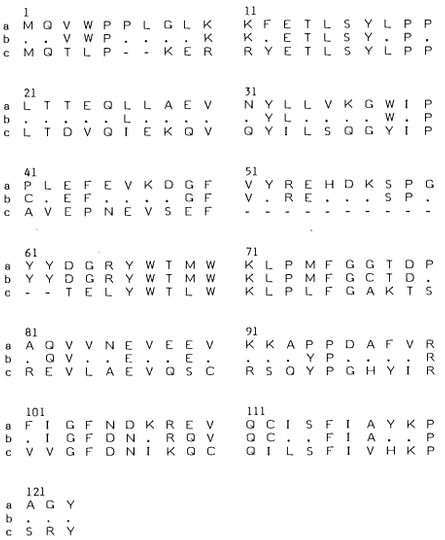

Fig. 6. Amino acid sequences of the small subunit of RuBisCo molecules. (**a**) Sequence of spinach S subunit (*6*). (**b**) Invariant residues in S subunits from other higher plants. [Data taken from the NBRF Protein Data Bank.] Residues that vary are indicated by dots. (**c**) Amino acid sequence of the S subunit from *Anabaena* RuBisCo (*12*). Residues 6, 7, and 51 to 62 are deleted.

without any prior knowledge of the protein structure, and therefore they provide independent evidence for a correct chain tracing if chemically proper side chains are found in the binding sites. Mercury compounds usually bind to accessible Cys residues. The Hg complexes that we used in spinach RuBisCo, ethyl mercury thiosalicylate and $K_2Hg(CN)_4$, bind to seven different sites, four in the L chain and three in the S chain. All four Hg binding sites in the L chain contain Cys residues; Hgl binds to Cys 84L and His 86L, Hg2 to Cys 172L as well as Cys 192L, Hg3 to Cys 427L and Met 387L, and Hg4 to Cys 459L. In the S chain we find that one Hg binds to Cys 112 as well as to the N atom of Trp 38. The other two Hg binding sites are adjacent to residues 41 and 77. Granted that these are Cys residues, the three different Hg atoms in the S subunit bind to the three Cys residues providing independent support for our chain tracing. Corresponding residues in the tobacco chain tracing are in quite different regions of the molecule (Fig. 4) and do not agree with our observed Hg positions.

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Receptor-Mediated Drug Delivery to Macrophages in Chemotherapy of Leishmaniasis

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Methotrexate coupled to maleylated bovine serum albumin was taken up efficiently through the "scavenger" receptors present on macrophages and led to selective killing of intracellular *Leishmania mexicana amazonensis* amastigotes in cultured hamster peritoneal macrophages. The drug conjugate was nearly 100 times as effective as free methotrexate in eliminating the intracellular parasites. Furthermore, in a model of experimental cutaneous leishmaniasis in hamsters, the drug conjugate brought about more than 90% reduction in the size of footpad lesions within 11 days. In contrast, the free drug at a similar concentration did not significantly affect lesion size. These studies demonstrate the potential of receptor-mediated drug delivery in the therapy of macrophage-associated diseases.

LEISHMANIASIS, A PARASITIC DISEASE, is estimated to affect 400,000 to 12 million people worldwide annually (*1, 2*). The causative agents of leishmaniasis, the various *Leishmania* species, reside and proliferate solely in mammalian host macrophages (*3*). Currently used drugs for chemotherapy of leishmaniasis, such as antimonials, amphotericin B, and pentamidine, can produce severe toxic side effects and relatively high relapse rates occur (*4, 5*). Such side effects are presumably due to the interaction of the drugs with different cell types of the host, including those not harboring the parasites. Antimonials, amphotericin B, and pentamidine encapsulated in liposomes were more effective than free drugs for the treatment of leishmaniasis in experimental model systems (*6–10*). Even in these earlier studies, liposomes that did not carry drugs showed appreciable toxic effects (*11*). In this report we describe an alternative modality for selective delivery of drugs to macrophages in which a cytotoxic drug, methotrexate (Mtx), was coupled to a macromolecular ligand, maleylated bovine serum albumin (MBSA), recognized by the "scavenger" receptors reported to be present primarily on the cells of macrophage lineage (*12–14*). The superior leishmanicidal activity of this drug conjugate in eliminating intracellular amastigotes of *Leishmania mexicana amazonensis* both in vitro and in vivo demonstrates the efficacy of this approach.

For preparation of the drug conjugate, water-soluble carbodiimide was used to couple MBSA chemically with unlabeled or tritiated Mtx (*15, 16*). Mtx-MBSA containing 35 moles of Mtx per mole of MBSA was used in these studies. The kinetics of uptake of free and conjugated Mtx by cultured macrophages derived from peritoneal fluid of hamsters are shown in Fig. 1. At an Mtx concentration of 3 μg/ml in the medium, either in free or conjugated form, the intracellular content of Mtx was 70 ng and 279 ng per milligram of cellular protein, respectively, after 3 hours of incubation. The uptake of Mtx-MBSA exhibited saturation

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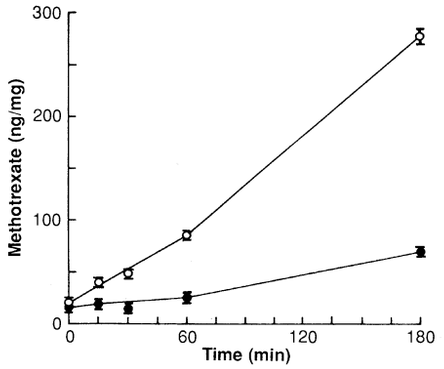

Fig. 1. Uptake of ^{3}H-labeled Mtx-MBSA (○) and ^{3}H-labeled Mtx (●) by hamster peritoneal macrophages at 37°C. Male or female young (70 to 75 g) hamsters (*Mesocricetus auratus*) were injected intraperitoneally with 2 ml of Brewer's thioglycollate medium. After 5 days, peritoneal fluid from five to ten hamsters (6×10^6 to 10×10^6 cells per hamster) was pooled. Cells were collected by centrifugation (400*g* for 10 min at 4°C) and suspended in medium A (RPMI 1640 medium containing 10% fetal calf serum, 2 mg of sodium bicarbonate, 100 IU of penicillin, and 100 μg of streptomycin sulfate per milliliter) at a final concentration of 2×10^6 cells per milliliter. One-milliliter portions of this cell suspension were dispensed into plastic petri dishes (35 by 10 mm) and incubated at 37°C for 2 hours in a humidified incubator containing 5% CO_2. Each dish was washed three times with phosphate-buffered saline (PBS) (0.15*M*) to remove nonadherent cells. The monolayers were incubated in medium A at 37°C. After 24 hours monolayers were washed twice with PBS and incubated at 37°C in the presence of 3 μg of [^{3}H]Mtx-MBSA or [^{3}H]Mtx (specific activity 51 cpm per nanogram of Mtx) in 1 ml of medium A with bovine serium albumin (1 mg/ml) instead of fetal calf serum. At indicated times, monolayers were washed three times with ice cold PBS; cells were dissolved in 1 ml of 0.1*N* NaOH, and the amount of radioactivity associated with the cells was measured. Results were expressed as nanogram of Mtx per milligram of cellular protein. Each value is the average of triplicate incubations ± SE.

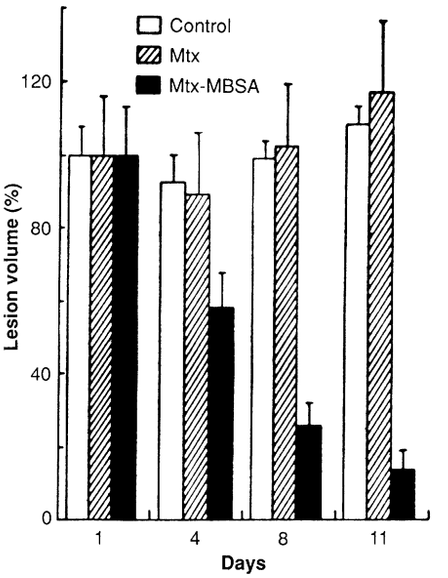

Fig. 3. Treatment of hamster footpad lesions of *L. mexicana amazonensis* with Mtx-MBSA or free Mtx, and untreated controls. Female hamsters (4 to 6 weeks old, weighing 125 to 150 g) were infected in each hind footpad with 1×10^6 *L. mexicana amazonensis* amastigotes isolated from donor hamster footpads. After 8 weeks (average weight 150 g), the hamsters were injected intramuscularly near the lesion of each hind footpad with Mtx-MBSA or Mtx (1 mg/kg) on days 1 to 4. Control animals received the same volume of PBS. The size of the lesion was measured with a dial caliper on indicated days (*20*). The volume of lesion before injection on day 1 was taken as 100% [400 ± 30 (SE) mm^3]. The size of a normal footpad was 36 ± 4 mm^3. Results are expressed as percentage of lesion volume ± SE for six footpads per group.

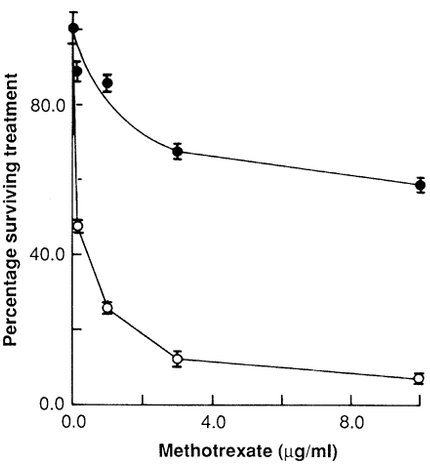

Fig. 2. Leishmanicidal activity of Mtx-MBSA (○) and Mtx (●) on *L. mexicana amazonensis*–infected macrophages at 37°C. *Leishmania mexicana amazonensis* (CII strain) were maintained at 25°C in a biphasic medium (*19*). Macrophage monolayers were prepared on glass cover slips (18 mm) as described for Fig. 1. After 24 hours at 37°C, the cells were washed twice with PBS and incubated in medium A with *Leishmania mexicana amazonensis* promastigotes at a ratio of ten parasites per macrophage at 37°C. After 2 hours, cells were washed three times with PBS to remove unattached parasites. The parasite-laden macrophages were incubated at 37°C with indicated concentrations of Mtx-MBSA or Mtx in 1 ml of medium A. After 3 hours, the cover slips were washed three times with PBS and incubated in drug-free medium A for 20 hours. Cultures were fixed by methanol and stained by Giemsa; the number of amastigotes in 100 macrophages in treated and control cultures was determined (*21*). The number of amastigotes present in untreated culture was taken as 100% [301 ± 12 (SE)], and results are expressed as an average of three determinations ± SE.

kinetics and was competitively inhibited by MBSA but not by free Mtx (*17*). These results indicated that Mtx-MBSA is taken up by the same receptor-mediated pathway as was shown for MBSA (*13*), leading to high intracellular accumulation of the drug.

Mtx-MBSA killed 70% of the amastigotes of *L. mexicana amazonensis* (CII strain) in cultured peritoneal macrophages at an Mtx equivalent concentration of 0.9 μg/ml, whereas free drug at the same concentration killed only 10% of the amastigotes (Fig. 2). When free Mtx was used at concentrations up to 10 μg/ml, only 35% of the maximal killing of amastigotes was observed. In contrast, 35% of amastigotes were killed by Mtx-MBSA at 0.1 μg/ml. At 3 μg/ml or above, Mtx-MBSA eliminated 90% of the amastigotes. These results show that Mtx-MBSA is nearly 100 times as effective as free Mtx at the lower concentration of the drug (0.1 μg/ml) and 3 times as effective as free Mtx at higher concentrations (3 to 10 μg/ml). Macrophages remained viable throughout the experiments as determined by trypan blue exclusion. Lysosomal degradation of the drug conjugate was necessary for eliciting the antileishmanial activity, because lysosomal inhibitors, chloroquine and monensin, effectively suppressed the leishmanicidal effect of the drug conjugate (*17*). The leishmanicidal product generated by lysosomal degradation of Mtx-MBSA has not been characterized yet. However, a metabolic antagonist of Mtx, folinic acid, suppressed the antileishmanial action of the drug conjugate (*17*). Thus, intracellular parasites residing in macrophages were selectively eliminated as a result of delivery of a drug through receptor-mediated endocytosis. Similar selective killing of *L. mexicana amazonensis* amastigotes by lysosomotropic amino acid esters, presumably taken up by macrophages through passive diffusion, has been reported (*18*).

The efficacy of the drug conjugate for the treatment of cutaneous leishmaniasis in vivo was determined by using hamster footpad lesions as a model system (*19*). Hamsters were infected in each hind footpad with *L. mexicana amazonensis* amastigotes as described under Fig. 3. The footpads increased to 400 ± 30 (SE) mm^3 8 weeks after infection. These animals were given intramuscular injections of free Mtx or Mtx-MBSA daily for four consecutive days. The drug was injected near the lesion site in each foot pad, at Mtx equivalent concentrations of 1, 3, and 10 mg per kilogram of body weight.

Mtx-MBSA therapy at 1 mg/kg per footpad for 4 days (total dose of Mtx administered per hind footpad was 150 μg × 4 = 600 μg) resulted in 45% reduction in the size of the footpad lesion on the fourth day (Fig. 3). Free Mtx did not cause any significant regression of the lesion at this dose. Furthermore, even at a free Mtx dose of 10 mg/kg per footpad, no significant reduction in lesion size

was observed. Finally, the conjugated drug caused 90% reduction in the size of the lesion 11 days after the initiation of drug treatment. The greatest effect on the regression of the lesions by the conjugated drug was observed at a dose of 1 mg/kg per footpad. The lack of effect at higher concentrations probably reflects saturation of the receptor-mediated uptake process for Mtx-MBSA. The footpad regressed to nearly normal size when Mtx-MBSA was used. In contrast, administration of free Mtx did not significantly affect the footpad lesion. The lesions did not reappear even 4 weeks after the last injection of Mtx-MBSA. During the experimental period all the animals remained healthy with no apparent weight loss. No antibody against MBSA or Mtx-MBSA was detectable in these animals after 3 weeks as determined by the Ouchterlony immunodiffusion technique.

In conclusion, our results show that effective delivery of drug to macrophages can be achieved by using the "scavenger" receptor-mediated endocytic pathway to achieve selective killing of intracellular parasites residing in macrophages, both in vitro and in vivo. A similar approach may be useful for effective delivery of drugs in the treatment of other diseases in which macrophages are the primary target, including tuberculosis, leprosy, monocytic leukemia, and heavy metal storage diseases.

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Studies of the HER-2/*neu* Proto-oncogene in Human Breast and Ovarian Cancer

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Carcinoma of the breast and ovary account for one-third of all cancers occurring in women and together are responsible for approximately one-quarter of cancer-related deaths in females. The HER-2/*neu* proto-oncogene is amplified in 25 to 30 percent of human primary breast cancers and this alteration is associated with disease behavior. In this report, several similarities were found in the biology of HER-2/*neu* in breast and ovarian cancer, including a similar incidence of amplification, a direct correlation between amplification and over-expression, evidence of tumors in which overexpression occurs without amplification, and the association between gene alteration and clinical outcome. A comprehensive study of the gene and its products (RNA and protein) was simultaneously performed on a large number of both tumor types. This analysis identified several potential shortcomings of the various methods used to evaluate HER-2/*neu* in these diseases (Southern, Northern, and Western blots, and immunohistochemistry) and provided information regarding considerations that should be addressed when studying a gene or gene product in human tissue. The data presented further support the concept that the HER-2/*neu* gene may be involved in the pathogenesis of some human cancers.

PROTO-ONCOGENES REPRESENT A family of normal cellular genes that were identified on the basis of their similarity to genetic sequences with known tumorigenic or transforming potential (*1*). Considerable circumstantial evidence now exists that alterations in either the structure, copy number, or expression of one or another of these genes may play a role in the pathogenesis of some human malignancies (*2*). One such gene, called HER-2/*neu* or c-*erb* B2, was first identified by transfection studies in which NIH 3T3 cells were transformed with DNA from chemically induced rat neuroglioblastomas (*3*). The gene encodes a protein that has extracellular, transmembrane, and intracellular domains (*4*) which is consistent with the structure of a growth factor reception.

Recently, we found a 28% incidence of amplification of HER-2/*neu* in 189 primary human breast cancers (*5*). Patients with multiple copies of the gene in DNA from their tumors had a shorter time to relapse as well as a shorter overall survival indicating that gene amplification was prognostic for disease behavior in these individuals. Moreover, multivariate survival analysis showed HER-2/*neu* amplification to be more predictive for clinical outcome than all other known prognosticators with the exception of positive lymph nodes (*5*). Since that initial report, a number of studies have been published on the amplification of this gene

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