riers that allow tumors to elude various antitumor agents. Thus, postsurgical immunotherapy might enhance the activity of cytotoxic drugs, monoclonal antibodies, and activators of immune effector mechanisms, each of which would otherwise be limited by the anatomic organization of micrometastatic nodules in situ.

To test this hypothesis, we injected strain 2 guinea pigs intravenously with 10^6 L10 cells (100 times the minimum lethal dose). Median survival was 56 days in the untreated animals. Treatment with therapeutic doses of cyclophosphamide (150 mg/kg) 1, 31, or 45 days after tumor cell injection resulted in no cures but increased the median survival time of the animals. Survival of guinea pigs treated with immunotherapy and then chemotherapy at the time of peak inflammatory disruption was significantly greater than that of animals treated with immunotherapy or chemotherapy alone. The survival rate of animals given sublethal doses of cyclophosphamide or *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea 21 days after the first immunization (31 days after tumor cell injection) was two to three times greater than that of animals given immunotherapy alone (Table 2). On the other hand, administration of cyclophosphamide before immunotherapy did not improve survival rates. The increase in survival obtained with the combined therapies diminished as the interval between immunotherapy and chemotherapy was extended. Thus, combination therapy apparently does not improve survival once the metastases have progressed to the more dense histiocytic (ischemic) configuration. When the inflammatory response was allowed to continue to this stage before cytotoxic drugs were administered, no improvement in the survival rate was observed.

There are several possible explanations of how cytotoxic drugs work when administered subsequent to immunotherapy. Tumor cells killed by drug therapy could shed tumor antigens, boosting the immune response. The tumor burden could be reduced or tumor-specific suppressor cells could be eliminated, allowing the immune response to be more effective (*7*). Antitumor drugs also could render tumor cells more susceptible to immune lysis; tumor cells treated in vitro with various cytotoxic drugs are more susceptible to killing by antibody and complement or by tumoricidal macrophages (*8*). Conversely, the immune reaction could injure tumor cells and make them more susceptible to chemotherapy (*9*). Our histological findings show that the immune reaction in the tumor can disrupt its normal anatomic architecture, rendering the tumor cells more vulnerable to subsequent, well-timed chemotherapy. These results provide a basis for reevaluating the sequence of multimodal treatments in humans.

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Lethal Effect of Phenothiazine Neuroleptics on the Pathogenic Protozoan *Leishmania donovani*

Abstract. *Phenothiazine drugs, which are widely used for their antipsychotic, antianxiety, and antiemetic effects, have been found to have protozoacidal effects on the human pathogen* Leishmania donovani. *These compounds are lethal to both the extracellular stage of the organism, which is inoculated into humans by the sand fly, and the intracellular stage, which is found solely in human macrophages during established infection.*

The phenothiazine neuroleptics, of which chlorpromazine is the prototype, are widely used for their antipsychotic, antianxiety, and antiemetic effects. Biological activities of these lipophilic compounds include modification of membranes and membrane constituents (*1*), alteration of cyclic nucleotide metabolism (*2*), intercalation into DNA (*3*), inhibition of actin polymerization (*4*), and destruction of various bacteria (*5*). In view of these effects, we investigated the possibility that phenothiazine neuroleptics might affect the pathogenic protozoan *Leishmania donovani*.

The causative agent of visceral leishmaniasis, *L. donovani* produces morbidity and mortality in many areas of the world. There is no universally effective, nontoxic form of chemotherapy against this protozoan, which exists in the phlebotomine sand fly as a flagellate, extracellular promastigote and in humans as an aflagellar amastigote in macrophages. We found that phenothiazine drugs kill promastigotes, amastigotes released from macrophages, and amastigotes in human monocyte-derived macrophages.

A Sudanese strain of *L. donovani* was maintained by serial passage of amastigotes in Syrian hamsters. Spleens from animals infected 4 to 8 weeks previously were removed and homogenized in a tissue grinder. Amastigotes were isolated (*6*) and used directly for study or allowed to convert to promastigotes at 26°C in modified (*6*) promastigote growth medium (*7*) to which were added 10 percent (by volume) heat-inactivated fetal calf serum, penicillin (100 U/ml), and gentamicin (50 μg/ml). The promastigotes were used after 3 to 8 days of in vitro cultivation and two washes in phosphate-buffered saline.

The effect of phenothiazines on promastigotes was assessed by incubating promastigotes (3×10^6 per milliliter) at 26°C with serial twofold dilutions of drug (0.78 to 50 μg/ml) in medium with 10 percent fetal calf serum and antibiotics. Experiments were done in duplicate. The minimum protozoacidal concentration (MPC) of each phenothiazine was defined as the lowest concentration that reduced the number of viable promastigotes by ≥ 90 percent after 18 hours, as assessed by flagellar motility and, in selected experiments with chlorpromazine, by uptake of the supravital stain neutral red (*6*).

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Table 1. The MPC of phenothiazine neuroleptics and analogs for *L. donovani* promastigotes and extracellular amastigotes. N.D., not determined.

Substance	Geometric mean MPC (μg/ml)	
	Promastigote	Amastigote
Chlorpromazine	13.6	13.2
Trifluoperazine	13.2	11.7
Butaperazine	31.5	14.9
Promethazine	25	N.D.
7,8-Dioxochlorpromazine	8.8	1.6
7,8-Dihydroxychlorpromazine	> 50	1.6
Promazine	35.4	50
2-Chlorophenothiazine	> 50	> 50
Phenothiazine	> 50	> 50
Chlorpromazine sulfoxide	> 50	> 50

The effect of phenothiazines on amastigotes was determined by incubating amastigotes (1×10^6 per milliliter) from infected hamsters with serial twofold dilutions of drug (0.78 to 50 μg/ml) at 37°C for 2 hours. These amastigotes were then washed twice in phosphate-buffered saline and allowed to convert to promastigotes in growth medium at 26°C. MPC was determined after 48 hours by counting the number of motile promastigotes.

The effect of phenothiazines on amastigotes in human macrophages was assessed by using a modification of the method of Berman *et al.* (*8*). Human mononuclear cells were obtained by Ficoll–sodium diatrizoate sedimentation of peripheral blood from healthy adult donors (*9*). Mononuclear cells (5×10^6) were placed on glass cover slips and incubated at 37°C in room air containing 5 percent CO_2. After 2 hours monolayers were suspended in 13 percent autologous serum, penicillin, and gentamicin. The monolayers were washed once after 24 hours to remove nonadherent cells, resuspended in fresh medium with serum and antibiotics, and incubated for 5 days. Adherent mononuclear phagocytes were then incubated with amastigotes at a parasite-to-phagocyte ratio of 10:1 in the presence of heat-inactivated, autologous human serum. After 2 hours the monolayers were washed in warm medium to remove residual extracellular amastigotes and suspended in medium containing 13 percent autologous serum and antibiotics. After 24 hours serial twofold dilutions of phenothiazines or medium were added to *Leishmania*-infected monolayers. Drug concentrations ranged from 6.2 to 25 μg/ml. The percentage of infected macrophages and the number of parasites per infected macrophage were determined 48 hours later by examining ≥ 200 macrophages on monolayers stained with Wright's and Giemsa.

Chlorpromazine and trifluoperazine were the most active phenothiazines against promastigotes and against extracellular amastigotes released from infected hamster spleens (Table 1). In studies with chlorpromazine, no difference was observed in the MPC when viability was assessed by flagellar motility and concurrently by uptake of neutral red. In addition, there was no change in the MPC when control and chlorpromazine-exposed promastigotes were washed three times in phosphate-buffered saline to remove residual phenothiazine and reincubated in promastigote growth medium for an additional 24 hours.

The killing of promastigotes appeared to be rapid. Exposure to chlorpromazine (50 μg/ml for 25 minutes) resulted in an irreversible loss of motility, and electron micrographs revealed loss of nuclear and cytoplasmic detail and, in some instances, disruption of the plasma membrane.

Structural changes in the chlorpromazine molecule were studied in an attempt to determine which residues on the tricyclic ring were responsible for the protozoacidal activity. Modification of chlorpromazine by removal of the 2-chloro moiety (promazine), removal of the aliphatic side chain from the ring nitrogen (2-chlorophenothiazine), removal of both (phenothiazine), or oxidation of the ring sulfur (chlorpromazine sulfoxide) resulted in decreased activity. Oxidation of the tricyclic ring to 7,8-dihydroxychlorpromazine or 7,8-dioxochlorpromazine produced compounds with differential activity against amastigotes and promastigotes. Both compounds were more potent against extracellular amastigotes than chlorpromazine and had an MPC of 1.6 μg/ml.

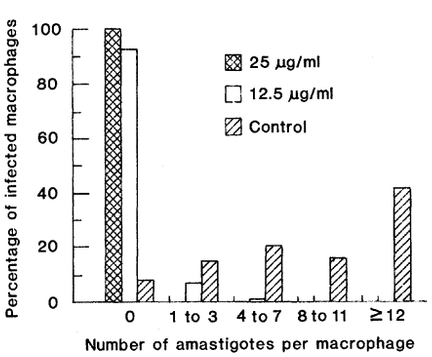

Fig. 1. Effect of chlorpromazine on *L. donovani* amastigotes in human monocyte-derived macrophages. The number of amastigotes per macrophage was determined 48 hours after exposure of infected macrophages to chlorpromazine at concentrations of 6.2, 12.5, or 25 μg/ml. Control macrophages were infected with amastigotes but were not exposed to chlorpromazine. Exposure to chlorpromazine at 6.2 μg/ml had no protozoacidal effect.

Of greatest importance was the finding that chlorpromazine kills amastigotes inside human monocyte-derived macrophages. This is crucial, since *L. donovani* exists solely in macrophages in its amastigote stage during established infection. As seen in Fig. 1, chlorpromazine (≥ 12.5 μg/ml) greatly reduced the number of parasites per infected macrophage. The geometric mean MPC of chlorpromazine against intracellular amastigotes was 15.8 μg/ml ($N = 3$). Intracellular amastigotes may have been killed directly by chlorpromazine, or the protozoacidal effect may have been mediated by changes in the infected macrophages. Chlorpromazine (12.5 μg/ml) caused no identifiable alteration in macrophage morphology or density on the monolayers, but at higher concentrations chlorpromazine produced rounding of macrophages and release of some from the monolayer. Chlorpromazine sulfoxide, which was ineffective against both extracellular amastigotes and promastigotes, and 7,8-dihydroxychlorpromazine and 7,8-dioxochlorpromazine, which killed extracellular amastigotes, had no effect on intracellular amastigotes.

Although the neuroleptic effects of the phenothiazine drugs have been extensively studied, little attention has been paid to their antiprotozoal activity since Guttman and Ehrlich's (*10*) observation in 1891 that methylene blue, a phenothiazine dye, is effective against vivax malaria. Pocztarska-Wegrzyn (*11*) observed that massive concentrations of chlorpromazine (2500 μg/ml) kill *Paramecium caudatum*, and Forrest *et al.* (*12*) found that chlorpromazine (50 μg/ml) is lethal to the ciliated extracellular protozoan *Tetrahymena pyriformis*.

It has been proposed that some of the pharmacological effects of phenothiazines are mediated by free radical formation (*13*). Although we did not investi-

gate the mechanism of the antileishmanial effect, the rapid immobilization and destruction of promastigotes by chlorpromazine suggest that this phenothiazine may act at the level of the protozoal membrane, possibly by inhibiting enzymes in one or more metabolic pathways (*14, 15*).

Whether phenothiazines can be used to treat visceral leishmaniasis or related protozoal infections remains to be determined. Chlorpromazine is highly protein bound; maximal concentrations of the parent compound in human serum are usually attained only after 2 weeks of oral administration. Furthermore, the concentration of chlorpromazine that kills parasites after a single dose in vitro is greater than the concentration achieved in the plasma of psychiatric patients (0.1 to 0.5 μg/ml). However, phenothiazines are lipophilic and concentrate in tissues, and in psychiatric patients their concentrations in serum are poorly correlated with clinical responses. In dogs, the tissue-to-plasma ratio of chlorpromazine is 68:1 in brain, 26:1 in spleen, and 13:1 liver (*16*). This characteristic of the phenothiazines is important. For example, we have found that chlorpromazine given by gavage (20 mg/kg per day for 14 days) to *L. donovani*–infected Syrian hamsters significantly reduces the number of amastigotes in the liver and spleen (*17*). Although the optimal dosage and duration of therapy required to obtain adequate protozoacidal levels in the human reticuloendothelial system are unknown, our data indicate that adequate concentrations may be achievable. Normally, the incidence of adverse effects from phenothiazines in humans is related to dosage and the duration of therapy. Additional studies are warranted to determine whether a phenothiazine such as chlorpromazine can be used safely and effectively in the treatment of visceral leishmaniasis or related protozoal diseases.

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Lethal Disruption of the Yeast Actin Gene by Integrative DNA Transformation

Abstract. *A mutant allele of the chromosomal locus corresponding to the cloned actin gene of the yeast* Saccharomyces cerevisiae *has been constructed by DNA transformation with a hybrid plasmid which integrates into, and thereby disrupts, the protein-encoding sequences of the gene. In a diploid strain of yeast, disruption of the actin gene on one chromosome results in a mutation that segregates as a recessive lethal tightly linked to a selectable genetic marker on the integrated plasmid. The actin gene, therefore, must encode an essential function for yeast cell growth.*

The genome of *Saccharomyces cerevisiae* contains a single actin gene (*1, 2*). This gene, which was cloned via its homology to actin genes of other eukaryotes, could encode a protein with extensive amino acid sequence homology with higher eukaryotic actins (*2, 3*). In addition, a protein that resembles actins from other sources in molecular weight, polymerization ability, and peptide map has

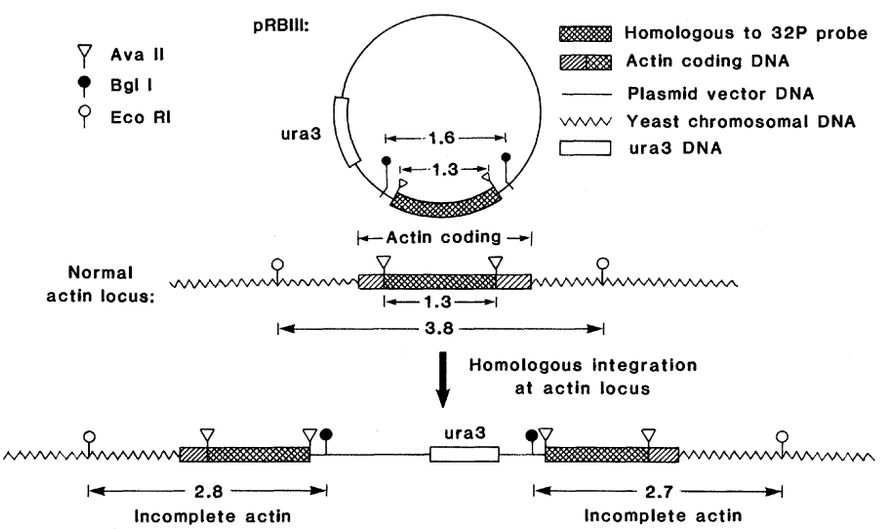

Fig. 1. Structure of the yeast actin locus before and after recombination with the hybrid plasmid pRB111. Positions of the Eco RI and Ava II restriction sites are taken from (*2*). Distances between relevant restriction sites are indicated in kilobase pairs. The structure predicted for the actin locus after recombination with pRB111 is confirmed by the data shown in Fig. 2. Plasmid construction involved ligation of the 1.3-kb Ava II fragment of the cloned actin gene (which contains the entire protein-encoding sequence except for deletion of the first $3\frac{1}{3}$ codons plus 50 base pairs of intervening sequence from the amino terminal end and the last nine codons from the carboxyl terminal end) into the yeast integrative cloning vector YIp-5 which had been linearized by Ava II cleavage in the presence of ethidium bromide (100 μg/ml). From among the amp^r tel^s bacterial transformants with this ligation mixture, pRB111 was identified as a hybrid plasmid with a single 1.3-kb fragment inserted into the Ava II site at position 1135 in the tetracycline resistance gene of pBR322.

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