presented here are an extension of this procedure, in which different detergents and several methods of detergent removal were used. Many of the preparations were examined by electron microscopy to determine a nominal average diameter (calibration of the gel filtration medium was one of the objectives), and chloride permeability measurements (*3*) were used to demonstrate the integrity of the vesicle membranes and the existence of a trapped volume of appropriate magnitude.

Two kinds of column were used, and both were presaturated with phospholipid to avoid adsorption during chromatography. Polystrene latex particles (Polysciences, Inc., Warrington, Pennsylvania) or multilamellar lipid vesicles were found to be suitable as void volume markers. Column A was designed for cursory examination of freshly prepared samples and was intended to provide (if necessary) a final fractionation step in the preparative procedure. The column dimensions were 50 by 0.9 cm; the void volume (V_0) was 14.8 ml; and the total volume (V_t) was 31.6 ml. The flow rate was 3 ml/hour, sample size was about 1 ml, and fractions of 0.5 to 1 ml were collected and analyzed. Column B was intended as a purely analytical column. The dimensions were 28 by 0.7 cm, $V_t - V_0$ was 6.7 ml, flow rate was 3 ml/hour, sample size was 0.2 ml, and the collected fraction size was 0.15 to 0.2 ml.

Figure 1a shows elution profiles obtained with four separate vesicle preparations on column A. Turbidity (absorbance at 335 nm) was used as a measure of vesicle concentration. Nominal diameters obtained by electron microscopy are shown for each sample.

Figure 1b shows elution patterns obtained with column B, in an experiment designed to investigate the effect of different methods and rates of detergent removal from initially similar lipid-detergent solutions. Organic phosphorus analysis (*4*) was used as a measure of lipid concentration for all fractions with significant absorbance at 230 nm (measured in a microcell). The data for column B show that multimodal size distributions are obtained under some conditions and are readily detected by this technique. This column was not calibrated: diameters shown for the principal peaks are based on the assumption that the calibration data for column A are applicable.

In order to show that the chromatographic behavior of Sephacryl S-1000 is similar to that of smaller pore resins, we have used the nominal average diameters obtained by electron microscopy and the peak positions of Fig. 1a to generate a

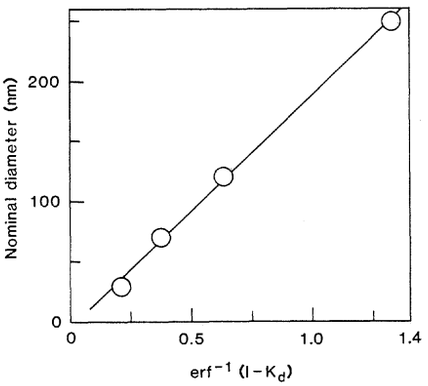

Fig. 2. Plot of the peak positions of Fig. 1 according to the equation of Ackers (*5*).

plot of the data according to the inverse error function (erf^{-1}) equation of Ackers (*5*). For spherical vesicles, the diameter should be precisely twice the Stokes radius, and Fig. 2 demonstrates that the linear relation between diameter and erf^{-1} $(1 - K_d)$ that is typical of earlier work with much smaller particles (*5*) applies here as well (*6*).

These results demonstrate the utility of gel exclusion chromatography as a quick and convenient technique for characterizing vesicle preparations. Provided that the eluting buffer and the internal vesicle solution are approximately isoosmotic, the technique can be applied to vesicles containing any desired trapped solute. Because sample size, flow rate, diffusion, and column dimensions influence the spread of an elution peak in a rather complex way (*7*), the chromatographic method is not well designed for a rigorous determination of particle size distribution. The narrow width for the second curve in Fig. 1b, however, suggests that size heterogeneity is the major determining factor (there is no reason to believe that the sample is truly monodisperse), so that peak width can be treated with confidence as at least a relative measure of polydispersity.

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Immunotherapy of Metastases Enhances Subsequent Chemotherapy

Abstract. *In many multimodal therapies of cancer, postsurgical chemotherapy is administered before immunotherapy for treatment of micrometastatic disease. This sequence may not be the most efficacious. Experiments in which strain 2 guinea pigs bearing syngeneic L10 hepatocarcinomas were given immunotherapy showed that infiltrating immune effector cells not only were tumoricidal but disrupted the characteristically compact structure of metastatic foci. When cytotoxic drugs were administered at the peak of this inflammatory response, the survival rate of the guinea pigs increased significantly. We conclude that postsurgical immunotherapy can enhance the effect of cytotoxic drugs administered subsequently.*

Considerable effort has been devoted to understanding how tumors avoid elimination by immunotherapy. Many mechanisms have been proposed, most of which attribute treatment failures to a compromised immune status of the host or a lack of tumor immunogenicity (*1*). Relatively little attention has been given to the possibility that anatomic characteristics of the metastases serve to protect them from therapy. Solid tumors as small as 1 mm in diameter contain areas of severe vascular insufficiency (*2*). These areas contain many viable but hypoxic cells. Even in culture, tumor cells grown as spheroids show evidence of hypoxia when the colony is larger than 0.35 mm in diameter (*3*). Thus, it is probable that most occult metastases have vascular insufficiencies and hypoxic cells, factors that could make metastases resistant to cytotoxic drugs or infil-

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Table 1. Survival of guinea pigs after immunotherapy of established metastases. Inbred strain 2 guinea pigs were given intravenous injections of 10^6 syngeneic L10 hepatocarcinoma cells. Each animal subsequently received two intradermal injections of 10^7 BCG admixed with viable but nontumorigenic L10 cells that had been exposed to x-rays (20,000 rads) and one injection of irradiated L10 cells alone. The data are pooled from three experiments (a total of 45 guinea pigs per group).

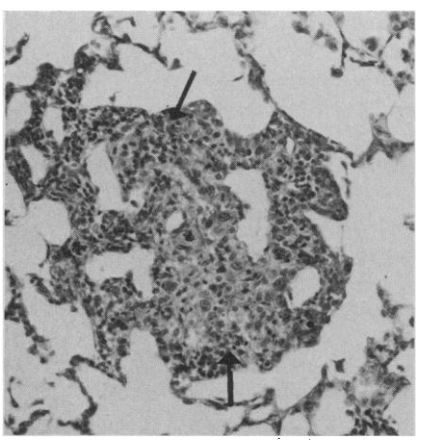
Vaccination schedule (days after tumor injection)*	Survival of guinea pigs (%)	Size of pulmonary metastases (day measured)
No vaccinations	0	
1, 7, 14	70	< 5 cells (day 1)
4, 11, 18	65	5 to 10 cells (day 4)
7, 14, 21	40	0.1 to 0.2 mm (days 7 to 10)
10, 17, 24	20	0.35 to 0.5 mm (days 21 to 24)

*One vaccination on the indicated days in the order described in the legend.

Table 2. Effect of combined immunotherapy and chemotherapy on the survival of guinea pigs bearing L10 tumors. Pulmonary L10 tumors were initiated on day 0 by the intravenous injection of 1×10^6 L10 tumor cells. Immunotherapy was administered on days 10 and 17 with an admixture of 10^7 BCG and 10^7 L10 cells. A booster injection of 10^7 L10 cells was given 7 days after the last treatment with vaccine or 10 days after the last chemotherapy treatment. Chemotherapy consisted of a single intraperitoneal injection of cyclophosphamide (150 mg/kg) or *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) (10 mg/kg); N.S., not significant.

Treatment				*P**	
Immunotherapy	Chemotherapy	Number of survivors	Percentage	Comparison with controls	Comparison with animals receiving immunotherapy alone
None	None	0 of 30	0		.003
Days 10, 17, 24	None	9 of 35	26	.003	
Days 10, 17, 24	Cyclophosphamide, day 31	15 of 26	58	< .001	.017
Days 10, 17, 41	Cyclophosphamide, day 31	15 of 24	63	< .001	.007
Days 10, 17, 24	BCNU, day 31	9 of 15	60	< .001	.028
Days 10, 17, 24	Cyclophosphamide, day 45	3 of 15	20	.032	N.S.
Days 10, 17, 24	Cyclophosphamide, day 90	6 of 21	29	.003	N.S.

*Fisher's two-tailed exact test.



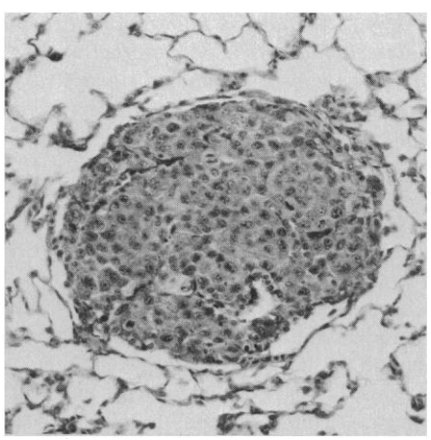


Fig. 1 (left). Metastatic L10 tumor nodule in the lung of a guinea pig 7 days after the first vaccination (14 days after tumor challenge). The nodule is being infiltrated by mononuclear cells. Arrows indicate lymphocyte and macrophage concentrations (hematoxylin and eosin, ×250). Fig. 2 (right). Metastatic nodule in the lung of a control guinea pig 21 days after intravenous injection of L10 cells. The nodule (diameter, 0.35 mm) has numerous mitotic figures and is highly vascularized. There is no evidence of host cell–mediated inflammation (hematoxylin and eosin, ×250).

tration by immune effector cells. In addition, fibrin, which can develop cocoon-like investments around tumors, not only retards the diffusion of tumor antigens to lymphoid tissues but also blocks penetration of the tumor by inflammatory cells (*4*). Hence, the number of metastatic foci may not always be the critical determinant of the efficacy of therapy; the size and anatomic structure of individual foci are also important.

We examined the ability of tumor-specific immune components to disrupt the anatomic architecture of small metastatic foci in guinea pigs (*5*). Significant numbers of animals were cured of established micrometastatic disease by vaccines consisting of the adjuvant bacillus Calmette-Guérin (BCG) admixed with viable but nontumorigenic L10 hepatocarcinoma cells (Table 1). Morphological studies revealed that characteristic host cell–mediated hypersensitivity reactions occurred at the sites of pulmonary metastases in the immunized guinea pigs (Fig. 1). There was an early, predominantly mononuclear, cell infiltration in these nodules consisting of lymphocytes and cells of the macrophage-histiocyte series. This infiltration disrupted the typical compact architecture of the tumor foci (Fig. 2).

Karyorrhexis and cytolysis were detected only after the inflammatory disruption of the established tumor nodules had occurred, suggesting that the tumor cells were killed by the same effector cells that had infiltrated and disrupted the nodules. Presumably, some of the infiltrating lymphocytes were sensitized to tumor-associated antigens. It has been shown (*6*) that these effector cells are not only tumoricidal but also produce lymphokines in the presence of specific antigens. These lymphokines are chemotactic for mononuclear cells and initiate inflammatory tissue reactions (*6*) which, we suggest, contributed to the disruption of the nodules. Eventually these tumor foci were transformed into chronic inflammatory lesions with histiocytosis and ischemic necrosis. Thus it appears that the observed tumor killing is correlated with the development of cell-mediated immune responses and anatomic disruption of the micrometastatic nodules. Nevertheless, as shown in both animals and humans given immunotherapy, the induction of tumor immunity is not always synonymous with protection.

These data, however, support a rationale for immunotherapy that has been overlooked. The disruption of metastases by induced cell-mediated hypersensitivity might overcome the anatomic bar-

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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