export, Georgetown, Guyana) (*8*), was fertilized by sperm from a Bolivian-type male (six acrocentric pairs of chromosomes; point of export, Santa Cruz, Bolivia). Four similar types of "hybrid" embryos have previously been produced in our laboratory from in vitro fertilization. One live birth has occurred of a "hybrid" squirrel monkey as a result of a natural mating.

With this demonstrated success with primate and hamster ova, xenogenous fertilization may be an alternative method for in vitro fertilization of a variety of species, including humans, and may be more effective and represent less risk to the embryo. It also may be useful as a technique for assessing the fertilizability of sperm samples independent of the need for in vitro or homologous in vivo fertilization.

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9. We thank T. J. Kuehl for developing the innovative techniques and procedures, as well as performing the experiments which led to the first evidence for xenogenous fertilization in a nonhuman primate, and M. W. Mitchell and P. J. Hirst for technical assistance. Supported by grants from the National Institutes of Health and a grant from the March of Dimes Birth Defects Foundation.

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Therapy of Spontaneous Metastases by Intravenous Injection of Liposomes Containing Lymphokines

Abstract. *Mice of two different strains were injected subcutaneously with spontaneously metastasizing syngeneic melanomas. After 4 to 6 weeks, the local tumors were removed and, 3 days after surgery, treatment of the metastases was initiated. The treatment consisted of intravenous injections of liposomes containing lymphokines or control supernatant fluids. Liposomes were injected twice weekly for 3 weeks, and the mice were killed 2 weeks later. Seventy-three percent of the mice injected with liposomes containing lymphokines were free of metastases, whereas only 10 percent of the mice treated with control liposomes were tumor-free. These experiments suggest that this form of therapy may provide a valuable addition to the more conventional approaches to the eradication of cancer metastases.*

Metastasis, the formation of secondary tumors at sites distant from the primary tumor, is responsible for most failures in cancer treatment. Recent studies suggest that neoplasms are heterogeneous with regard to many phenotypic characteristics, including metastatic potential (*1*), and that metastases may result from the proliferation of a minor subpopulation of cells preexisting within the primary tumor. These studies imply that a successful therapy of metastases will be one that circumvents this problem of cellular diversity between primary cancers and their metastases and among various metastases.

Tumoricidal macrophages distinguish between normal and cancerous cells by some as yet unknown mechanism. Further, the destruction of cancer cells, at least in vitro, occurs independently of such phenotypic characteristics as antigenicity, invasiveness, metastatic potential, and drug sensitivity. Although tumor cells resistant to most other toxic regimens have been described, attempts to select tumor cells that are resistant to macrophage cytotoxicity in vitro have been unsuccessful (*2*).

Normal macrophages can be rendered tumoricidal in vitro by activation with a variety of agents, including macrophage-activating factor (MAF), a lymphokine released by sensitized lymphocytes during their interaction with antigens or mitogens in culture (*3*). Evidence of the effectiveness of tumoricidal macrophages in controlling cancer metastasis in vivo was obtained from studies in which macrophages activated in vitro were injected intravenously into syngeneic mice bearing pulmonary tumors (*4*). This approach, however, has serious clinical limitations, such as the need to transfuse large numbers of autologous or histocompatible macrophages. Although macrophages from tumor-bearing animals can respond to MAF to become tumoricidal (*5*), it would be preferable to activate these cells by delivering the activating agent to them in vivo. Advances in liposome technology (*6*) have provided a mechanism for accomplishing this task. Recent studies demonstrated that MAF encapsulated within liposomes is much more efficient at activating macrophages in vitro than free MAF (*7*). This raises the possibility that liposome-encapsulated MAF might also be highly efficient in activating macrophages in vivo (*8*), thereby providing a new approach for the treatment of metastases.

To test this possibility, we used a variant line of the C57BL/6 B16 melanoma, B16BL-6 (*9*), and a clone of the recently derived C3H K-1735 melanoma (*10*). In a preliminary study, both metastasized to pulmonary and other sites in over 85 percent of mice in which the tumors were grown subcutaneously. Syngeneic mice (C57BL/6 and C3H) were injected subcutaneously in the footpad or external ear with 2.5×10^4 living tumor cells grown in vitro in a volume of 0.1 ml of Hanks balanced salt solution (HBSS). Four or 6 weeks later, the mice injected with B16 or K-1735, respectively, were anesthetized by methoxyflurane inhalation, and the foot or ear, bearing tumors ranging in diameter from 10 to 15 mm, was amputated. Liposome treatment began 3 days later. Each treatment consisted of an intravenous injection of 5 μmole of multilamellar large vesicle (MLV) liposomes suspended in 0.2 ml of HBSS. The liposomes consisted of chromatographically pure egg phosphatidylcholine and bovine brain phosphatidylserine at a 1 : 1 mole ratio and contained either MAF, normal lymphocyte supernatant (NLS), or supplemented Eagle's minimum essential medium (SMEM) (*11*). The liposomes were injected twice weekly for 3 weeks, and the mice were killed 2 weeks after the final injection. The number of pulmonary tumor colonies in each animal was counted in double-blind fashion under a dissecting microscope by two independent observers. All suspected pulmonary and extrapulmonary metastases were confirmed by microscopic examination of fixed histological sections.

Spontaneous pulmonary metastases were well established in the animals at the time liposome treatment was initiated. Many consisted of hundreds of tumor cells. Some superficial metastases could even be detected under a dissecting microscope (Fig. 1a). Without therapy, these metastases developed into macroscopic tumor nodules by the end of the experiment (Fig. 1b). How-

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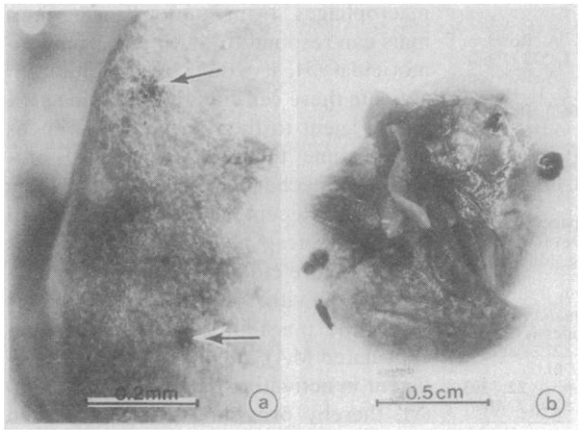

Fig. 1. Spontaneous metastases in lungs of syngeneic C57BL/6 mice produced by B16BL-6 melanoma cells. Metastases are shown (a) at start of liposome therapy and (b) at the end of the experiment in control mice.

ever, the majority of the animals treated with liposome-encapsulated MAF had no macroscopically or microscopically detectable metastases.

Table 1 summarizes the results of three experiments with the B16BL-6 tumor. In two experiments, B16BL-6 cells were injected into the footpad; in the third, they were injected into the external ear. The results of the experiments were quite similar and, therefore, were analyzed together. The B16BL-6 cells formed macroscopic metastases in 26 of 28 untreated control mice. The multiple intravenous injections of unencapsulated (free) MAF or NLS (data not shown) or of liposomes containing SMEM or control supernatant did not reduce the incidence of metastasis or the median number of pulmonary metastases per mouse. In contrast, the multiple intravenous injections of liposomes containing MAF brought about a dramatic decrease in the incidence of spontaneous metastasis. At the time they were killed, 22 of 30 mice were free of disseminated tumors ($P < .005$, χ^2 test). There was also a significant reduction in the number of pulmonary metastases per animal in the liposome-encapsulated MAF-treated group relative to all other treatment and untreated control groups (Mann-Whitney U test) (*12*).

Similar success in treating established pulmonary metastases was obtained in C3H mice bearing the syngeneic K-1735 melanoma. The results clearly demonstrate that injections of liposome-encapsulated MAF lead to a significant reduction in the incidence of metastasis compared to that in control mice or in animals injected with liposomes containing control supernatant fluids (Table 1).

In the experiments with the B16BL-6 tumor, five mice with metastases (treatment failures) in the group treated with liposome-encapsulated MAF had a recurrence of the primary tumor. In a separate set of experiments, no eradication of metastases by liposome-MAF therapy occurred when the subcutaneous tumors were not excised surgically, but were allowed to grow progressively. Thus the success of liposome-MAF therapy may be limited when the tumor burden is large. However, this problem does not present an immediate obstacle to therapy, since in clinical practice, extensive surgical resection of the primary tumor is carried out routinely. Furthermore, the optimal conditions for liposome therapy have not yet been defined. The mice in these studies were given only six injections of liposome-encapsulated MAF. Future studies should determine whether increasing the number of such treatments could lead to eradication of all metastases or be effective against increased tumor burden.

We used phosphatidylcholine-phosphatidylserine MLV liposomes as carriers for MAF for several reasons. First, studies of the body distribution of liposomes of differing size and phospholipid composition demonstrated that optimal localization and retention of liposomes in the lungs were achieved with these negatively charged liposomes at a 1:1 mole ratio (*13*). Second, at the dose used here, MAF-containing liposomes of this composition are not toxic and activate alveolar macrophages in mice and rats to become tumoricidal after being injected intravenously (*14*).

In conclusion, the systemic administration of liposomes containing a macrophage activator can eradicate established pulmonary metastases originating from a subcutaneous melanoma. Since, at least in vitro, all tumor cells seem to be susceptible to destruction by activated tumoricidal macrophages, resistant clones may be less likely to emerge during this form of therapy than during chemo- or radiotherapy. In any event, this biological approach may provide a valuable addition to the therapeutic regimens currently in use.

Table 1. Effects of treating spontaneous metastases in C57BL/6 and C3H mice by intravenous injection of liposomes containing lymphokines.

Liposome treatment	Median number of pulmonary metastases per mouse	Range	Number of mice positive for pulmonary metastasis/ total mice
C57BL/6 mice given B16BL-6 tumor			
Control	8	0–68	26/28
SMEM	4	0–44	23/29
NLS	5	0–61	23/27
MAF	0	0–12	8/30*
C3H mice given K-1735 tumor			
Control	3	0–10	9/10
NLS	9	0–31	9/10
MAF	0	0–5	3/10†

*$P < .005$ compared to controls and $< .01$ compared to the groups given SMEM and NLS. †$P < .01$ compared to control or NLS.

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11. Phosphatidylcholine and phosphatidylserine were purchased from Avanti Biochemicals. Liposomes were prepared as described in (*7*). Supernatant fluids containing MAF were obtained from cultures of normal F344 rat lymphocytes incubated in vitro for 48 hours with concanavalin A bound to Sepharose beads (Pharmacia). Normal lymphocyte supernatant fluids came from F344 rat lymphocytes cultured for 48 hours in Eagle's MEM supplemented with 5 percent fetal calf serum, sodium pyruvate, nonessential amino acids, L-glutamine, and vitamin solution (Flow Laboratories). In some experiments, concanavalin A was added to NLS 10 minutes before harvest. The efficacy and activity of MAF and NLS from these sources are described in (*8*). Encapsulation of MAF-containing lymphocyte supernatant fluids within liposomes was achieved as follows. A mixture of the lipids in chloroform was evaporated to dryness under nitrogen, resuspended in the MAF supernatant

fluids or control solutions, and vortex-shaken. The solution was centrifuged at 2000*g* for 20 minutes, and the liposome pellet was resuspended in HBSS to the final injection volume.
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Niemann-Pick Disease: A Genetic Model in Siamese Cats

Abstract. *Three Siamese cats were found to have a progressive neurological disease that became obvious when they were 4 to 5 months of age. Their brains contained an excess of G_{M2} and G_{M3} gangliosides, and their livers a nine- to tenfold excess of sphingomyelin and cholesterol. A total deficiency of lysosomal (pH 5.0) sphingomyelinase was found in the leukocytes, liver, and brain of the cats, although the activity of the microsomal (pH 7.4, magnesium-dependent) sphingomyelinase was normal in brain. These cats appear to have a genetic disease identical to Niemann-Pick disease type A.*

Niemann-Pick disease (NPD) constitutes a group of recessively inherited syndromes in which sphingomyelin and, secondarily, other lipids, are stored in various organs of the patients. In NPD type A, hepatomegaly and slowing of motor and mental progress become evident early in the first year of life (*1*). Deterioration continues until the patients reach a vegetative state, and death usually occurs before 3 years of age. The liver and spleen contain foam cells filled with sphingomyelin and cholesterol. In the brains of these children there is an increase in G_{M2} and G_{M3} gangliosides (*2*). There is a pronounced deficiency of lysosomal acid (*p*H 5.0) sphingomyelinase (E.C. 3.2.4.12) activity, but the brain contains normal levels of a nonlysosomal magnesium-dependent neutral (*p*H 7.4) sphingomyelinase activity (*3*). Patients and carriers can be identified by examination of leukocytes and cultured skin fibroblasts. Although prenatal diagnosis is available for couples at risk, no treatment is available for children affected with this fatal autosomal recessive disease. We now report a genetic disease in Siamese cats that is identical in pathological and biochemical findings to human NPD type A.

Tissues from three affected cats from three unrelated litters were examined. The tissues from the first two cats had already been fixed in Formalin. These cats were 4 to 5 months old when the owners noted tremors, ataxia, hind-leg weakness, lack of appetite, and lack of interest in their surroundings. Treatment with antibiotics and corticosteroids provided only transitory improvement, and the cats were humanely killed at about 6 months of age. Examination of the tissues by light microscopy revealed the cytoplasmic vacuolization of neurons, hepatocytes, and cells of the reticuloendothelial system suggestive of a lipid storage disease. Because G_{M1} and G_{M2} gangliosides have been reported in Siamese cats, the brain ganglioside pattern was examined, and an excess of G_{M2} and G_{M3} gangliosides was found. The Formalin-fixed liver of one of these cats was available, and qualitative analysis on thin-layer chromatography showed the great excess of sphingomyelin and cholesterol consistent with NPD.

When a third cat was brought to the veterinarian with similar symptoms some months later, blood samples were obtained and leukocytes were prepared for assays of lysosomal enzyme activities as described previously (*4*). The leukocytes showed no measurable sphingomyelinase activity when this was measured at *p*H 5.0 (11 controls averaged 4.97 nmole of substrate hydrolyzed per milligram of protein per hour with a range of 2.99 to 7.20 nmole/mg-hour); an unaffected littermate had a value of 2.63 nmole/mg-hour and is considered a heterozygote for this defect. The parents of these two cats are not available for study.

β-Galactosidase activities were normal in the third affected cat and in its littermate, with values of 42.1 and 40.8 nmole of substrate hydrolyzed per milligram of protein per hour, respectively (14 controls averaged 30.1 nmole/mg-hour with a range of 21.4 to 64.3 nmole/mg-hour). The activities of β-*N*-acetylglucosaminidase (and the percentage activity of hexosaminidase A) were also normal in the affected cat and the unaffected littermate with values of 1332 nmole/mg-hour (57 percent) and 965 nmole/mg-hour (38 percent), respectively [14 controls averaged 864 nmole of substrate hydrolyzed per milligram of protein per hour (67 percent) with a range of 423 to 1928 (50 to 83 percent hexosaminidase A)]. The cat continued to deteriorate clinically until it was 13 months old when it was unable to stand or to feed itself. It was humanely killed with an injection of sodium barbital and the tissues were immediately removed for light and electron microscopy, as well as for measurement of the lipid

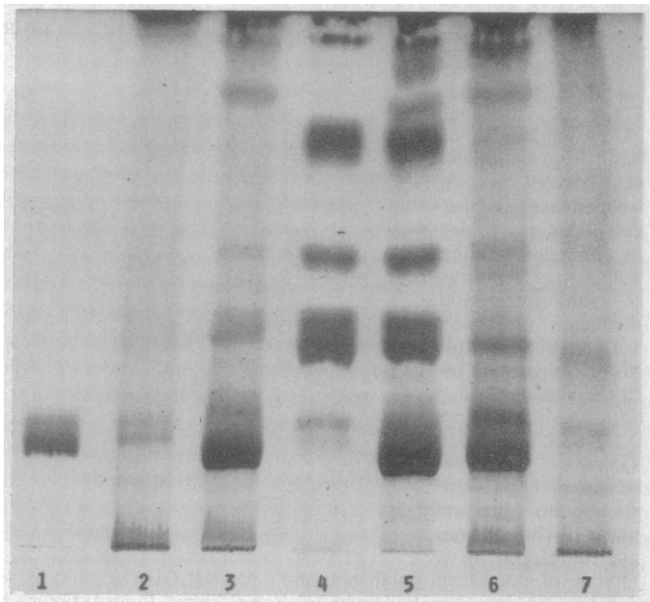

Fig. 1. Thin-layer chromatography of liver phospholipids. Liver samples were homogenized in ten volumes of a mixture of chloroform and methanol (2:1, by volume) and the extract was washed with one-fifth volume of 0.1*M* KCl; the lower phase was washed again with the Folch upper phase. The lower phase was dried with nitrogen, and the residue was dissolved in one volume of the chloroform-methanol mixture. Portions of equal size (on the basis of the wet weight of liver) were spotted on silica gel plates (Brinkmann) and developed in a mixture of chloroform, methanol, acetic acid, and water (75:45:12:6, by volume). The plates were dried and the lipids were visualized by spraying with 50 percent sulfuric acid and heating at 100°C for 20 minutes. Lane 1, sphingomyelin standard; lane 2, Formalin-fixed control cat liver; lane 3, Formalin-fixed liver from affected cat; lane 4, fresh frozen control cat liver; lane 5, fresh frozen affected cat liver; lane 6, fresh frozen liver from human with NPD type A; and lane 7, fresh frozen control human liver.

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