However, this assay is not as specific as an MAb-directed radioimmunoassay because many different isozymes of cytochrome P-450, including constitutive forms, are AHH-active. To compare the usefulness of the radioimmunoassay and of the assay for AHH activity, we examined the stability of placental cytochrome P-450 as measured by the two methods (Table 1). Measurements were made with microsomes stored at 21°C or 4°C. The AHH activity for both samples declined substantially under the conditions of the test, but little change was observed in the radioimmunoassay measurements. The catalytic activity of the AHH-active cytochrome P-450 is therefore more unstable than the MAb-specific epitope detected by the radioimmunoassay. This assay is therefore both more reliable and more specific than measurements of catalytic activity of cytochrome P-450 in tissues.

We next applied the radioimmunoassay to lymphocytes, which are more readily available than placentas and thus more useful for studies of biochemical epidemiology and for phenotyping human tissues. Basal lymphocytes and those induced with benz[*a*]anthracene from different individuals were examined. Blood samples (50 ml) were obtained from each individual; these yielded enough lymphocytes for duplicate assays with different amounts of cell homogenate. Treatment with benz[*a*]anthracene resulted in a greater amount of MAb-specific cytochrome P-450, as revealed by the radioimmunoassay (Fig. 2). Because only about 6 ml of blood is needed per duplicate measurement of both basal and induced cells, this assay is well suited for screening studies of humans.

A relation between the inducibility of AHH in lymphocytes and the incidence of lung cancer has been suggested, but the data are conflicting (*11*). The various results of previous studies (*12*) may be due to differences in sample handling or possible loss of enzyme activity during preparation. Our MAb-based assay may help in resolving the controversy because it is rapid and sensitive and bypasses these problems. It may be more applicable for screening studies of appropriate populations and a more suitable and specific method than the conventional enzyme assay, especially for detecting human cytochromes P-450 that are unstable or present at low levels of enzyme activity.

The specificity of MAb's to individual epitopes makes them useful for detecting single enzymes or classes of enzymes within a system comprising multiple enzymes. The cytochromes P-450 are a paradigm of such a system and are also the enzymatic interface between xenobiotics and higher organisms and because they metabolize endobiotics. The cytochrome P-450 profile of a tissue may determine differences within an individual in the metabolism of cytochrome P-450 substrates. The MAb-directed radioimmunoassay for cytochrome P-450 should also be useful for relating tissue cytochrome P-450 phenotype and drug and carcinogen sensitivity and for detecting polymorphisms.

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19 October 1984; accepted 22 January 1985

Two Types of Somatostatin Receptors Differentiated by Cyclic Somatostatin Analogs

Abstract. *Somatostatin receptors in rat brain, pituitary, and pancreas were labeled with two radioiodinated analogs of somatostatins 14 and 28. Two cyclic analogs of somatostatin, SMS201–995 and cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys), showed biphasic displacement of binding to somatostatin receptors by these radioligands. In contrast, all other somatostatin analogs, including somatostatin-14, competed for the receptor sites with monophasic displacement of radioligand receptor binding. Thus two types of somatostatin receptors were identified. It was found that the pituitary and pancreas have predominantly one type of somatostatin receptor whereas the brain has both, and that different regions of the brain have various proportions of the two types. These findings suggest methods to characterize other types of somatostatin receptors subserving somatostatin's diverse physiological functions, including a potential role in cognitive function and extrapyramidal motor system control.*

Somatostatin is a tetradecapeptide widely distributed in the mammalian central nervous system and peripheral tissues where it exerts a wide variety of physiological effects (*1*). Somatostatin concentration is markedly increased in the basal ganglia in Huntington's disease (*2, 3*), whereas it is decreased in the cortex in Alzheimer's disease (*4*). Thus somatostatin may play a significant role in extrapyramidal motor system and cognitive functions.

Somatostatin stimulates release of [^{3}H]dopamine from the striatum in vivo and in vitro (*5*) and increases dopamine turnover rates without affecting other biogenic amines (*6*). These effects are probably mediated through specific somatostatin receptors. Somatostatin receptors in the rat have been studied in great detail (*7–10*) by using binding to brain membranes. An autoradiographic study (*11*) showed that somatostatin receptors are preferentially distributed in the ventral medial portion of the caudate nucleus compared to its dorsolateral region. Dopamine turnover is also highest in the nucleus accumbens and ventromedial striatum (*12*), and dopamine receptors follow the same distribution (*13*), suggesting a possible interaction between dopamine and somatostatin. So-

matostatin receptors are also concentrated in the substantia nigra (*14*). Thus somatostatin and its receptors assume key anatomical positions to modulate nigral-striatal dopaminergic activities. Somatostatin might therefore play a substantial role in the extrapyramidal motor dysfunction seen in Parkinson's and Huntington's diseases. Of the neurotransmitters studied in Alzheimer's disease only acetylcholine and somatostatin are decreased in the cerebral cortex.

It is conceivable that somatostatin's effects on cognitive and extrapyramidal motor systems are mediated by distinct subsets of somatostatin receptors, just as opiates are thought to deliver their analgesic, mood-elevating, and respiratory suppressive effects through the μ, δ, and κ receptors, respectively (*15*). We report here the characterization of two distinct types of somatostatin receptors in the rat brain.

Cyclic analogs of somatostatin have selective effects in inhibiting growth hormone release, suggesting that they may be useful in differentiating subclasses of somatostatin receptors. SMS201–995 is a very potent octapeptide analog of somatostatin which is resistant to proteolysis (*16*). It is 700 times more potent than somatostatin-14 (SS-14) in the inhibition of growth hormone release in vitro. Moreover, when administered to rats subcutaneously or intravenously, its median inhibitory dose for the inhibition of growth hormone release is 0.08 μg/kg, whereas the glucose-induced insulin secretion is unaffected by doses up to 1 mg/kg. Cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys) is a conformationally constrained bicyclic hexapeptide analog of somatostatin (*17*) that is two to three times more potent than SS-14 in the inhibition of glucagon, insulin, and growth hormone secretion. It is, however, 20 times less potent than SS-14 in the inhibition of pentagastrin-stimulated gastric secretion.

Somatostatin receptors in rat brain membranes were labeled with ^{125}I-[Tyr^{11}]SS-14 by a modification of the method described by Srikant and Patel (*7*). The ^{125}I[Tyr^{11}]SS-14 was prepared (*18*) and purified on a C_{18} reversed-phase column (*19*). Binding of ^{125}I[Tyr^{11}]SS-14 to somatostatin receptors was rapid and reversible, with the association following pseudo first-order kinetics. The half-life of association at 37°C was 13 minutes; apparent equilibrium was attained at 45 minutes and remained at that plateau for at least 2 hours. At 37°C the kinetic constant of dissociation K_{-1} was 0.026 per minute, corresponding to a half-life of dissociation of 32 minutes.

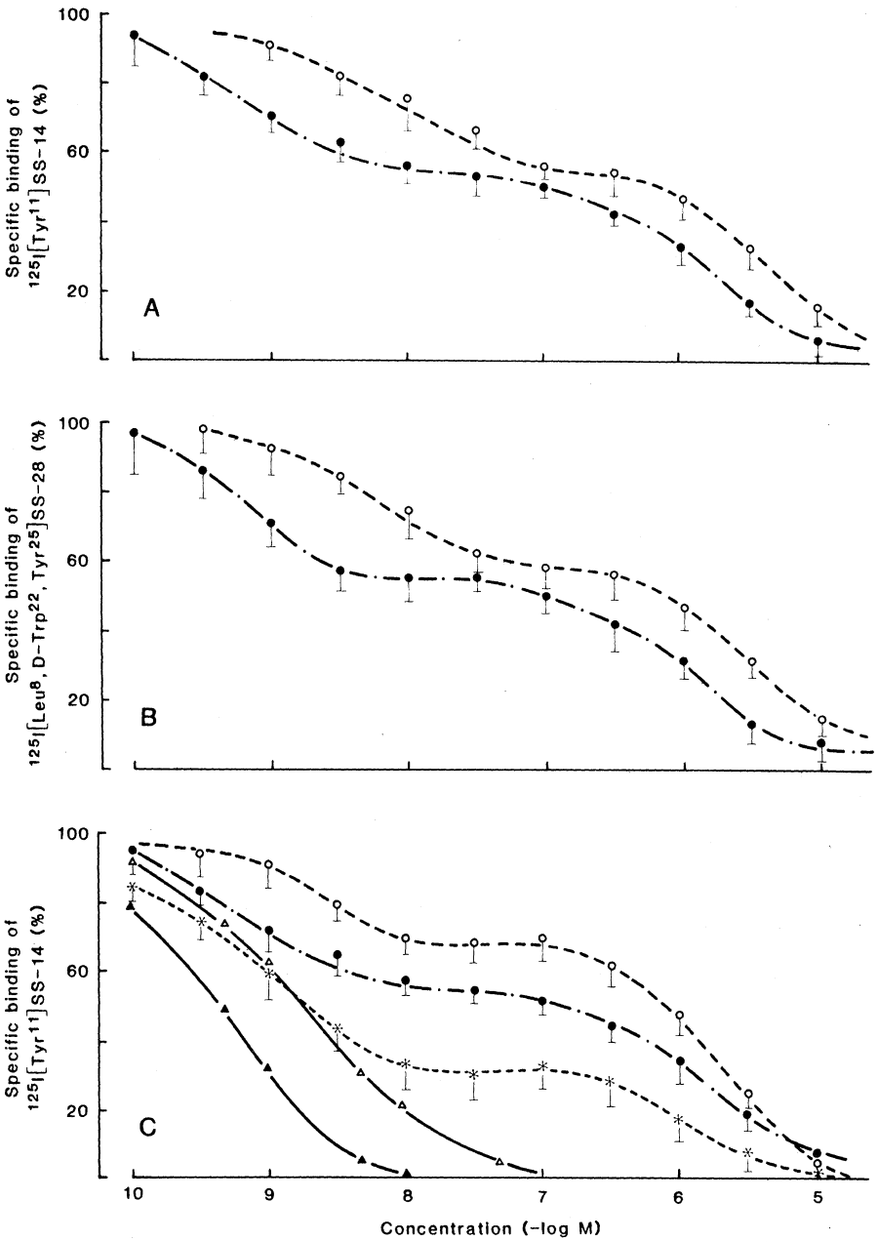

Fig. 1. Competition for specific ^{125}I[Tyr^{11}]SS-14 and ^{125}I[Leu^8,D-Trp^{22},Tyr^{25}]SS-28 binding by SMS201–995 and cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys). Iodination of [Tyr^{11}]SS-14 and [Leu^8,D-Trp^{22},Tyr^{25}]SS-28 was performed by the chloramine-T method (*9, 19*). ^{125}I[Tyr^{11}]SS-14 and ^{125}I[Leu^8,D-Trp^{22},Tyr^{25}]SS-28 were purified by high-performance liquid chromatography (HPLC) on a μBondapak C_{18} reversed-phase column eluted isocratically with 0.25*M* TEAF buffer (*p*H 3.5) and 15.5 percent *n*-propanol (*19*). Both ^{125}I[Tyr^{11}]SS-14 and ^{125}I[Leu^8,D-Trp^{22},Tyr^{25}]SS-28 then eluted from the uniodinated peptides with good separation. Binding of ^{125}I[Tyr^{11}]SS-14 and ^{125}I[Leu^8,D-Trp^{22},Tyr^{25}]SS-28 to somatostatin receptors was performed in a final volume of 250 μl of tris-HCl with 0.1 percent bovine serum albumin, 0.1 percent bacitracin, and 5 m*M* Mg^{2+}. Tissue (2 to 8 mg, wet weight) was incubated with 0.08 n*M* radioligand (corresponding to 60,000 count/min) and various concentrations of displacers. Nonspecific binding was determined in the presence of 2×10^{-7}*M* SS-14. The reaction was allowed to proceed for 60 minutes at 27°C. Bound and free radioactivity were separated by filtration under suction onto GF/C filters presoaked in 0.3 percent polyethyleneimine for at least 3 hours. In routine experiments with 2 mg of rat cortex, total binding was 7800 count/min and nonspecific binding 2100 count/min for ^{125}I[Tyr^{11}]SS-14. The corresponding values were 5800 and 550 count/min for ^{125}I[Leu^8,D-Trp^{22},Tyr^{25}]SS-28. Degradation of the radioligands was negligible under our binding conditions. This was ascertained by extraction of ^{125}I[Tyr^{11}]SS-14 from the incubation and rechromatography (HPLC). (A) Displacement of ^{125}I[Tyr^{11}]SS-14 binding to rat brain cortex by SMS201–995 (●) and cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys) (○). Both analogs displaced ^{125}I[Tyr^{11}]SS-14 binding in a biphasic manner. The high-affinity component, the SS_A receptors, represented 45 percent of the total binding. (B) Repetition of the experiment with ^{125}I[Leu^8,D-Trp^{22},Tyr^{25}]SS-28 as the ligand. Similar results were obtained. (C) Regional and organ variation of the relative proportions of SS_A and SS_B receptors. The SS_A receptors represented 45 percent of the total binding for the cortex (●), 30 percent for the hippocampus (○), 70 percent for the striatum (*), and 100 percent for the pituitary (▲) and pancreas (△). Data points are the means for five separate experiments.

Table 1. Differentiation of the two types of somatostatin receptors by SMS201-995. Somatostatin receptors were labeled with 0.08 n*M* ^{125}I[Tyr^{11}]SS-14. The percentage of specific binding that remained after displacement by SMS201-995 was plotted against the negative logarithm of the concentration, yielding a biphasic curve. Dissociation constants for the receptors were estimated from these curves. Experiments were repeated five times in triplicate; values are means ± standard errors.

Tissue	Dissociation constants		Percentage of total binding	
	SS_A (n*M*)	SS_B (μ*M*)	SS_A	SS_B
Cortex	0.5 ± 0.3	1 ± 0.5	45 ± 6	55
Hippocampus	0.6 ± 0.2	0.5 ± 0.5	30 ± 7*	70
Striatum	0.8 ± 0.3	1.5 ± 0.4	70 ± 8†	30
Pituitary	0.5 ± 0.2		100	0
Pancreas	1.0 ± 0.3		100	0

*Significantly different from corresponding value for cortex ($P < 0.01$, two-tailed *t*-test). †$P < 0.001$.

Scatchard analysis of specific binding of ^{125}I[Tyr^{11}]SS-14 to membranes from rat cerebral cortex revealed a single saturable component with an apparent dissociation constant of 0.17 n*M* and maximum binding of 5.2 fmol per milligram of tissue (wet weight). The total number of receptor sites varied in accordance with the brain regions examined. The cortex and hippocampus contained three times as many binding sites as the striatum, which had twice the number of binding sites as the midbrain and hypothalamus.

Binding of this radioligand to a physiologically relevant somatostatin receptor is supported by the low median inhibitory concentrations (IC_{50}'s) (0.5 to 5 n*M*) shown for each of the biologically active somatostatin analogs. Thus the affinities of various somatostatin analogs in vitro are closely correlated with their reported endocrinologic activities in vivo (*20–25*). [Tyr^{11}]SS, with an IC_{50} of 0.5 n*M*, is two to three times more potent than SS-14 (IC_{50}, 0.3 n*M*), [D-Trp^{8}]SS (1.6 n*M*), [Tyr^{1},D-Trp^{8}]SS (1.2 n*M*), and [Leu^{8},D-Trp^{22},Tyr^{25}]SS-28 (1.4 n*M*), which are in turn two to three times more potent than [D-Cys^{14}]SS (2.9 n*M*) and SS-28 (5 n*M*). In contrast, the alanine substituted analogs, which are relatively inactive in the inhibition of growth hormone release, were also inactive in competing for binding, showing IC_{50}'s of less than 1 μ*M*. Peptides unrelated to somatostatin, including substance P, Met- and Leu-enkephalin, bradykinin, neurotensin, cholecystokinin octapeptide, rat growth hormone–releasing factor, angiotensin II, and arginine vasopressin, did not compete for binding. All analogs, except for SMS201–995 and cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys), competed for specific ^{125}I[Tyr^{11}]SS-14 binding with Hill coefficients of 1, demonstrating that they are unable to discriminate among subsets of somatostatin receptors.

Biphasic displacement of specific ^{125}I[Tyr^{11}]SS-14 binding was observed with both SMS201–995 and cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys). In the cortex the displacement of binding by SMS201–995 began at a concentration of 10^{-10}*M* and reached an intervening plateau at about 10^{-8}*M*. This plateau was maintained for SMS201–995 concentrations from 10^{-8} to 10^{-7}*M*. When the concentration of this analog was increased above 10^{-7}*M*, a second phase of displacement was demonstrated until virtually complete inhibition of binding was achieved at 10^{-5}*M*. Similar results were obtained with cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys) (Fig. 1A). To examine whether the binding characteristics of these two analogs were dependent on the ligands with which somatostatin receptors were labeled, we repeated the displacement studies when somatostatin receptors were labeled with ^{125}I[Leu^{8},D-Trp^{22},Tyr^{25}]SS-28 (*11*). Virtually identical patterns of displacement were observed (Fig. 1B). We propose to call those somatostatin receptors that are displaceable by both SMS201–995 and cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys) with high affinity SS_A receptors and those showing low affinity SS_B receptors.

The relative abundance of SS_A and SS_B receptors varied with the brain region and organ examined (Table 1 and Fig. 1C). In the cortex, hippocampus, and striatum the percentages of receptor sites characterized as SS_A were 45, 30, and 63, respectively. These differences are statistically significant. In the pituitary and pancreas the displacement of ^{125}I[Tyr^{11}]SS-14 binding by SMS201–995 showed only a single component with nanomolar affinity, indicating that there are predominantly SS_A receptors in these two organs. The proportions of SS_A and SS_B receptors in the cerebral cortex did not change with different concentrations of ^{125}I[Tyr^{11}]SS-14. The proportion of SS_A receptors in rat brain cortex determined with ^{125}I[Tyr^{11}]SS-14 was similar to that observed when ^{125}I[Leu^{8},D-Trp^{22},Tyr^{25}]SS-28 was used.

The seemingly discrepant results obtained from Scatchard analysis (showing a single saturable component) and displacement studies (showing biphasic displacement) can be explained by the hypothesis that both ^{125}I[Tyr^{11}]SS-14 and ^{125}I[Leu^{8},D-Trp^{22},Tyr^{25}]SS-28 bind to the two types of receptors with identical or virtually identical dissociation constants that cannot be discriminated by Scatchard analysis. This contention is supported by the finding that the ratio of SS_A receptors to SS_B receptors remains unaffected by changing the concentration of ^{125}I[Tyr^{11}]SS-14 and by the fact that this ratio is independent of whether ^{125}I[Tyr^{11}]SS-14 or ^{125}I[Leu^{8},D-Trp^{22},Tyr^{25}]SS-28 is used as the radioligand.

We have described direct biochemical evidence that there are at least two types of somatostatin receptors, SS_A and SS_B, which are characterized by their markedly different affinities for SMS201–995 and cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys). The SS_A receptor has nanomolar affinity while the affinity of the SS_B receptor is micromolar. The relative proportions of SS_A and SS_B receptors vary with the brain region and organ examined. At least in the cortex, the proportions are similar when SMS201-995 or cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys) is used as the discriminating analog. Similarly, results are independent of the radioligand used to label the receptors. Srikant and Patel (*9*) and Reubi *et al.* (*8, 10*) suggested that there are two classes of somatostatin receptors based on their findings that SS-14 and SS-28 had reversed rank orders of potency in the cortex and pituitary. It is not clear how the two classes of somatostatin receptors reported here relate to those proposed by those investigators or to the receptors subserving the myriad of physiological responses. However, further investigations with these conformationally constrained cyclic analogs may be illuminating.

Our results suggest methods for further studying these two types of somatostatin receptors. Radioactively labeled SMS201–995 and cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys) could be used to selectively label SS_A receptors, and SS_B receptors could be characterized by studying those ^{125}I[Tyr^{11}]SS-14 or ^{125}I-[Leu^{8},D-Trp^{22},Tyr^{25}]SS-28 bindings that remain intact in the presence of concentrations of cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys) or SMS201–995 sufficient to displace the high-affinity SS_A component. These approaches would allow analysis of the pharmacological and biochemical properties of the two classes of

somatostatin receptors and of the functional significance of altered somatostatin concentrations in pathological conditions.

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26. We thank D. Romer and P. Marbach for generously supplying SMS201-995, D. F. Veber for cyclo(Ala-Cys-Phe-D-Trp-Lys-Thr-Cys), and J. Rivier for [Leu^8,D-Trp^{22},Tyr^{25}]SS-28. This study was supported by NIH grant NS16367 (Huntington's Disease Center Without Walls) and the Julieanne Dorn Fund to J.B.M.

16 May 1984; accepted 3 January 1985

Protection of Mice Against Fatal Herpes Simplex Type 2 Infection by Liposomes Containing Muramyl Tripeptide

Abstract. *Intravenous administration of liposomes containing muramyl tripeptide phosphatidylethanolamine, a lipophilic derivative of muramyl dipeptide that activates macrophages to a cytolytic state in situ, significantly protected mice against lethal challenge with herpes simplex virus type 2. These findings suggest that the systemic activation of macrophages by liposomes containing an immunomodulator can lead to prophylaxis of severe infections caused by herpesviruses.*

There is increasing evidence that macrophages play an important role in host defense against viral infections, in particular, those caused by herpesviruses (*1*). Macrophages activated to become cytotoxic by a wide range of immunomodulators acquire the ability to discriminate between uninfected cells and cells infected with herpes simplex virus (HSV) (*2*). The data have stimulated interest in the synthesis of compounds capable of promoting macrophage-mediated destruction of virus-infected cells. *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide; MDP), which can now be synthesized, is the minimal unit of the mycobacterium cell wall with immune potentiating activity (*3*). Although MDP is a potent macrophage activator in vitro (*4*), pharmacokinetic studies have indicated that the drug is excreted from the body within 60 minutes after parenteral administration (*5*), thereby limiting its efficacy for therapy in vivo (*6*).

The use of multilamellar phospholipid vesicles (MLV, liposomes) offers a way to overcome the problem of rapid excretion of soluble MDP (or other macrophage activators) from the body (*6, 7*). Upon entering the circulation, liposomes are rapidly cleared by free and fixed phagocytic cells. This clearance provides an approach for the targeting of macrophage-activating agents to monocytes and macrophages in vivo. In this regard, Fidler and colleagues have demonstrated that MLV containing MDP or its lipophilic derivative, D-isoglutamyl-L-alanyl-2-(1′,2′-dipalmitoyl-*sn*-glycero-3′-phosphoryl)ethylamide (MTP-PE), can efficiently activate macrophages in situ to destroy spontaneous pulmonary and lymph-node metastases in mice implanted with malignant melanoma. We recently reported that MLV containing MTP-PE–activated human monocytes in vitro to destroy HSV type 2 (HSV-2)–infected cells without lysing uninfected cells (*8*). The present study was designed to determine whether MLV containing MTP-PE could also protect mice against a lethal systemic infection with this virus.

Male BALB/c mice (6 to 8 weeks old) free of specific pathogens were obtained from the Animal Production Area, NCI–Frederick Cancer Research Facility. The mice were injected intravenously or intraperitoneally with 1×10^4 plaque-forming units of HSV-2 strain 333 (*9*). For protection studies, mice were injected intravenously with free (unencapsulated) MTP-PE (*10*), with control liposomes, or with liposomes containing MTP-PE. Injections were administered 2 days prior to virus infection, on the day of virus infection, and 2 days after virus infection. The liposomes consisted of an admixture of phosphatidylcholine (PC) and phosphatidylserine (PS) (*11*), at 5 μmol of phospholipid per dose. Mice were examined daily for signs of disease, for example, ruffled fur and paralysis. Survival indices were monitored for a period of up to 42 days after infection. The Duncan multiple-range test (*12*) was used to evaluate the significance of differences in survival time between experimental and control mice. The Newman-Keuls multiple-range test (*13*) was used to analyze survival percentage.

The ability of liposomes containing MTP-PE to protect BALB/c mice from a lethal intravenous HSV-2 infection is shown in Fig. 1. In this study, mice were treated with various preparations on days −2, 0, and +2 after infection. Practically all the mice treated with phosphate-buffered saline (PBS) (28 of 30) died by day 28 after infection. Eight of 30 mice treated with MLV-PBS were alive by day 42 after infection. Treatment of mice with free MTP-PE (10 μg per dose) led to the survival of 11 of 30 mice ($P < 0.02$, Newman-Keuls multiple-range test, in comparison with PBS-treated controls). Increasing the dose of free MTP-PE to 100 μg per mouse did not affect the prophylactic efficacy of the free drug. A most significant increase in survival was observed in mice treated with MLV-MTP-PE, where 16 of 30 animals were alive by day 42 after infection ($P < 0.005$; Newman-Keuls multiple-range test, in comparison with PBS-treated controls).

In the above experiments, HSV-2 was injected intravenously. Under these conditions, HSV-2 causes focal necrosis of the liver and subsequently disseminates to the spleen, lung, and central nervous system; death is caused by encephalitis. Since mice are more resistant to HSV-2 infection by the intravenous route than the intraperitoneal route (*14*), we compared the efficacy of liposome-mediated prophylaxis of HSV-2 infection against