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

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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

these two routes of virus inoculation. The data in Fig. 2 show that MLV containing MTP-PE protect the murine host against lethal challenge with HSV-2 by either route and that protection is greatest when the virus is administered intraperitoneally (80 percent survival versus 10 percent for the control group).

Liposomes are cleared from the circulation by the phagocytic cells of the reticuloendothelial system (*6, 7, 15*). Distribution studies with radiolabeled MLV have shown that most liposomes are found in the liver 4 to 24 hours after intravenous injection (*15*). Since Kupffer cells of the liver can be activated in situ by liposomes containing MTP-PE to lyse tumorigenic cells while leaving nontumorigenic cells unharmed (*16*), we assumed that activation of these cells before they were challenged with HSV-2 could also enhance their antiviral capacity. We found that mice infected with HSV-2 and treated with liposomes containing MTP-PE had a significantly greater survival rate than control mice. When serum samples from the surviving mice were tested for antibodies to HSV-2 by immunoprecipitation with HSV-2 glycoproteins, all samples, with the exception of those from one mouse, contained the antibodies. Thus, although virus-induced encephalitis and subsequent death was inhibited by liposomes containing MTP-PE, sufficient virus replication occurred to stimulate an immunological response. Since MDP has immune adjuvant properties in addition to its macrophage-activating capability (*3*), the antibody response might have been due to low levels of virus replication coupled with the adjuvant effects of the drug.

The ability of liposomes containing immunomodulators to activate macrophages (*6, 7, 15*) and protect mice against HSV-2 infections may have important implications for patients undergoing immunosuppressive therapy. For example, to prevent potentially fatal viral infections, bone marrow transplantation patients are being treated with drugs such as acyclovir (*17*). Antiviral chemoprophylaxis, however, is inherently subject to the problem of drug resistance (*18*). In contrast, virus-infected cells are less likely to be resistant to lysis by activated macrophages (*19*). The demonstration by Osada *et al.* (*20*) that nonspecific host resistance to opportunistic infections can be stimulated even in immunocompromised hosts further supports the potential use of liposomes containing immunomodulators to achieve macrophage activation and thus prophylaxis of systemic viral diseases.

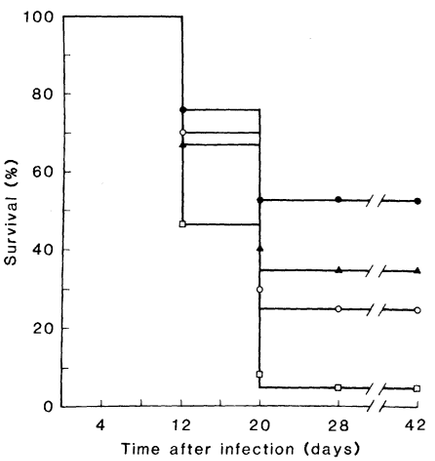

Fig. 1. Protection of mice against a fatal HSV-2 infection by free and liposome-entrapped MTP-PE. Mice were injected intravenously with 1×10^4 plaque-forming units of HSV-2. Free MTP-PE (10 μg per mouse), control liposomes (5 μmol per mouse) or liposomes containing MTP-PE (5 μmol with 40 μg MTP-PE per mouse) were injected intravenously on days −2, 0, and +2 of viral infection. Each treatment group consisted of 30 mice. The survival of mice treated with MTP-PE and with MLV-MTP-PE differed significantly from the survival of the controls ($P < 0.02$ and $P < 0.005$, respectively; Newman-Keuls multiple-range test). Significant differences were also observed in the median survival time of animals succumbing to HSV-2 infection. For mice treated with PBS (□), it was 15 ± 2 days (standard deviation of data obtained from a representative experiment); MLV-PBS (○), 19.5 ± 6; free MTP-PE (▲), 20 ± 2 ($P < 0.05$); and MLV-MTP-PE (●), 22 ± 3 ($P < 0.001$). This experiment was carried out four times with 20 to 30 mice per group. In each experiment the number of mice that were alive 28 days after infection was as follows: for PBS control, 2 of 30, 1 of 20, 1 of 20, and 3 of 30; for MLV, 8 of 30, 7 of 20, 2 of 20, and 10 of 30; for free MTP-PE, 11 of 30, 8 of 20, 3 of 20, and 12 of 30; for MLV-MTP-PE, 16 of 30, 12 of 20, 10 of 20, and 19 of 30. These data are very similar, and thus in Fig. 1 we show a representative study.

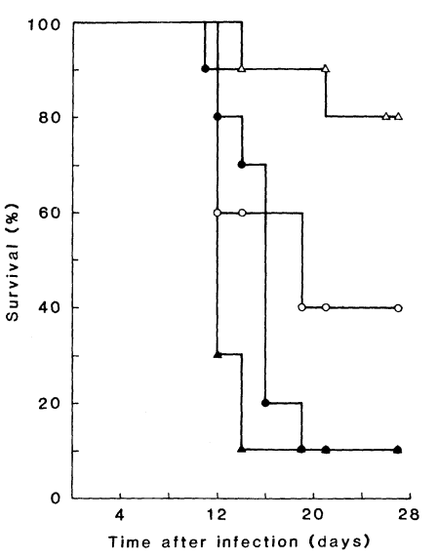

Fig. 2. Protection of mice from intravenous or intraperitoneal HSV-2 infection by liposomes containing MTP-PE. Mice were infected with 1×10^4 plaque-forming units of HSV-2 either by intravenous or intraperitoneal administration. Each treatment group consisted of ten mice. All mice were treated on days −2, 0, and +2 of infection. Symbols: (△) mice infected intraperitoneally and treated with MLV-MTP-PE; (▲) mice infected intraperitoneally and treated with PBS; (○) mice infected intravenously and treated with MLV-MTP-PE; (●) mice infected intravenously and treated with PBS. This represents three replicate experiments. In each experiment the number of mice that were alive 28 days after infection was as follows: for intraperitoneal PBS, 1 of 10, 3 of 10, and 1 of 10; for intraperitoneal MLV-MTP-PE, 8 of 10, 7 of 10, and 7 of 10; for intravenous PBS, 1 of 10, 1 of 10, and 2 of 10; and for intravenous MLV-MTP-PE, 4 of 10, 4 of 10, and 6 of 10.

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per milliliter. Inoculum dose was 0.2 ml per mouse (10 μg of MTP-PE).
11. Chromatographically pure egg PC and beef-brain PS were purchased from Avanti Polar Lipids, Inc., Birmingham, Ala., and stored in sealed ampules at −70°C. MLV containing Ca^{2+}- and Mg^{2+}-free PBS or MTP-PE were prepared from a mixture of PC and PS (8:2 molar ratio) as described in (*6*). MTP-PE was incorporated into the liposomes in a ratio of 8 μg per micromole of phospholipid. Preparations of liposomes in PBS were adjusted to concentrations of 25 μmol of lipid per milliliter of solution. Inoculum dose was 0.2 ml, which contained 5 μmol of phospholipids and 40 μg of MTP-PE.
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Interleukin-1 Stimulation of Astroglial Proliferation After Brain Injury

Abstract. *The interleukins, which have a regulatory role in immune function, may also mediate inflammation associated with injury to the brain. In experiments to determine the effect of these peptide hormones on glial cell proliferation in culture, interleukin-1 was a potent mitogen for astroglia but had no effect on oligodendroglia. Interleukin-2 did not alter the growth of either type of glial cell. Activity similar to that of interleukin-1 was detected in brains of adult rats 10 days after the brains had been injured. These findings suggest that interleukin-1, released by inflammatory cells, may promote the formation of scars by astroglia in the damaged mammalian brain.*

The interleukins are a family of peptide hormones that take part in the regulation of immune function (*1*). Interleukin-2 (IL-2) is released by activated T cells and has a strict T-cell tropism (*2*). Interleukin-1 (IL-1), however, is not only secreted in considerable quantities by monocytes and macrophages (*3*) but is also released by various other cells (*4–6*). IL-1 affects a wide range of target tissues and is thought to be an important mediator of the inflammatory response by acting as a growth factor for fibroblasts (*7*), by inducing the release of prostaglandin E_2 (*8*), and by stimulating the secretion of proteases (*9*). It is possible that interleukins also mediate inflammatory responses that occur after injury to the brain (*6, 10*). Because one cellular response to brain injury is proliferation of glial cells, we tested the ability of interleukins to stimulate growth of brain glia.

Glia from brains of newborn rats were grown in dissociated cell cultures in a defined medium on cover slips coated with poly-L-lysine (*11*). Indirect immunofluorescence techniques were used to identify cell-specific markers, galactocerebroside (GC) for oligodendroglia and glial acidic fibrillary protein (GFAP) for astroglia (*12*). Mean cell numbers were determined for each culture by counting ten randomly selected fields (0.314 mm^2) of specifically stained cells as viewed by fluorescence microscopy. Control cultures with added phosphate-buffered saline (PBS) were prepared in an identical fashion. A minimum of three cultures was used for each data point.

Conditioned medium containing IL-1 was prepared from acute monocytic leukemia (AMoL) cells from patients receiving therapeutic leukapheresis (*13*). The IL-1 was purified from the conditioned medium by ultrafiltration and diafiltration (*14*) and by either anion-exchange chromatography or isoelectric focusing (*15*). Activity of IL-1 was measured by the murine thymocyte assay (*16*). The activity of a preparation of recombinant human IL-2 (Sandoz) was

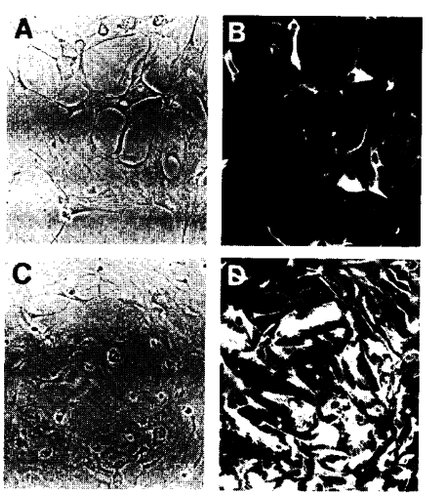

Fig. 1 (left). Photomicrographs of astroglia after treatment with partially purified human IL-1. Phase microscopy (A and C) shows the effects of IL-1 on cultures of rat brain glial cells. Cultures were treated for 3 days with either 5 percent (by volume) of the 10- to 30-kD partially purified IL-1 (C and D) or PBS (A and B). As revealed by immunofluorescence staining for glial fibrillary acidic protein, IL-1 stimulated the number of astroglia (D). Scale bar, 25 μm.

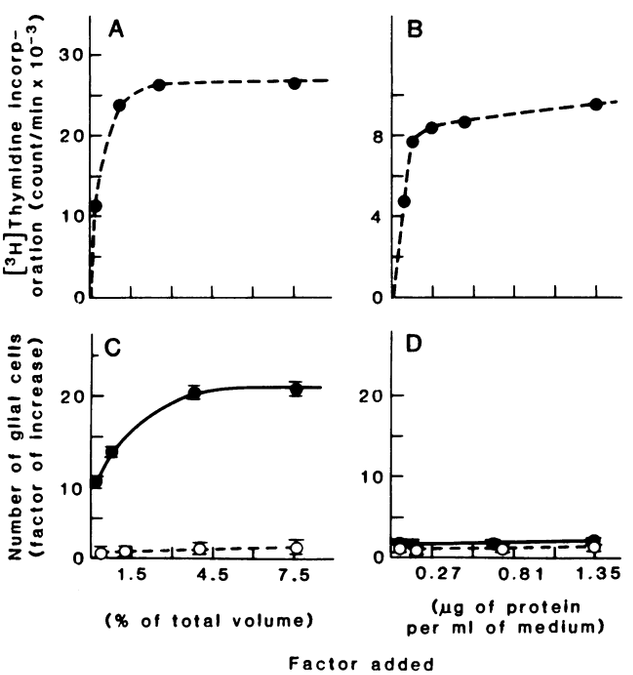

Fig. 2 (right). Dose-response curves showing the effects of partially purified IL-1 (A and C) and recombinant IL-2 (B and D) on responsive cell types. (A) The mitogenic effect of human IL-1 purified by ultrafiltration and isoelectric focusing upon murine thymocytes after 3 days of culture. The conditions for the preparation and purification of the IL-1 have been described (*14, 15*). The direct mitogenic effect of human IL-1 on murine thymocytes is well established (*16*) and is shown here as reference for the comparable effect upon astroglia (C). (B) The mitogenic effect of recombinant human IL-2 on the responsive HT-2 cell line (*2*) is shown as reference for subsequent experiments (D). (C) Concentrations of partially purified IL-1, which were shown to increase incorporation of ^3H-labeled thymidine by murine thymocytes, were also shown to increase the number of astroglia (●), but not oligodendroglia (○), in culture 3 days after the addition of IL-1. (D) Concentrations of recombinant IL-2, which increased incorporation of ^3H-labeled thymidine by the responsive HT-2 cell line, were not able to stimulate the growth of either astroglia or oligodendroglia in culture.