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Antigen-Presenting Liposomes Are Effective in Treatment of Recurrent Herpes Simplex Virus Genitalis in Guinea Pigs

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The therapeutic and immunologic effects of a liposome preparation containing both a macrophage activator, muramyl-tripeptide-phosphatidylethanolamine, and a recombinant antigen, glycoprotein D of herpes simplex virus type 1, have been investigated. This preparation was tested in vitro for the ability to stimulate peripheral blood lymphocytes and in vivo for the control of recurrent herpes genitalis in guinea pigs. Our results show that the liposome-antigen-adjuvant preparation is capable of enhancing antigen-specific lymphocyte stimulation, which may be related to the observed 75% suppression of the frequency and severity of reactivation of recurrent herpes simplex virus type 2 genitalis compared with that of placebo controls.

Herpes simplex virus (HSV) infections continue to be a serious and widespread health problem of epidemic proportion (9, 10) owing to the proclivity of the virus to establish a latent infection and thereafter to produce spontaneous recurrent disease. It has been estimated that up to 50% of teenagers, from a range of socioeconomic backgrounds and demographic locations, will be HSV seropositive by the age of 16 (27). This indicates that they have a past history of either HSV type 1 (HSV-1) or type 2 (HSV-2) infection. Therapy with the nucleoside analog, acyclovir, is effective for primary infections (8, 24). In addition, continuous acyclovir therapy will suppress the frequency of recurrences (14, 15, 19, 25, 36), but the drug effect requires continuous administration. The rate of recurrence rebounds to the pretherapy rate immediately after cessation of continuous acyclovir therapy (14, 15, 19, 25, 36). There is hope that immunization could be effective in protection against this disease (5, 32, 39). Therefore, attempts have been made to develop a subunit vaccine composed of HSV proteins, prepared either from virus-infected cell extracts or by using recombinant DNA technology. Although immunization has been successful for protection against primary HSV infection in animals (13), mixed results have been obtained in the immunotherapeutic treatment of recurrent disease (6, 33; L. R. Stanberry, C. J. Harrison, D. I. Bernstein, R. L. Burke, R. Schukla, G. Ott, and M. G. Myers, Antiviral Res., in press). Beneficial treatment of recurrent herpetic disease depended on the adjuvant used with HSV antigen and the route of vaccine administration (6, 33; Stanberry et al., in press). Therefore, development of an effective adjuvant without side reactions is needed for safe and effective immunotherapy against recurrent disease in humans. For example, the best currently available adjuvant is complete Freund adjuvant, which causes both skin necrosis and potentially more serious sequelae such as encephalitis.

In this report, a novel approach to immunotherapy is presented which employs a recombinant HSV surface glycoprotein incorporated into a liposome-adjuvant carrier that is both effective and potentially safe for use in humans. The adjuvant in this new formulation contains liposomes which can potentially deliver the HSV antigen to antigen-presenting cells and also carry a biologic modifier that could activate these target cells to enhance the presentation of ingested antigen to T lymphocytes. Since T helper cells are involved in other immune cell functions, such as generation of cytotoxic T cells and antibody-producing plasma cells (21, 38), HSV antigen delivered by this strategy could enhance these functions as well. This could potentially lead to an improved efficacy in the clinical outcome as reflected in the control of recurrent disease. Hence, liposomes containing muramyl-tripeptide-phosphatidylethanolamine (MTP-PE) were used as an inert carrier for passively targeting encapsulated HSV antigen to the mononuclear phagocytic system, including antigen-presenting cells such as macrophages (26).

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine and dioleoyl phosphatidylglycerol were purchased from Avanti Polar Lipids, Inc. (Pelham, Ala.), *N*-acetyl-muramyl-L-alanyl-D-isoglutaminyl-L-alanine-(1′-2′-dipalmitoyl-*sn*-glycero-3′-hydroxyphosphoryloxy)-ethylamine (MTP-PE) was kindly provided by CIBA-GEIGY Corp., Basel, Switzerland, and other reagents were of analytical grades.

Animals. Female guinea pigs of 250 to 300 g (Hartley strain) were purchased from EZH Caviary (Williams, Calif.). They were maintained in the isolated animal care facility at Stanford according to the guidelines of the National Institutes of Health.

Liposome preparation. Routinely, 2.0 mg of lipids, composed of egg phosphatidylcholine and dioleoyl phosphatidylglycerol (9:1 [wt/wt]) in either $CHCl_3$ or $CHCl_3$-CH_3OH, were dried under N_2 gas, vacuum desiccated for 30 min, and suspended in 2 ml of injection-grade H_2O (Abbott Laboratories, North Chicago, Ill.) with 10 μl of sterile phosphate-buffered saline. For in vitro and in vivo studies, 2.5 or 10% (wt/wt) of MTP-PE, respectively, were added to the organic solvent. After the mixture was vortexed, it was sonicated as described previously in a bath-type sonicator (Laboratory Supplies, Inc., Hickville, N.Y.) for 15 min (16, 17) to achieve a translucent liposome suspension. Then it was sterilized with a 0.2-μm-pore-size filter, and 120 μg of recombinant HSV-1 glycoprotein D (rgD-1) was added to achieve an

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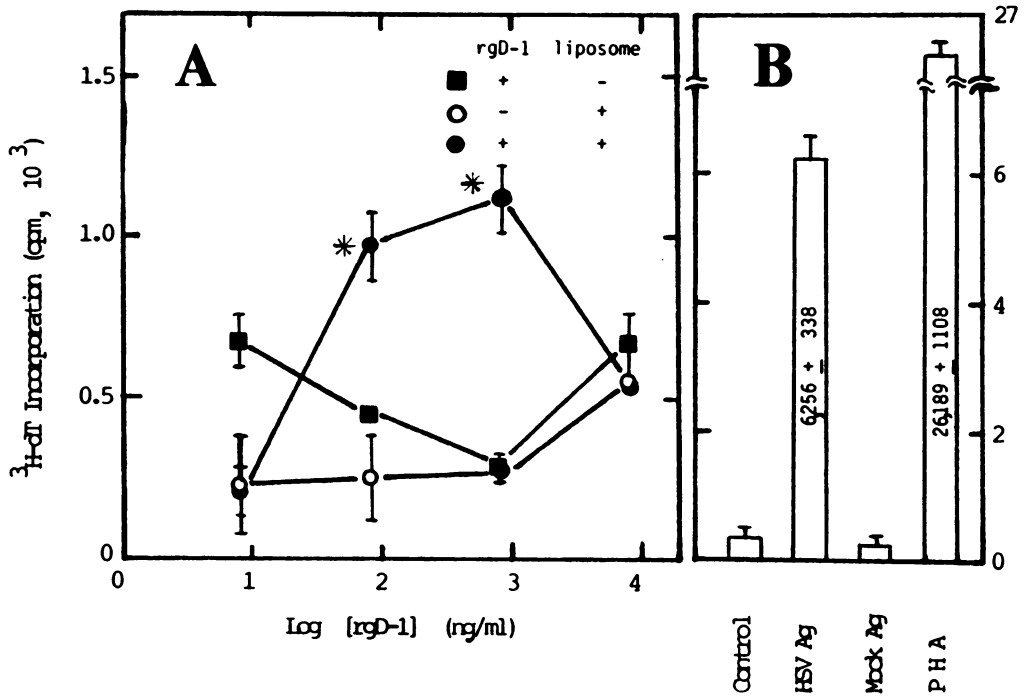

FIG. 1. Effect of liposome encapsulation of rgD-1 on the stimulation of peripheral blood lymphocytes. Peripheral blood lymphocytes from HSV-2-infected animals were isolated more than 90 days postinfection by Ficoll gradient centrifugation and prepared by previous published methods (35). (A) Isolated lymphocytes (2×10^5) were stimulated with either free rgD-1 (■), liposome-encapsulated rgD-1 (●) (for preparation of liposomes, see Materials and Methods), or equivalent lipid-containing empty liposomes (○). (B) Total HSV antigens equivalent to 3×10^6 PFU/ml before UV inactivation (HSV Ag), equivalent cellular lysate (Mock Ag), 0.03% phytohemagglutinin (PHA), or tissue culture fluid (Control) were also tested for their ability to stimulate the isolated lymphocytes. These lymphocytes were incubated at 37°C in a CO_2 incubator for 6 days in RPMI 1640 medium supplemented with 10% fetal calf serum and the indicated stimulant (40). On the sixth day, lymphocytes were pulsed with 1 μCi of tritiated thymidine for 6 h and then harvested onto glass-fiber filters. Incorporation of tritiated thymidine (^{3}H-dT) was measured as an index of lymphocyte proliferation (see the footnotes of Table 2). The asterisk indicates those samples with an LPI of ≥ 3, defined as a positive response. Samples were done in triplicate with the results expressed as mean ± standard deviation.

osmolar value equivalent to that of 0.1 phosphate-buffered saline. The mixture was frozen and lyophilized in a Virtus (The VirTis Co., Inc., Gardiner, N.Y.) overnight (20, 30). When needed, the lyophilized liposomes were rehydrated in 0.2 ml of injection-grade H_2O, vortexed, and allowed to stand at room temperature for 15 min. Finally, 1.8 ml of injection-grade normal saline was added. Each injected dose of 0.2 ml of rgD-1-containing liposomes contained 12 μg of rgD-1 (liposome encapsulated and unencapsulated) and 20 μg of MTP-PE. Values for percent trapping of rgD-1 in these liposomes were determined by using ^{125}I-labeled rgD-1 and ^{3}H-labeled lipids, to be 47% ± 6% at zero time and 41% ± 4% after 72 h. Endotoxin contents, determined by *Limulus* amebocyte assay (Whittaker Bioproducts, Inc.), were less than 0.21 endotoxin units of rgD-1 per μg and nondetectable for liposomes.

MTP-PE/LO preparation. Routinely, about 15 doses of MTP-PE emulsified in low oil (MTP-PE/LO) was prepared by mixing 180 μg of rgD-1, 12 μl of squalene, 24 μl of 1% Tween 80, and 750 μg of MTP-PE in 3 ml of sterile phosphate-buffered saline. After the mixture was heated in a 45°C water bath for 5 min, it was emulsified by passage through a 26.5-gauge needle six times and then a 200-μl dose was immediately injected into each animal. Each dose of MTP-PE/LO contains 12 μg of rgD-1, 50 μg of MTP-PE, 4% squalene (vol/vol), and 0.008% Tween 80.

RESULTS AND DISCUSSION

To determine whether rgD-1 can function in liposomes as an in vitro immunostimulant, peripheral blood lymphocytes were isolated from guinea pigs that had acquired immunity to HSV. These animals had been infected by intravaginal HSV-2 (10^4 PFU) inoculation and immunity was boosted by frequent recurrences of herpes genitalis. These lymphocytes were stimulated with various concentrations of either liposome-encapsulated or soluble rgD-1 (29), and the degree of lymphocyte proliferation was determined. In this study, about 50% of the rgD-1 was found to be associated with liposomes and the remaining unencapsulated antigen was not removed for the experiments described in this report. Representative experiments are depicted in Fig. 1. Lymphocytes from animals which exhibited cell-mediated immune (CMI) responses to total HSV antigen gave a positive rgD-1 response only when it was presented in liposomes. Neither the free, soluble rgD-1 nor the empty liposome containing MTP-PE provided a significant lymphocyte stimulation. Thus, only liposomes containing MTP-PE and antigen enhanced antigen presentation. A total of 18 animals were tested on three separate occasions (Table 1). Of animals with CMI response to total HSV antigen, 53% (9 of 17) exhibited CMI responses to liposome-encapsulated rgD-1 (Table 1). Neither liposome carrier alone nor liposomes containing irrelevant

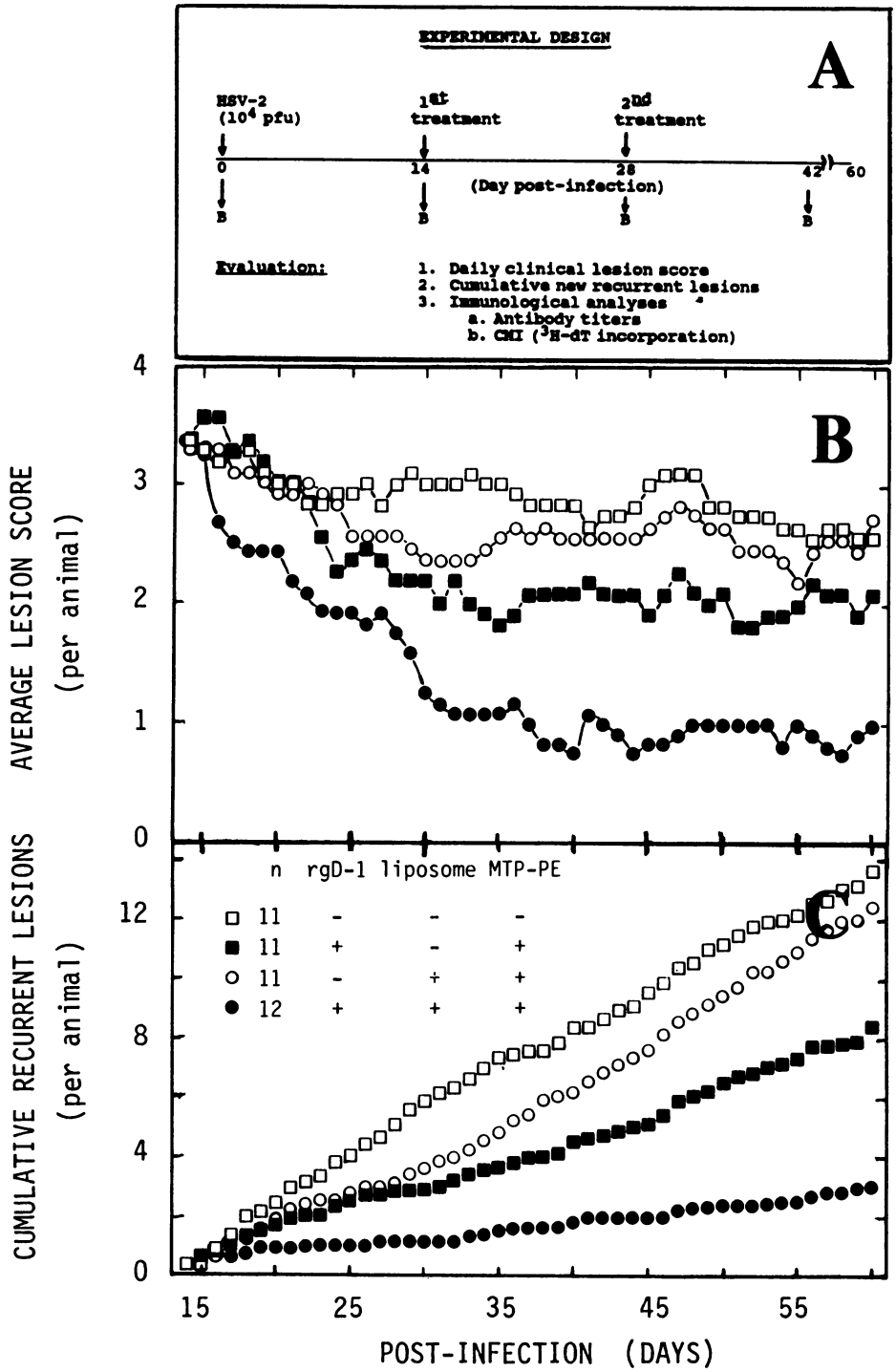

FIG. 2. Clinical effect of treatment with rgD-1-containing liposome formulation in vivo. Sixty-five female guinea pigs of 250 to 300 g (Hartley strain; EZH Caviary) were utilized in this study. (A) Animals were intravaginally infected with 10^4 PFU of HSV-2 (strain MS) on day 0 and allowed to recover from primary infection. On day 14 postinfection, the animals were treated with MTP-PE/LO (containing 12 μg of rgD-1 [see Materials and Methods for details]) (■), empty liposomes (with MTP-PE) (○), rgD-1-containing liposomes (with MTP-PE) (●), or saline (placebo) (□). The second treatment was given on day 28. Immunological analyses were performed on the blood samples (indicated as B's) collected on days 0, 14, 28, and 42 by intracardiac puncture under general anesthesia (40). Some animals (30%) were excluded from the final analyses due to either lack of primary HSV lesion or mortality before the completion of experiments. (B) Average daily lesion score per animal. (C) Group average of the number of cumulative recurrent lesions. For details of the method of scoring the clinical lesions and the new recurrent lesions, see reference 40. Statistical analyses were done using two-tailed *t* distribution for data in this figure at day 60 (see Table A1 for details).

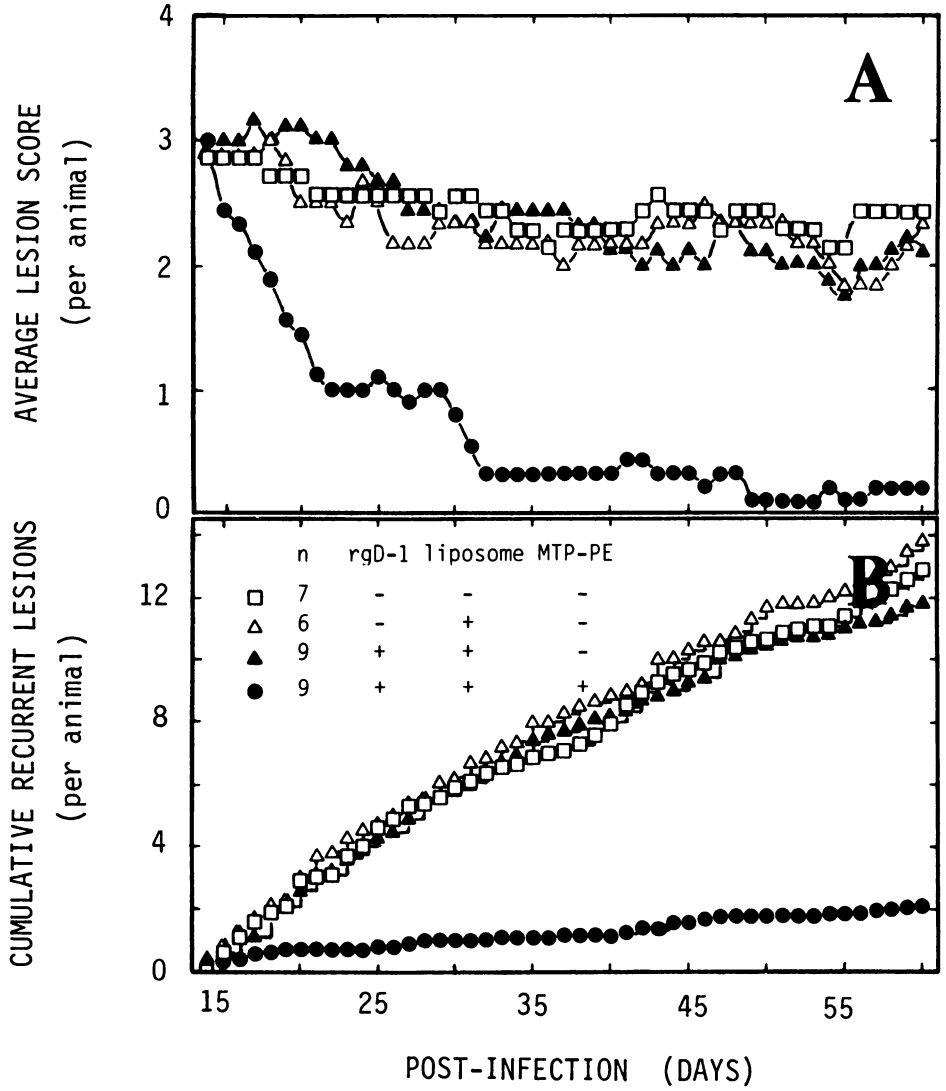

FIG. 3. Requirement of MTP-PE in the antigen-presenting liposomes. Forty animals were enrolled in this study. The same experimental design as described in Fig. 2A was used. On day 14 postinfection, the indicated numbers of animals (*n*) were treated with empty liposomes without MTP-PE (△), rgD-1-containing liposomes without MTP-PE (▲), rgD-1-containing liposomes with MTP-PE (●), or saline (placebo) (□). The rest of the protocol and scoring was done exactly the same as described in the legend to Fig. 2. Statistical analyses of the data shown in this figure at day 60 are summarized in Table A2.

TABLE 1. Cellular immune responses to free and liposome-encapsulated rgD-1[a]

Expt no. (no. of animals tested)	% of animals with positive CMI responses[b] to:		
	rgD-1		HSV crude antigen
	Free	Liposome encapsulated	
Expt 1 (3)	0	67	100
Expt 2 (9)	0	44	89
Expt 3 (6)	17	50	100

[a] Peripheral blood lymphocytes from HSV-2-infected guinea pigs were isolated by Ficoll gradient centrifugation. After the cells were washed with tissue culture fluid, 2×10^5 cells were suspended with the indicated antigen at various concentrations and allowed to grow for 6 days. Triplicate samples were then pulsed with tritiated thymidine for 6 h on the sixth day and harvested with a cell harvester. A typical titration of rgD-1 is shown in Fig. 1.

[b] For determination of percent positive CMI responses, see footnote *b* of Table 2. None of the animals had a positive CMI response to empty liposomes with MTP-PE; all of the animals had positive CMI responses to phytohemagglutinin.

protein lysozyme (data not shown) elicited a measurable response, and soluble rgD-1 stimulated lymphocytes from only 1 of 17 (6%) animals, demonstrating the superiority of rgD-1 presentation by liposomes.

The enhanced presentation of HSV antigen in liposomes might result clinically in an improved efficacy of controlling HSV recurrent disease with immunotherapy. It has been shown that systemically injected liposomes are rapidly taken up mainly by the mononuclear phagocytic system, including circulating macrophages (26). Hence, the introduction of HSV antigen in this carrier may improve the antigen uptake, proteolytic processing, and presentation by the antigen-presenting cells. Antigen presentation by macrophages to helper T cells has been shown to require association of the macrophage processed-antigen with the histocompatibility antigen, Ia (2, 3). In addition, increased expression of Ia has been shown to be associated with the activation of macrophages (21, 38). Therefore, inclusion of MTP-PE with its

TABLE 2. CMI responses after treatment

Expt no. and treatment	28 days postinfection			42 days postinfection		
	n	LPI,[a] mean (SD)	% Positive animals[b]	*n*	LPI, mean (SD)	% Positive animals
Expt 1						
Placebo	10	3.5 (1.7)	60	10	4.5 (2.9)	60
rgD-1 (MTP-PE/LO)	10	3.6 (3.2)	40	10	4.6 (3.2)	70
Empty liposomes with MTP-PE	11	10.4 (13.0)	73	10	4.9 (4.7)	60
rgD-1-containing liposomes with MTP-PE	6	8.4 (2.8)	100	5	6.9 (2.8)	100
Expt 2						
Placebo	9	8.9 (8.9)	67	8	3.7 (1.9)	75
Empty liposomes without MTP-PE	6	4.5 (3.4)	57	6	23.6 (20)	67
rgD-1-containing liposomes without MTP-PE	9	4.4 (3.8)	56	9	2.9 (1.5)	44
rgD-1-containing liposomes with MTP-PE	9	22.8 (16.4)	100	9	5.2 (2.4)	100

[a] LPI, Lymphocyte proliferation index, which is defined by taking the ratio of tritiated thymidine incorporation in the HSV antigen (3×10^6 PFU/ml; UV inactivated)-mediated lymphocyte proliferation over that of mock antigen (40).

[b] LPI of ≥ 3 is taken as a positive CMI response. Percent positive animals is defined as the number of animals that exhibit positive CMI responses over the total number of animals tested. Refer to the legend of Fig. 1 for details of the lymphocyte proliferation assay procedure.

macrophage-active component of muramyl dipeptide (1) in the liposome membrane could enhance Ia expression and provide increased Ia-associated processed antigen. As a result, a significant improvement in presentation of HSV antigen might be achieved which could affect the extent of disease recurrence.

To evaluate the effects of the rgD-1-containing liposomes in vivo, guinea pigs recovering from primary herpes genitalis and subject to recurrent disease (34, 35, 40) were treated with liposomes containing rgD-1 and MTP-PE. Infection in this animal model was chosen because of its clinical similarity to HSV recurrences in humans (18). The treatment schedule is indicated at the top of Fig. 2A. Briefly, intravaginally infected female guinea pigs were divided into four treatment groups that received rgD-1-containing liposomes or antigen-free liposome control via intracardiac injection and saline or rgD-1 (MTP-PE/LO formulation) via intramuscular injection. The intracardiac route was chosen for liposome-encapsulated rgD-1 to parallel intravenous administration to human subjects in order to target the liposomes to antigen-presenting cells. Animals in the study were monitored daily for the severity of lesions as well as the appearance of the new lesions. The scale of clinical lesion scores is the same as that of previously published methods (40). Liposome-encapsulated-rgD-1 treatment was the most effective of all the treatments (Fig. 2B and C). About a 75% lower daily lesion score (Fig. 2B), and about a 80% lower rate of recurrences was documented by the end of day 60 (Fig. 2C). This difference is statistically significant compared with that for animals in the placebo group ($P < 0.005$). In comparison, the MTP-PE/LO formulation (aqueous rgD-1 emulsified with squalene and MTP-PE) was less efficacious; only a 30% reduction in the severity of the clinical lesion score ($0.2 < P < 0.3$) and a 40% reduction in the recurrence rates ($0.01 < P < 0.025$) was observed, despite the fact that the MTP-PE/LO formulation contains the same dose of rgD-1 as the rgD-1-containing liposome dosage. A slight clinically beneficial effect was also noted with liposome carrier containing MTP-PE (without rgD-1) ($P < 0.4$). This effect of MTP-PE-containing liposomes could be due to a nonspecific activation of macrophages. MTP-PE-containing liposomes at higher doses have been shown previously to be beneficial in treating HSV-1 pneumonitis in young mice (J. D. Gangemi, E. Mayer, and A. Ghaffar, Abstr. J. Cell. Biochem. Suppl. **12B**:255, 1988).

To determine whether the presence of MTP-PE is essential to achieve the enhanced immunotherapeutic effect, liposome-encapsulated rgD-1 was prepared either with or without MTP-PE in the membrane. Then, guinea pig treatment experiments as described in the legend to Fig. 2, were repeated. The clinical outcome of treatment with liposome-encapsulated rgD-1 was dependent on the presence of MTP-PE, since elimination of MTP-PE abrogated the potent therapeutic effect of the rgD-1-containing liposomes (Fig. 3). This impact was evident both in the disease severity profile as well as in the recurrence rates. Therefore, the potent adjuvant effect provided by the liposomes requires the presence of MTP-PE.

To examine the mechanism by which this adjuvant effect occurs, the cellular and humoral immune responses of the treated animals were monitored (Table 2). In experiment 1, all of the animals receiving rgD-1-containing liposomes exhibited positive HSV-specific cellular immune response as early as 14 days posttreatment. Despite a lower lesion score and fewer recurrences by day 42 (Fig. 2), all animals in the rgD-1-containing liposome treatment group had CMI responses. This finding was confirmed in experiment 2 by the rgD-1-containing liposome (containing MTP-PE) group (Table 2). The clinically less efficacious MTP-PE/LO treatment gave fewer cellular immune reactive animals compared with the rgD-1-containing liposome group. Although administration of empty liposomes containing MTP-PE produced a somewhat higher number (73%) of cellular immune reactive animals at day 28 compared with that for the placebo group (60%), it was less than that for the group treated with rgD-1-containing liposomes (100%). In comparison, the percent of cellular immune reactive animals in the MTP-PE/LO group and the placebo group were about the same at day 28. On day 42, after the second treatment, additional animals in the MTP-PE/LO group became cellular immune reactive. However, a positive CMI response at day 42 did not appear to be associated with an improved clinical outcome of the recurrent disease (Table 2; Fig. 2 and 3). This could be due to a time-dependent difference in the activation of different subsets of lymphocytes that are specific for specific HSV antigens.

The antibody titer to the total HSV antigen (employing infected-cell lysate) was determined by an enzyme-linked-immunosorbent assay. No difference was found in total anti-HSV immunoglobulin G (IgG) titer among the different treatment groups (data not shown). However, a moderate titer of anti-rgD-1 IgG was noted for most of the animals treated with rgD-1-containing liposomes at 14 days posttreatment (28 days postinfection) (Table 3). Compared with placebo treatment, administration of rgD-1-containing liposomes to guinea pigs apparently increased the anti-rgD-1 IgG response at day 28 after HSV infection: 7 ± 2 versus 120 ± 26 in experiment 1 and 0 ± 0 versus 7 ± 2 in experiment 2 (Table 3). However, combining experiments 1 and 2 gave a value that is not statistically significant. This value was constant even after the second boost with the rgD-1-containing liposomes. The failure of rgD-1-containing liposomes to enhance antibody titer against rgD-1 may actually reflect a requirement by the plasma cells for recognition of glycoprotein D antigen to be in a native conformation. The majority of liposomal rgD-1, administered to guinea pigs, is likely to be ingested by macrophages and presented in a form of processed or degraded antigen. In contrast, MTP-PE/LO administration gave a lower anti-rgD-1 IgG response at day 28, although the titer at later times after second injection was the highest of all treatments (Table 3).

These data suggest that rgD-1-containing liposome treatment appears to be exerting the most potent therapeutic effect on HSV recurrent disease by enhancing the cellular immune response of these animals early in treatment. This can be seen by the fact that CMI was sustained in these animals even in the absence of frequent recurrences as natural immune stimulants. Also, there may be more rapid recognition of the antigen presented within the liposome carriers compared with aqueous antigen (MTP-PE/LO formulation). The role of MTP-PE in the liposome membrane for proper antigen presentation is further reflected in the analysis of CMI response (Table 2, experiment 2). If MTP-PE was eliminated in the liposome-adjuvant, the number of animals exhibiting CMI responses, tested on days 28 and 42 postinfection, was the same as for the placebo control group. Therefore, it is concluded that MTP-PE, which has been shown to activate the macrophages both in vitro and in vivo (1), is required in the liposome formulation to achieve the potent immunostimulation of encapsulated rgD-1. Whether another macrophage stimulant such as interleukin-1, various colony stimulation factors, gamma interferon, or tuftsin can replace the MTP-PE in our liposome formulation remains to be seen.

How the three active components, liposome, MTP-PE, and rgD-1, exert their effect is not presently clear. We do know that MTP-PE is essential for providing proper immune responses that lead to the improved clinical outcome. It is possible that MTP-PE, together with gamma interferon, produced in response to viral infection (11, 12, 37), may be synergistically stimulating surface Ia expression of macrophages (4, 31) which, in turn, may lead to an improved antigen presentation. Alternatively, activation of macrophages by MTP-PE alone may be sufficient to increase Ia expression on the cell surface. These and other possibilities still remain to be investigated. Regardless of the mechanism of macrophage activation by MTP-PE, potentiation of antigen presentation was seen with rgD-1-containing liposomes in vitro (Fig. 1; Table 1), as well as in vivo (Fig. 2; Tables 2 and 3). This probably resulted in rapidly enhanced and sustained immune surveillance to HSV, which was reflected in a control of the recurrent herpetic lesions (Fig. 2 and 3). Understanding of the molecular and cellular mechanisms underlying these processes could lead to improved vaccines.

It is not clear whether the soluble rgD-1 present in our MTP-PE-containing liposome preparation contributes to the observed immunotherapeutic effects. Only future studies with purified liposome-encapsulated antigen will determine the contribution of each physical form of rgD-1 in our preparation. Whether all surface-adsorbed or soluble rgD-1 can be removed from liposome-encapsulated rgD-1 is an unresolved issue.

Potential human use of MTP-PE-containing liposomes as an adjuvant should be considered. Liposomes containing phosphatidylcholine and phosphatidylglycerol appear neither toxic nor pyrogenic even at high doses (24, 41). In addition, MTP-PE-containing liposomes composed of phosphatidylserine, currently being used in phase I studies in humans, shows minor side effects only at much higher doses (J. R. Hanagan, H. Frost, P. Trunet, D. LeSher, and K. Andrejcio, Abstr. J. Cell. Biochem. Suppl. **12B**:251, 1988; P. J. Creaven, D. E. Brenner, J. W. Cowens, B. Dadey, R. Huben, C. Karakousis, S. Arbuck, M. K. Cushman, T. Han, and K. Andrejcio, Abstr. J. Cell. Biochem. **12B**:262, 1988) than those used for this study. Furthermore, the pyrogenicity observed above may be attributed to the phosphatidylserine in these liposomes (7). The low doses of MTP-PE and lipid used in the antigen-presenting liposomes should give only minimal side effects after administration to humans.

Antigen-presenting liposomes may be useful as immunotherapeutic agents for treating both infectious and noninfectious diseases. For noninfectious diseases such as cancer, incorporation of tumor-associated antigens, such as carcinoembryonic antigen, into these liposomes may be beneficial by activating the immune surveillance against the antigen-expressing tumor cells (22; N. C. Phillip. Abstr. J. Cell. Biochem. Suppl. **12B**:257, 1988). For infectious diseases such as human immunodeficiency virus infection, presentation of cell surface viral antigen, i.e., glycoprotein 120, glycoprotein 41, etc., may help to eliminate the virus-

TABLE 3. IgG titers to rgD-1 after treatments[a]

Expt no. and treatment	*n*	Antibody titer, geometric mean (SE)	
		28 days postinfection	42 days postinfection
Expt 1			
Placebo	10	7 (2)	7 (2)
rgD-1 (MTP-PE/LO)	10	35 (6)	1,314 (262)
Empty liposomes with MTP-PE	11	19 (3)	20 (4)
rgD-1-containing liposomes with MTP-PE	6	120 (26)	185 (48)
Expt 2			
Placebo	6	0 (0)	111 (45)
Empty liposomes without MTP-PE	6	0 (0)	0 (0)
rgD-1-containing liposomes without MTP-PE	9	33 (10)	40 (13)
rgD-1-containing liposomes with MTP-PE	9	7 (2)	115 (35)

[a] Anti-rgD-1 IgG antibody titers were determined by enzyme-linked immunosorbent assay. This is done by adsorbing 6 μg of rgD-1 in the microwells as an immobilized antigen source. Plasma samples (1:1 [vol/vol]) from guinea pigs were diluted threefold serially. Half the maximum endpoint was used as a cutoff value as previously described in detail (29).

TABLE A1. Statistical analysis of data in Fig. 2

Treatment	n	Avg lesion score (Fig. 2B)			New recurrent lesions (Fig. 2C)		
		Avg	SD	P value	Avg	SD	P value
Placebo	11	2.55	1.03		13.3	0.3	
Empty liposomes with MTP-PE	11	2.72	1.19	$0.4 > P > 0.3$	12.3	5.1	$0.4 > P > 0.3$
rgD-1 (MTP-PE/LO)	11	2.09	1.51	$0.3 > P > 0.2$	8.3	4.7	$0.025 > P > 0.01$
rgD-1-containing liposomes with MTP-PE	12	1.0	1.13	$P < 0.005$	2.8	1.9	$P < 0.005$

TABLE A2. Statistical analysis of data in Fig. 3

Treatment	n	Avg lesion score (Fig. 3A)			New recurrent lesions (Fig. 3B)		
		Avg	SD	P value	Avg	SD	P value
Placebo	7	2.43	0.79		13.0	5.4	
Empty liposomes without MTP-PE	6	2.33	1.37	$P > 0.4$	13.7	8.5	$P > 0.4$
rgD-1-containing liposomes without MTP-PE	9	2.11	1.05	$0.3 > P > 0.2$	11.7	6.9	$0.4 > P > 0.3$
rgD-1-containing liposomes with MTP-PE	9	0.22	0.44	$P < 0.005$	2.1	1.4	$P < 0.005$

infected cells via a rapid stimulation of CMI responses (28).

APPENDIX

Statistical analyses of the data shown in Fig. 2 and 3 were done, using two-tail t distribution. Results of statistical analyses are summarized in Tables A1 and A2.

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