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Physical and biological characterization of antigen presenting liposome formulations: relative efficacy for the treatment of recurrent genital HSV-2 in guinea pigs

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Summary

Antigen presenting liposomes (APLs) containing both liposome encapsulated (44%) and free (56%) recombinant glycoprotein D of herpes simplex virus type-2 (rgD-2) were characterized with respect to the interaction of the antigen with the lipid bilayer and the biological activities provided by each form of rgD-2. We found that free rgD-2 added externally to empty liposomes exhibited some biological activities both in vitro and in vivo, although we could not detect any significant adsorption and/or insertion of this form of rgD-2 into the lipid bilayer. Compared to APLs containing both forms of rgD-2, purified liposomes containing only encapsulated rgD-2 gave only 50% of the relative activity in vitro as measured by their ability to stimulate rgD-2 specific lymphocyte proliferation, and 67% of the relative activity in vivo as measured by their immunotherapeutic effect on recurrent genital HSV-2 disease in guinea pigs ($P < 0.05$). These data indicate that while liposome encapsulated rgD-2 is essential for the elicitation of immunogenic responses, the free soluble rgD-2 in the APL formulation also acts in concert to generate an optimum immunotherapeutic efficacy.

Immunotherapy; HSV; Liposome; Recurrence; Antigen presentation

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Introduction

Herpes simplex virus (HSV) infection continues to be a disease of increasing prevalence in the United States (Corey and Spear, 1986). In susceptible individuals, herpesvirus infection may lead not only to symptomatic primary disease but also to the appearance of recrudescent lesions due to reactivation of latent virus. In fact, the primary reservoir of the transmissible virus is probably that produced by viral replication in recurrent epithelial lesions or that shed asymptomatically from latently infected individuals (Corey and Spear 1986; Rooney et al., 1986). The recurrent nature of this disease can cause significant morbidity particularly for the immunosuppressed individuals.

The antiviral drug acyclovir is effective for treatment of acute episodes of HSV infection but suppression of recurrent disease requires continuous daily therapy. Currently it is recommended that such treatment be limited to a period of six months. After the cessation of drug treatment, recurrence rates immediately return to the pre-therapy levels (Bryson et al., 1983; Dorsky and Crumpacker, 1987; Douglas et al., 1984; Kinghorn et al., 1985; Mertz et al., 1982; Mindel et al., 1988; Straus et al., 1984). Host factors that determine differences in the recurrence rates of individuals are not clear but may partly be due to differences in HSV-specific immune responses. High titers of HSV antibody are apparently not sufficient to control recurrences, since HSV lesions recur despite the presence of high HSV antibody titers that do not change with recurrences (Corey and Spear, 1986). On the other hand, patients treated with immunosuppressive drugs such as cyclosporin A, have relatively unaltered levels of HSV-specific antibody but are deficient in immune-reactive T lymphocytes (Rand et al., 1976). These patients often have severe HSV reactivation (Calne et al., 1979) indicating the important role of cell mediated immunity.

Recently recombinant HSV glycoproteins have been used as an immunotherapeutic agent to suppress HSV recurrences (Stanberry et al., 1988; Ho et al., 1989). In addition, the efficacy of HSV-glycoprotein preparations have been shown to depend on the adjuvants employed for both prophylaxis and immunotherapy (Sanchez-Pescador et al., 1988; Burke et al., 1989; Ho et al., 1989; Stanberry et al., 1989). In this regard, we have recently shown that antigen presenting liposomes containing a lipophilic adjuvant, muramyl tripeptide attached to phosphatidylethanolamine (MTP-PE), and the HSV glycoprotein D (rgD) can be used as a potent formulation that may also be safe for human administration (Ho et al., 1989). The glycoprotein antigen in these antigen presenting liposomes (APLs) was approximately equally distributed between liposome-encapsulated and unencapsulated forms: 47% of the rgD was encapsulated in liposomes while the other 53% was present as free antigen in solution. These APLs enhanced rgD specific lymphocyte proliferation in vitro, and suppressed recurrent, genital HSV-2 when administered to HSV-infected guinea pigs.

In this report, we further characterize the physical association of rgD with the APL membrane and determine the relative role of the free vs. encapsulated antigen in mediating the immunobiological effects observed in vitro and in vivo. We

show that both forms of rgD, free and encapsulated, contribute to the enhanced lymphoproliferative responses observed in vitro as well as to the reduction in recurrent disease frequency observed in vivo.

Materials and Methods

Materials

Egg-phosphatidylcholine and dioleoyl phosphatidylglycerol were purchased from Avanti Polar Lipids, Inc. (Pelman, AL), *N*-acetylmuramyl-*L*-alanyl-D-isoglutaminyl-*L*-alanine-2-(1,2-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy) ethylamide (MTP-PE) was kindly provided by Ciba-Geigy (Basel, Switzerland), trypsin and trypsin-inhibitor were obtained from Sigma (St. Louis, MO). All other reagents were analytical grade.

Animals

Female guinea pigs of 250–300 g (Hartley strain) were purchased from EZH caviary (Williams, CA). They were maintained according to NIH guidelines in a isolated animal care facility at Stanford University. Guinea pigs were infected by intravaginal inoculation with 10^4 pfu of HSV-2 MS strain. They were monitored daily for 14 days following HSV-2 infection for the severity of acute HSV disease. At day 14, after the resolution of the primary infection, animals were sorted into groups with similar average lesion scores for the acute disease (Weinberg et al., 1986). On days 14 and 28 post-infection the indicated treatment was administered intra-cardially (under anesthesia) in a 200 μl volume. Animals were monitored daily for clinical lesions during the recurrent disease phase from day 14 to 60. For the scoring of recurrent disease, HSV lesions detected on perineum as swelling or vesicles limited to about 1/6 equivalent to perineum area is given a score of 1, 1/6 to 1/3 is given a score of 2, 1/3 to 2/3 is given a score of 3, and greater than 2/3 is given a score of 4. The appearance of new vesicles, papules or bleeding of the old lesion crust are scored as a new recurrent episode and this score is compounded daily. Animals were bled prior to HSV infection, and on days 14, 28 and 42 post-infection to measure lymphoproliferative responses and antibody titers.

Liposome preparation

Freeze-dried multilamellar liposomes containing both liposome-encapsulated and unencapsulated rgD-2 were prepared as described (Ho et al., 1989). Upon rehydration per 200 μl dose, 200 μg of lipid (egg-phosphatidylcholine:dioleoyl phosphatidylglycerol 9:1, w/w), 20 μg MTP-PE and 12 μg rgD-2 in PBS. Empty liposomes were prepared by the same procedure except that no rgD-2 was added. For some experiments, the liposome-encapsulated rgD-2 was separated from the unencapsulated or free rgD-2 form by pelleting the liposomes by centrifugation at

15000 × *g* for 30 min. The supernatant containing soluble, unencapsulated rgD-2 was removed, and the liposome pellet was washed three times with PBS. To prepare the soluble rgD-2 plus the empty liposome mixture, rgD-2 was added to the empty liposome preparation. The final amount of rgD-2 per 200 μl dose were 12, 5 and 12 μg/ml for the unseparated gD-2 liposome mixture, the liposome encapsulated rgD-2 (without free rgD-2) and the empty liposomes plus soluble rgD-2 mixture, respectively.

Physical association of rgD-2 to liposome membranes

The extent of membrane association of rgD-2 was determined using ^{125}I-labeled rgD-2 as a tracer. rgD-2 was iodinated using the chloramine T method (Hunter and Greenwood, 1962). Twenty-four microgram of [^{125}I]rgD-2 was incubated with 2 mg of hydrated empty liposomes in 0.4 ml PBS overnight at 4°C. The liposomes were then separated from soluble rgD-2 by centrifugation. APLs prepared in the presence of [^{125}I]rgD-2 were used as a positive control and empty liposomes without rgD-2 addition were used as a negative control. The efficiency of lipid recovery from the centrifugation process was routinely about 80–90%.

Tryptic digestion of liposome encapsulated rgD-2

rgD-2 liposomes containing a total of 20 μg of liposome encapsulated as well as unencapsulated rgD-2 and purified liposome-associated rgD-2 containing about 8.3 μg of encapsulated rgD-2 were digested with 5 μg of trypsin added in 5 μl volume. The mixture was incubated at 37°C for either 2 or 10 min, immediately followed by the addition of 25 μg of trypsin inhibitor to stop the reaction. As a positive control, 10 μg of soluble rgD-2 was also subjected to a similar proteolytic digestion. For the 0 time point, trypsin inhibitor was added prior to trypsin addition. All samples except soluble rgD-2 were extracted with $CHCl_3$:CH_3OH (2:1, v/v) prior to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Protein bands were visualized by staining with Coomassie blue.

Cell-mediated immune response

The cell-mediated immune response was determined by the proliferative response of peripheral blood lymphocytes (PBLs) to HSV antigen as described previously (Ho et al., 1989). For the preparation of UV-inactivated HSV antigen, the titer of HSV prior to inactivation was 3×10^6 PFU/ml; no infectious virus detected following UV treatment. The lymphocyte stimulation index (SI) was defined as the ratio of ^{3}H-dT incorporated in the samples stimulated with HSV antigen to that of mock antigen (Weinberg et al., 1986). Animals exhibiting a stimulation index greater than 3 were considered to be cell-mediated immune (CMI) responsive animals.

Anti-gD-2 antibody titer

rgD-2 specific antibody titers were determined by enzyme-linked immunosorbent assay. The titer is the reciprocal of the dilution of serum yielding an absorbance value equal to one half the maximum end point. Antibody titers are expressed as geometric mean ± SE.

Results

Physical association of rgD-2 to liposomes

To determine whether the recombinant, truncated HSV-2 glycoprotein D (rgD-2) (Burke et al., manuscript in preparation), physically associates with the liposome membrane, ^{125}I-labeled rgD-2 was incubated overnight with preformed empty liposomes followed by the separation of liposomes from the solution by centrifugation. As shown in Table 1, rgD-2 remained free in solution and was not associated with the liposome membrane. The percent encapsulation of rgD-2 when it was present during rehydration of the liposome was 44%, a value very similar to that previously reported for rgD-1 liposome preparations (47%) (Ho et al., 1989).

The physical location of encapsulated rgD-2 within the multilamellar liposome membrane was investigated to determine if liposome-associated rgD-2 molecules are completely encapsulated, or are inserted into the lipid bilayer and partially exposed on the outer membrane surface. Such a partially surface exposed rgD-2 molecule would be accessible to proteolytic digestion. Enzymatically cleaved rgD-2 is separable from intact rgD-2 by polyacrylamide gel electrophoresis (SDS-PAGE). Proteolytic treatment of liposome encapsulated rgD-2 revealed that the liposome associated rgD-2 was completely encapsulated since rgD-2 molecules were inaccessible to trypsin digestion (Fig. 1, lanes 10–12). This result was not due to the inability of the trypsin to digest rgD-2 as free, soluble rgD-2 (lanes 4–6) as well as the rgD-2 liposome preparation containing both unencapsulated and liposome encapsulated rgD-2 (lanes 7–9) were susceptible to trypsin cleavage. In addition,

TABLE 1

Physical association of rgD-2 to liposomes[a]

Sample	Percent of total rgD-2 encapsulated Avg ± SD
rgD-2 prepared as liposome formulation	44.2 ± 3.1
rgD-2 added to preformed empty liposomes	0.3 ± 0.3
rgD-2 (without liposomes)	0.5 ± 0.2
Preformed empty liposomes	ND[b]

[a]rgD-2 encapsulated in liposomes was separated from free rgD-2 by centrifugation. The percent encapsulation was determined by counting the ^{125}I-rgD-2 recovered with the liposome pallet.
[b]ND, non-detectable level.

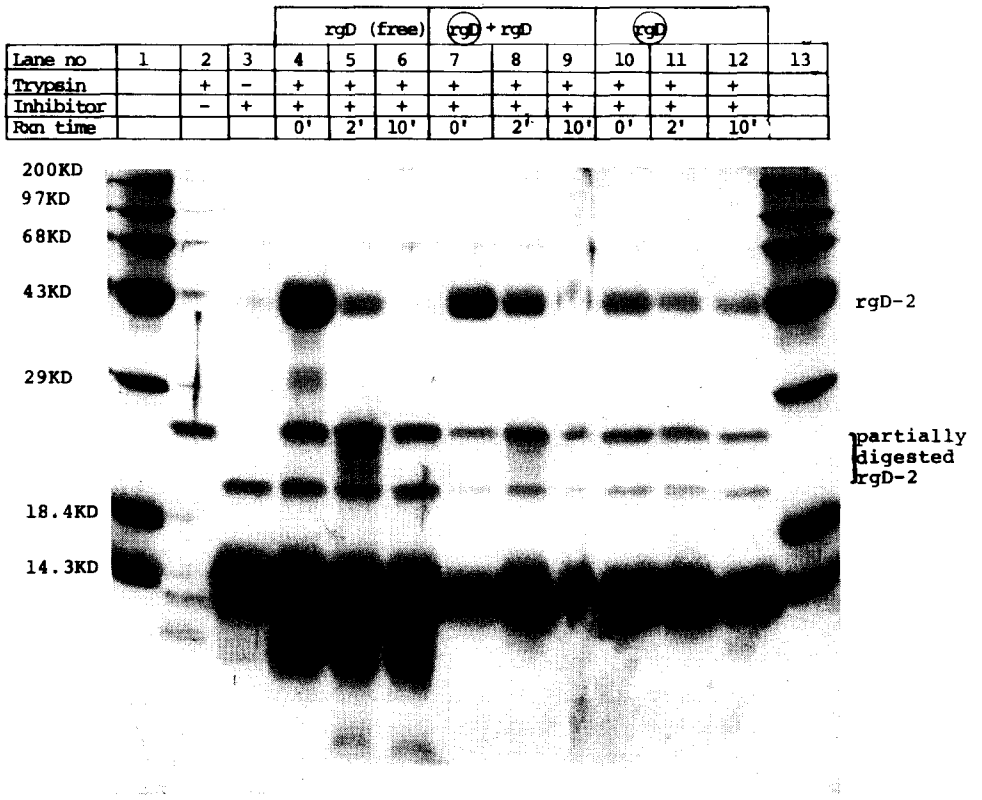

Fig. 1. Protection of rgD-2 from tryptic digestion by liposomes. Antigen-presenting liposomes containing rgD-2 in the form of (i) free plus liposomes encapsulated antigen (APL formulation) (lanes 7–9), (ii) purified liposome-encapsulated antigen (lanes 10–12) or (iii) free antigen (lanes 4–6) were subjected to trypsin digestion for 0 min (lanes 4, 7 and 10), 2 min (lanes 5, 8 and 11) or 10 min (lanes 6, 9 and 12). The tryptic reaction was stopped by the addition of trypsin inhibitor at the indicated time and the mixture was separated by SDS-PAGE on a 12% polyacrylamide gel. Lanes 2 and 3 contain trypsin and trypsin inhibitor, respectively. Partially digested rgD-2 bands can be detected between the trypsin (lane 2) and trypsin inhibitor (lane 3) bands. Lanes 1 and 13 contain the molecular weight standards: myosin, 200 kDa phosphorylase B, 97.4 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbamylase, 29 kDa; β-galactosidase, 18.4 kDa; lysozyme, 14.3 kDa.

about an equal amount of trypsin-resistant rgD-2 was observed with the rgD-2 liposome preparation and the purified liposome associated rgD-2 (in lanes 8 and 11), indicating the proteolytic protection provided by the liposome membranes. Therefore, we conclude that rgD-2 liposomes prepared as described, produced liposomes that truly encapsulated rgD-2 such that rgD-2 was not accessible to the protease, trypsin. Moreover, the free, unencapsulated rgD-2 in the mixture did not adsorb onto liposomal membranes as it remained susceptible to proteolytic cleavage.

Immunogenicity of various rgD-2 liposome formulations in vitro

In order to compare the potential of the two physical forms of rgD-2 (free and liposome-encapsulated forms) in APLs to increase the antigenicity of rgD-2, we tested various rgD-2 liposome preparations for their ability to enhance rgD-2-specific lymphocyte-proliferation in vitro. Peripheral blood lymphocytes (PBLs) of 8 HSV-infected guinea pigs, exhibiting HSV-specific cell-mediated immune (CMI) responses, were tested and the results are summarized in Fig. 2. Of the 8 HSV-CMI responsive animals tested on day 90 post HSV-2 infection, 6 also responded to rgD-2 liposomes containing both free and encapsulated forms of rgD-2. 3/8 responded to rgD in the form of either liposome encapsulated or added exogenously to the empty liposomes (rgD-2 plus empty liposomes). These 3 animals were a subset of the 6 animals responding to rgD-2 liposomes. Hence, liposome encapsulated rgD that contains 0.4 equivalent the amount of rgD-2 protein as the rgD-2, in APL preparation with both free and liposome-encapsulated antigen, exhibited 50% of the adjuvant activity of the latter. This effect was not due merely to the reduced protein in the liposome-encapsulated formulation since a mixture of an equivalent amount of rgD-2 with empty liposomes such that the final concentration of rgD-2 equals that in the APL formulation, also exhibited 50% of the APL-mediated activity. Therefore, while liposome encapsulation of rgD-2 is essential, the unencapsulated rgD-2 in APL preparation must also have contributed to the total liposome mediated adjuvant activity.

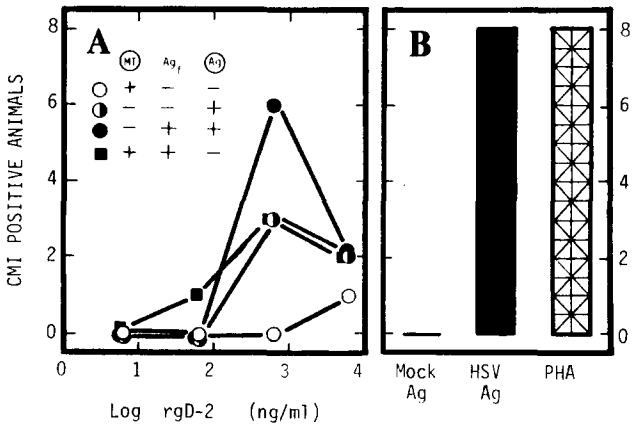

Fig. 2. Proliferation of guinea pig lymphocytes stimulated by various forms of rgD-2. Peripheral blood lymphocytes of HSV immune animals (90 days post-infection) were stimulated with rgD-2 in the APL formulation (●) (containing both free and liposome-encapsulated forms of rgD-2), liposome-encapsulated rgD-2 alone (◑), free rgD-2 added to empty liposomes (■) or empty liposomes (○). For liposome-encapsulated rgD-2, the actual rgD-2 concentration was 0.4 that of the indicated value. CMI positive animals are defined as animals with a stimulation index (SI) ≥ 3 (panel A). At 666 ng/ml (log 2.8) of rgD-2, the mean SI and standard error for rgD-2 in APL's = 4.4 ± 0.8 (N=8); liposome-encapsulated rgD-2 = 2.3 ± 0.4 (N=8); rgD-2 plus empty liposomes = 2.4 ± 0.8 (N=8); empty liposomes = 1.5 ± 0.1 (N=8). UV inactivated HSV antigen, mock antigen equivalent, and PHA were also tested in the same experiment as controls (panel B).

Efficacy of various physical forms of rgD-2 for controlling guinea pig recurrent genital HSV-2

Our previous report showed that intravenously infected rgD-1 in the APL formulation modified the clinical severity and also reduced the recurrence rate of genital HSV-2 by over 75% compared to control, untreated animals. This immunotherapeutically effective preparation of liposomes contained about 47% liposome encapsulated rgD-1 and 53% free rgD-1 (Ho et al., 1989). We employed the rgD-1 liposome preparation directly without separating free from liposome-encapsulated rgD-2 in the APL preparation, based on the idea that upon systemic administration, most of the liposome-encapsulated rgD-1 would be readily taken up by macrophages while soluble rgD would not be taken up as efficiently. As a result free rgD would be effectively separated from liposome encapsulated rgD in vivo. It is important to determine whether these two forms of antigen, free vs. liposome-encapsulated rgD, both contribute to the immunobiological effects responsible for control of HSV-2 recurrent disease in guinea pigs.

In the following experiments, we used rgD-2 instead of rgD-1 as our preliminary studies indicated that they exhibited similar efficacies in this animal treatment model (data not shown). As shown in Fig. 3, treatment of recurrent HSV disease with either liposome-encapsulated rgD-2 or with free rgD-2 administered with the empty liposome preparation was not as effective as the rgD-2 liposome (APL) preparation containing both free and encapsulated rgD-2 ($P < 0.05$ for lesion score at day 60, Fig. 3A; $P < 0.005$ for recurrent score at day 60, Fig. 3B). This result was apparent upon analyses of the daily lesion scores (a measure of the disease severity) (Fig. 3A), and the cumulative new recurrence rates (a measure of the disease frequency) (Fig. 3B). Although we cannot rule out a dose effect for the observed 67% relative efficacy for the purified liposome-encapsulated rgD-2 compared to rgD-2 in the APL formulation (5 μg vs. 12 μg/dose), if the liposome-encapsulated rgD-2 in APLs was the major active component, then it should exhibit about equivalent potency as 12 μg of liposome antigen mixture. Thus, the inability of purified, liposome-encapsulated rgD-2 (5 μg/dose) to afford an equivalent immunotherapeutic efficacy, and the partial treatment efficacy observed with the rgD-2 added to empty, preformed liposomes during the early recurrent phase suggest that free rgD-2 in the mixture also contributes to the therapeutic effects observed. After day 35, the rate of recurrences appeared to be the same for all treatment groups (Fig. 2B). This is inconsistent with the increase in both cellular and antibody responses seen 14 days after the second treatment (Table 2).

In order to understand the differences of these various rgD-2 preparations to stimulate HSV-specific immune responses, we determined the rgD-2 specific antibody titers and cell mediated immune (CMI) responses on the guinea pig blood samples collected on days 28 and 42 post-infection. CMI responses were evaluated using UV inactivated HSV antigen to stimulate peripheral blood lymphocytes (PBLs). As shown in Table 2, by day 28 post HSV-infection (14 days after 1st treatment), all animals treated with both the rgD-2 APL preparation (free and liposome-encapsulated rgD-2 mixture), or liposome encapsulated rgD-2 alone be-

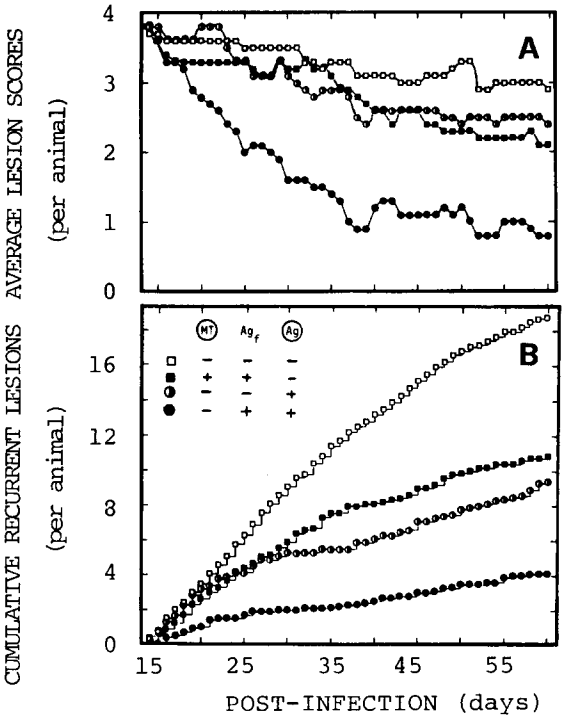

Fig. 3. Clinical efficacy of different physical forms of rgD-2 liposomes. After recovery from acute intra-vaginal HSV infection, guinea pigs were treated by systemic administration on day 14 and day 28 of rgD-2 in the APL formulation containing both free and liposome-encapsulated rgD-2 (●) (N=11), or liposome-encapsulated rgD-2 alone (◑) (N=8), or free rgD-2 added to empty liposomes (■) (N=9). As a control, animals in the placebo group were treated with saline (□) (N=10). Panel A shows the average daily lesion scores of the group, and panel B shows the group-average, cumulative recurrent lesion rates. Statistical analyses were done using two-tail t-distribution at day 60 to compare each treatment group with the placebo control and the results are summarized as follows:

TABLE 3

Statistical analysis of data in Fig. 3

Treatment	N	Avg lesion scores (panel A)			New recurrent lesions (panel B)		
		Avg	SD	P value	Avg	SD	P value
Placebo	10	2.9	0.9	–	18.9	8.6	–
rgD-2-liposome formulation	11	0.8	1.3	<0.005	4.1	2.6	<0.005
rgD-2 plus empty liposomes	9	2.1	1.5	0.03<P<0.05	10.8	5.5	0.01<P<0.03
rgD-2 liposomes w/o free rgD-2	8	2.4	1.4	0.15<P<0.2	7.4	4.2	0.005<P<0.01

came CMI-reactive. In contrast, only 67% of animals treated with rgD-2 added to empty liposomes showed HSV-specific CMI responses. Except for animals in the rgD-2 plus empty liposome group, a similar distribution was observed at day 42 post-infection. All animals treated with rgD-2 plus empty liposomes became CMI reactive by day 42, 14 days after second treatment. These data suggest that rgD-2 encapsulated in liposomes augments the development of CMI responses early in the course of therapy.

However, rgD-2 administered with empty, preformed liposomes appeared to be better in boosting rgD-2 antibody titers than other antigen preparations (Table 2). On days 28 the highest antibody titers were achieved in this treatment group ($P < 0.05$ compared to the other two treatment groups). At day 42 this group still had the highest antibody titers but the differences with other treatment group were not statistically significant. The rgD-2 in APL formulation and liposome-encapsulated rgD-2 alone elicited similar antibody titers even though a lower dose of total rgD-2 (0.4) was given for the liposome-encapsulated protein alone. Taken together, these data suggest that liposome-encapsulated fraction of rgD-2 was the major contributor to the enhancement of early (day 28) CMI responses while the free fraction of soluble rgD-2 presented together with liposomes was important for boosting the antibody responses observed in the treatment of guinea pig recurrent genital HSV-2.

Discussion

Based on our current understanding of antigen recognition, we have designed an antigen presenting vehicle consisting of liposomes plus adjuvant that may be efficiently taken up by antigen presenting cells, thus enhancing the presentation of a given antigen such as HSV recombinant rgD. The enhanced presentation mediated by these antigen presenting liposomes (APLs) was detected in vitro as an increased frequency of rgD-1 specific lymphocyte proliferation of guinea pig PBLs (Ho et al., 1989). Intravenous injection of these APLs to HSV-infected guinea pigs for immunotherapeutic treatment resulted in over a 75% reduction of the rate of recurrent HSV-2 (Ho et al., 1989).

In the initial APL preparations, soluble, unencapsulated rgD-1 was not separated from liposome-encapsulated rgD-1 as we assumed that the liposome-unencapsulated fraction of rgD-1 would be biologically inert. We have now further investigated the mechanism of APL immuno-enhancement. A detailed analysis of each form of rgD-2 in the APL formulation indicates that both the liposome encapsulated fraction as well as the free antigen fraction contribute to the observed efficacy both in vitro as well as in vivo. The liposome-dependent enhancement provided by the free rgD admixed to the empty liposomes was not due to nonspecific adsorption of rgD to the liposome membrane since even prolonged incubation of rgD with liposomes did not result in detectable association of the rgD to liposomes (Table 1). Although it is possible that both free rgD and empty liposomes in the mixture could be adsorbed by the same antigen presenting cell (i.e.

TABLE 2

Immune responses of guinea pigs enrolled in therapeutic treatments

Treatment	*N*	Day 28			Day 42		
		Anti-gD[a] titers	CMI		Anti-gD[a] titers	CMI	
			SI[b]	% Positive animal[c]		SI[b]	% Positive animal[c]
Placebo	10	25±6	20.2±20	60	29±13	8.6±11	60
rgD-2 liposome formulation	11	76±28	15.2±17	100	251±62	36.1±31	100
rgD-2 plus empty liposomes	9	165±36	20.7±24	67	300±115	39.3±37	100
rgD-2 liposomes without free rgD-2	8	69±25	34.5±26	100	205±48	19.2±15	100

[a]Anti-gD (IgG) antibody titers were determined by ELISA and expressed as geometric mean ± SE.
[b]SI, stimulation index to HSV antigen expressed as average ± SD for each treatment group.
[c]Animals with SI ⩾ 3 were considered to be positive CMI responses. The percent CMI positive animals was defined as the fraction of animals that exhibit positive CMI responses for each treatment group × 100.

a single macrophage) for subsequent antigen presentation in vitro, such events are expected to be rare in vivo. The systemically injected empty liposome and rgD mixture will probably be separated by the dynamic forces of the fluid flow in guinea pigs. This inference is supported by the fact that free rgD-2 admixed with empty liposomes elicited strong early antibody responses (day 28) while liposome-encapsulated rgD-2 enhanced early CMI responses in vivo (Table 2). These results indicate that the free rgD in the rgD-2 liposome mixture may be involved preferentially in B cell stimulation with less contribution to the CMI responses (Table 2). Liposome-encapsulated rgD-2 was not as efficient in boosting antibody responses as free rgD plus empty liposomes although it enhanced CMI responses as effectively as the rgD-2 in the APL formulation which contains both forms of rgD-2 (Table 2). Therefore, it is probable that liposome-encapsulated rgD-2 in the original rgD-2 liposome formulation enhances CMI responses while the free, soluble fraction of rgD in the mixture boosts the Ig responses. The additional boosting of antibody responses observed with the empty liposomes plus rgD mixture (Table 2) may be due to the higher amount of antigen given per dose. Regardless, these data suggest that rgD-2 formulated in an APL preparation enhances both cellular and humoral immune responses and that both liposome-encapsulated and unencapsulated fractions of rgD-2, contribute to the observed immunotherapeutic effects on recurrent HSV-2 genitalis in guinea pigs.

The exact mechanism of liposome-mediated immune enhancement is not clear at present. It is likely that a significant amount of systemically injected, liposome-encapsulated rgD-2 will be taken up by the antigen presenting cells (APCs) (i.e. macrophages) in circulation as these liposomes have been shown by others to be

mainly taken up by the mononuclear phagocyte systems including circulating macrophages (Post et al., 1985). At the same time, the soluble rgD in circulation may be bound to the B cells expressing the proper receptor. The rgD taken up by the APCs can then present the antigen to T_h cells, an essential step for the activation of the cellular immune cascade. It is probable that the liposome may mediate its effects by enhancing the loading efficiency of the APC. It has been demonstrated that delivery of a soluble molecule in a liposome-encapsulated form, through the IV route, improves the loading efficiency of such molecules to macrophages by 10–100-fold (Post et al., 1985).

Additional immune enhancement mediated by the APL formulation is probably due to the adjuvant activity of MTP-PE. As a lipophilic derivative of muramyl dipeptide which is the smallest component of bacterial lipopolysaccharide required to stimulate both cellular and humoral immune responses, MTP-PE was shown in our previous report to be essential to obtain immunological enhancement in treating HSV-2 recurrent disease of guinea pigs (Ho et al., 1989). Deletion of MTP-PE in the formulation abrogated the liposome-mediated therapeutic effects indicating that improving APC uptake alone is not sufficient to suppress the recurrent disease of genital HSV-2 (Ho et al., 1989). At present, however, it is not clear how the mixture of empty MTP-PE liposomes plus soluble rgD exerts its effects on rgD specific humoral response.

In the previous report, we showed that APL mediated rgD specific enhancement of CMI responses seen in vitro paralleled the immunotherapeutic efficacy observed upon treatment of guinea pigs for recurrent genital HSV-2 disease (Ho et al., 1989). Similarly, the relative biological potency of different forms of rgD in the rgD liposome preparation determined in vitro using lymphocyte stimulation assays (Fig. 2) appears to predict the outcome of animal experiment (Fig. 3). Therefore, if this correlation is confirmed by future experiments, the lymphocyte proliferative assay may be used as a preliminary indicator to aid in selecting APL formulations to be tested in the therapeutic animal model system.

In addition to determining the mechanism of actions, the APL formulations must be optimized for clinical use. We used MTP-PE as a convenient lipophilic immune stimulant of APC that can easily be incorporated in the liposome formulation. The use of other immune stimulants such as IL-1, tuftsin, etc. remain to be tested. In addition, the optimum combination of lipid, protein, and adjuvant also remain to be determined. Whether the same approach can be used for other HSV antigens such as gB is currently under our investigation. We are also comparing alternate routes of APL administration with the present systemic route of administration.

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