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In Vivo Induction of Anti-Herpes Simplex Virus Immune Response by Type 1 Antigens and Lipid A Incorporated into Liposomes

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To establish the requirements for a potential subunit vaccine against herpes simplex virus (HSV), we analyzed the effects on immunogenicity of incorporating detergent-extracted glycoprotein-enriched HSV type 1 (HSV-1) antigens into liposomes alone or with the adjuvant lipid A. Incorporating HSV-1 antigens into liposomes enhanced their immunogenicity for antibody production as detected by radioimmunoassay. Antibody levels to free and liposome-bound antigens were enhanced by administering lipid A as an adjuvant. The maximum immunogenic effect was obtained by incorporating lipid A into liposomes containing the HSV-1 proteins. Such liposomes induced secondary antibody responses higher than those engendered by virus infection. Whereas infectious virus induced cell-mediated immunity detectable by the delayed-type hypersensitivity reactions and cytotoxic T lymphocyte production, none of the liposome preparations induced cell-mediated immunity.

The trend in vaccine development is to identify the component antigens of viruses and bacteria that are responsible for stimulating protective immune responses. Usually these nonreplicating subunit vaccines are poor immunogens and require administration with an appropriate immunopotentiator, especially if economically acceptable amounts of antigen are to be used. The candidate components for inclusion in subunit vaccines for use against herpes simplex virus (HSV) are one or more of the five glycoproteins found in the viral envelope and which are the only viral antigens found at the cell surface of infected cells (27). By in vitro approaches we have shown that antigen preparations enriched for HSV glycoproteins are poor immunogens but their immunogenicity can be markedly improved by incorporation into liposomes (17). Furthermore, studies with hepatitis B viral components and several nonviral antigens (9, 21, 31; E. K. Manesis, C. H. Cameron, and G. Gregoriadis, Fed. Proc. **102**:107, 1979) have shown that incorporating these antigens into liposomes leads to an enchanced immune response in vivo. It has also been shown that the additional incorporation of an adjuvant, such as lipid A, into the antigen-containing liposome further enhances the immune response against the antigen (8, 32). In this communication we evaluate the use of liposomes to improve the in vivo immune response against HSV antigens.

MATERIALS AND METHODS

Viral antigens. HEp-2 cells grown in Dulbecco medium with 5% donor calf serum (Flow Laboratories, McLean, Va.) in roller bottles were infected with HSV type 1 KOS (HSV-1) at a multiplicity of infection of 1. The virus stocks were grown in human embryonic lung cells (MRC5), as previously described by Bone and Courtney (4), and virus growth was allowed to continue for four viral generations (48 h). Tritium-labeled glucosamine hydrochloride (Amersham Corp., Arlington Heights, Ill.) (specific activity, 38 Ci/mmol) was added to the infected cells at 4 h postinfection to label glycoproteins. After 48 h, the infected cell medium was collected, and whole cells and large debris were removed by centrifugation at 2,000 × *g* for 15 min. The clarified cell medium was then centrifuged for 1 h at 100,000 × *g* to pellet virus particles. The viral pellet was suspended in a small volume of reticulocyte standard buffer, applied to a linear sucrose gradient (20 to 60%), and centrifuged at 100,000 × *g* for 12 h at 4°C. After centrifugation, the gradient was fractionated into 0.5-ml fractions, and portions were monitored for radioactivity. The virus-containing fractions were pooled and diluted 10-fold with phosphate-buffered saline (PBS) (pH 7.4), and the virus particles were pelleted by centrifugation at 100,000 × *g* for 1 h at 4°C. The resulting viral pellet was suspended in a small volume of PBS and mixed with enough 5% deoxycholate (DOC) to produce a final DOC concentration of 2%. Virus-DOC mixtures were allowed to incubate for 1 h at room temperature with continual agitation, after which the DOC-insoluble material was removed by centrifugation at 100,000 × *g* for 1 h at 4°C. The DOC-solubilized material was checked for viral proteins by

sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography (17). The type of gel profile obtained for such preparations was published previously (17) and routinely shows predominantly virion glycoproteins, having molecular weights of approximately 58,000, 88,000, and 120,000. The protein concentration was determined by the method of Lowry et al. (19).

Lipid A. The lipid A used in this study was a generous gift from David Morrison, Emory University, Atlanta, Ga. It was extracted from *Salmonella minnesota* R595, as described by Galanos et al. (10) with modifications of Skelley et al. (27), yielding a glycolipid containing the lipid A moiety and the trisaccharide 2-keto-3-deoxyoctosonate (KDO), but lacking both the terminal portion of the core polysaccharide and the entire O-antigen oligosaccharide (2, 27). The lipid A was suspended in PBS at 800 μg/ml and stored at −20°C until use. Lipid A was quantitated by the thiobarbituric acid used to detect KDO (KDO assay) (7).

KDO assay. Samples of lipid A or liposomes containing lipid A, approximately 200 μl, were first treated with 10 μl of 0.4 N H_2SO_4 and heated for 8 min at 100°C. The treated samples were allowed to cool to room temperature before the addition of 200 μl of 25 mM sodium periodate in 0.125 N H_2SO_4. The samples were incubated for 20 min at room temperature followed by the addition of 400 μl of 2% sodium arsonite in 0.5 N HCl with rapid mixing. Subsequently, 1.6 ml of 0.3% 2-thiobarbituric acid was added, and the sample was heated for 12 min at 100°C. Quantitation was achieved by monitoring samples for optical density at 550 nm and comparing the liposome-lipid A preparations to the standard curve obtained for lipid A. This standard curve gave an optical density at 550 nm of 0.009/mg of lipid A.

Plasma membrane isolation. Plasma membranes were isolated from RDM-4 cells grown in McCoy 5A plus 10% donor calf serum. The cells were harvested by centrifugation at 2,000 × *g* for 2 min. The cells were then washed three times in PBS, finally suspended in cold 1/10 PBS to allow osmotic swelling, and incubated on ice for 10 min. The swollen cells were then disrupted by homogenization in a 15-ml Dounce homogenizer, using 15 strokes and monitoring disruption under the microscope. The disrupted cells were then centrifuged for 30 s at 2,000 × *g* to remove whole cells, nuclei, and large debris, after which the resulting supernatant was layered over a linear 20 to 60% sucrose gradient and centrifuged at 100,000 × *g* for 14 h at 4°C. Those fractions containing plasma membrane (38 to 42% sucrose) were collected, and the membranes were washed twice with PBS by centrifugation. The final membrane pellet was suspended in PBS containing 1 μM phenylmethylsulfonyl fluoride at 1 to 3 mg of protein per ml and stored at −70°C less than a month. The protein concentration was determined by the method of Lowry et al.

Lipids. Phosphatidylcholine (PC) used in this study was purified from egg yolk (13). [^{14}C]PC was purchased from Amersham at 114 mCi/mmol. Cholesterol (chol) was purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation of liposomes. PC and chol dissolved in $CHCl_3$ at a mole ratio of 7:2 (PC/chol) were divided equally among test tubes, evaporated to dryness under N_2 gas, and vacuum-desiccated for 30 min. The dried lipids were suspended in PBS by bath sonication to a final concentration of 20 mg of lipid per ml. The suspended lipids were subsequently probe-sonified to clarity with a Heat Systems sonicator (model W-375) at setting 2.5. Liposomes used in assessing HSV-1 proteins and lipid A incorporation were prepared with tracer [^{14}C]PC added to the original lipid mixture (126 μCi/mmol) before drying to serve as the lipid marker. The sonicated lipids were then mixed with appropriate quantities of HSV-1 proteins in 2% DOC, lipid A, 5% DOC, and PBS to give a final solution of lipid, HSV-1 proteins, and lipid A at a lipid-to-protein ratio of 5:1 (wt/wt) and a final DOC concentration of 1%. The lipid, HSV-1 proteins, lipid A, and detergent mixture was then dialyzed for 72 h against PBS (pH 7.4) containing 2.0 μM phenylmethylsulfonyl fluoride and 0.02% sodium azide, yielding oligolamellar liposomes containing lipid A and the HSV-1 antigens (Fig 1).

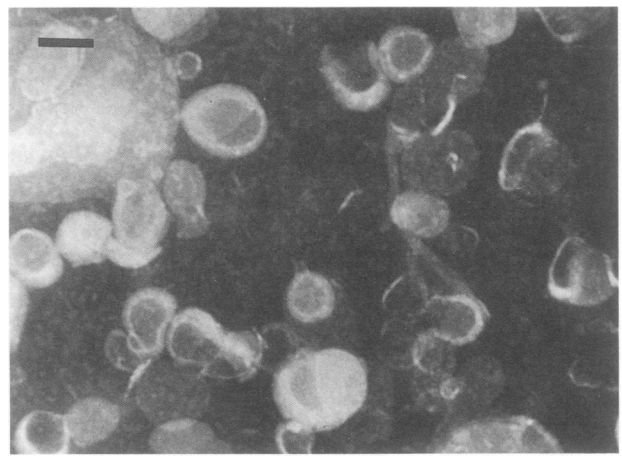

FIG. 1. Electron micrograph of PC/chol (7:2 mole ratio) liposomes containing HSV-1 antigens and lipid A. Bar, 77 nm.

Liposomes containing HSV-1 antigens alone or lipid A alone or containing RDM-4 plasma membranes were also prepared in the same manner.

Electron microscopy. One drop of liposome suspension was placed onto a 400-mesh grid previously coated with 0.5% Formvar and carbon and allowed to settle on the grid for 2 min. Excess liposome solution was then blotted away with a filter paper. Immediately, a drop of 1% phosphotungstic acid solution (pH 7.6) was placed on the grid and allowed to stain for 2 min. The excess stain was removed, and the grid was air-dried before viewing in a Hitachi H-600 electron microscope.

Analysis of liposome composition. To insure that the lipid A and HSV-1 antigens were incorporated into the liposomes, sucrose density gradient centrifugation of liposomes was carried out. Briefly, 500 μl of liposomes or liposomal components was placed on a 5-ml linear 5 to 20% sucrose gradient and centrifuged for 5 h at 200,000 × *g* in a Beckman SW50.1 rotor. The gradients were fractionated, and all fractions were monitored for ^{3}H-labeled HSV-1 proteins, [^{14}C]PC, and lipid A.

Immunization and collection of sera. Young female C3H/HeJ mice were obtained from the University of Tennessee Memorial Research Center. BALB/c and C3H/Anf mice were purchased from Cumberland View Farms, Clinton, Tenn. Mice were injected intraperitoneally, unless otherwise indicated, with appropriate inocula on day 1. In all cases where the mice were to receive two types of inocula, they received two simultaneous but separate injections. On day 10, the mice were bled from the retro-orbital sinus. This first bleeding yielded serum that we refer to as primary (1°) serum, and it was tested for antibody activity as described below. The bled mice were subsequently reinoculated with appropriate antigens on the same day. On day 20, the mice were bled again via the other retro-orbital sinus, to provide sera to test for secondary (2°) antibody responses. These mice were then subjected to the delayed-type hypersensitivity test on day 21. On day 22 the mice were sacrificed, and their spleens were removed and prepared for in vitro culturing as previously described (18).

Antibody determinations. To measure antibodies, we used a modification of the solid-phase radioimmunoassay (RIA) described by Zollinger et al. (33). In brief, HSV-1 viral stocks, grown in HEp-2 cells, were diluted to 10^{7} PFU/ml with distilled water. The diluted virus stock was used to coat the wells of a plastic microtiter plate (Cooke Engineering Co., Alexandria, Va.), which served as the immobilized antigen source. Each microtiter well was partially filled with 0.1 ml of the diluted virus and incubated for 1 h at 37°C to allow virus adsorption to the plate. After the incubation period the unbound virus was washed from the wells with five washes of PBS containing 2% Tween 20 and 1 mg of bovine serum albumin per ml (PBS-Tween). Sera to be tested for anti-HSV-1 antibodies were serially diluted with PBS-Tween, and 0.1-ml samples of each dilution were placed in different wells of HSV-1-bound microtiter plates. The plates were then incubated for 2 h at 4°C. After the incubation period, the plates were washed five times with PBS-Tween to remove any unbound antibody. The antigen-antibody complexes were then detected by adding ^{125}I-labeled protein A (Zymed Labs) to each well. The reagent was labeled by the chloramine-T method (15) with ^{125}I (5 to 9 mCi/mg). After incubation for 2 h at 4°C, the unbound material was removed by five washes with PBS-Tween. The wells from each plate were then cut out and counted in a gamma counter to assess the amount of protein A bound in each well. Each dilution was tested in triplicate, and the data were plotted as radioactivity in counts per minute versus antiserum dilution. The midpoint of the descending linear portion of the curve was identified. RIA units were calculated by multiplying the counts per minute by the dilution factor at this midpoint (1). The RIA results will depend upon several factors, including the specific activity of the label, the antigen preparation used, and other factors. Therefore, the RIA units enumerated in Table 1 are not absolute values and should not be used for comparison between different assays. However, these values can be compared within a given assay to reflect the relative antibody titers of different antisera, as has been done in Table 1.

An identical protocol was used to test sera for antilipid antibodies and anti-HEp-2 cell antibodies, except that in the antilipid antibody test, the microtiter plates were allowed to adsorb 50 μg of PC per well instead of virus stock. This amount of lipid is estimated to be 10 times the amount of the PC present in the virus stock. The anti-HEp-2 cell antibody test was done by first adsorbing detergent-solubilized extracts of noninfected cells to the microtiter plates in identical quantities as in the virus stock.

Cytotoxicity assay. The assay for cytotoxic T lymphocytes (CTL) was carried out as previously described (18). Spleen cells were cultured for 5 days in vitro with 10^{7} PFU of HSV-1 to induce the secondary CTL. Cells were assayed with syngeneic and allogeneic virus-infected and uninfected target cells at effector-to-target-cell ratios of 100:1, and the results are reported as percent specific lysis.

Measurement of DTH. The protocol employed for the measurement of delayed-type hypersensitivity (DTH) was a modification of the method reported by Crowle (6). In brief, mice were inoculated with liposomes or virus as previously mentioned and after their last bleeding were challenged in the right hind footpad with 0.025 ml of HSV-1 (1×10^{7} PFU) and in the left hind footpad with Hanks balanced salt solution. Control mice received Hanks balanced salt solution in both footpads. The thickness of the footpads was measured with calipers 24 h after challenge. The DTH response was expressed as a percentage of footpad increase at 24 h. Significance was determined by the Student *t*-test at 95% confidence limits.

RESULTS

Incorporation of lipid A and HSV-1 antigens into liposomes. To ascertain whether the HSV-1 antigens and lipid A were incorporated into the liposomes, the liposomes and the separate liposomal components were centrifuged through linear sucrose gradients, and the distribution profile was analyzed. The data in Fig. 2A indicate that there were two types of liposome populations in this preparation and that the HSV-1 antigens and lipid A were incorporated into these liposomes. The bulk of the HSV-1 antigens

TABLE 1. Induction of anti-herpesvirus immune responses by HSV-1 antigens: dependence on form of antigen presentation and the adjuvant lipid A

Inoculum[a] (amt)	Antibody titer[b] (RIA units × 10^{-3})		DTH	2° CTL (% specific ^{51}Cr release)[c]
	1°	2°		
HSV[d] Ag (5 μg)	0	7.6	−	0
HSV Ag (1 μg)	0	0	−	0
HSV Ag (0.2 μg)	0	0	−	0
PC/chol[e]-HSV Ag (5 μg)	0	24.5	−	0
PC/chol-HSV Ag (1 μg)	0	5.8	−	0
PC/chol-HSV Ag (0.2 μg)	0	2.7	−	0
HSV Ag (5 μg) + lipid A (2 μg)	0	122.8	−	0
HSV Ag (1 μg) + lipid A (2 μg)	0	20.0	−	0
HSV Ag (0.2 μg) + lipid A (2 μg)	0	0	−	0
PC/chol-HSV Ag (5 μg) + lipid A (2 μg)	6.0	622.8	−	0
PC/chol-HSV Ag (1 μg) + lipid A (2 μg)	4.7	312.0	−	0
PC/chol-HSV Ag (0.2 μg) + lipid A (2 μg)	0	142.0	−	0
PC/chol-HSV Ag (5 μg)-lipid A (2 μg)	7.1	2,320.0	−	0
PC/chol-HSV Ag (1 μg)-lipid A (2 μg)	4.2	703.0	−	0
PC/chol-HSV Ag (0.2 μg)-lipid A (2 μg)	0	240.0	−	0
HSV-1 HI[f] (10^7 PFU)	6.1	129.0	−	0
HSV-1 (10^7 PFU)	33.6	880.0	+	70

[a] BALB/c mice were injected intraperitoneally with 200 μl of inoculum on day 1, bled 10 days later and reinoculated, and bled 10 days later and tested for antibody titer, DTH, and CTL activity. Each experimental group consisted of six mice. Ag, Antigen.

[b] Selected sera with high titer were also tested against cells sensitized with HEp-2 cell antigens, the cell type in which the HSV-1 virus was produced. None of the sera bound. Sera also failed to bind to wells sensitized with PC.

[c] Secondary CTL directed against HSV-1 virus were assayed against HSV-1-infected L-cells and A-31 cells, noninfected L-cells, and A-31 cells as targets. Only the results for HSV-1-infected A-31 cells are reported, as all other control target cells were not killed under any conditions tested.

[d] Detergent-extracted HSV-1 virus was dialyzed to remove detergent forming aggregates of antigen.

[e] Mole ratio, 7:2.

[f] Virus was heat-inactivated (HI) by heating for 1 h at 56°C.

and lipid A appeared in the faster-migrating peak, which contained approximately 80 to 85% of the initial material. The appearance of these two liposome peaks reflects the heterogeneity of this preparation, as seen in Fig. 1. The preparation contains both oligolamellar and unilamellar liposomes, with the oligolamellar ones being greater in number. The remaining panels in Fig. 2 show that both HSV-1 (Fig. 2B) and lipid A (Fig. 2C) incorporate into liposomes by themselves with a slightly slower-migrating profile than the liposomes containing both components. Figure 2D through F shows that the individual liposomal components band quite differently by themselves as opposed to the banding observed when they are incorporated into liposomes. In Fig. 2E (HSV-1) and Fig. 2F (lipid A), both liposomal components smear across the bottom of the gradient, most likely as a function of aggregate size. Figure 2D shows that liposomes made of lipid alone float on top of the gradient and only upon incorporation of lipid A or HSV-1 antigen will they migrate into the gradient.

Induction of immune responses. To assess the adjuvant effect of liposomes and lipid A, experiments were performed to compare the anti-HSV-1 immune responses in groups of BALB/c mice injected with various doses of free viral antigens or the same quantity of antigens incorporated into liposomes. Experiments were also done with free and liposome-incorporated HSV-1 antigens in the presence of free or liposome-incorporated lipid A. The results of these experiments are shown in Table 1. All sera shown in Table 1 were assayed for antibody concurrently. This table shows that although free HSV-1 protein aggregates were not very immunogenic by themselves, their immunogenicity was enhanced at least threefold by incorporating the antigens into liposomal bilayers. It can also be seen that the addition of the adjuvant, lipid A, to the free antigens stimulated even higher antibody titers than those induced by the liposome-incorporated viral antigens. Five micrograms of free viral protein plus 2 μg of lipid A gave approximately a 16-fold higher antibody titer than free viral protein alone and a fivefold higher response than the liposome-incorporated HSV-1 antigens. However, still higher responses occurred when the HSV-1 antigens were incorporated into lipo-

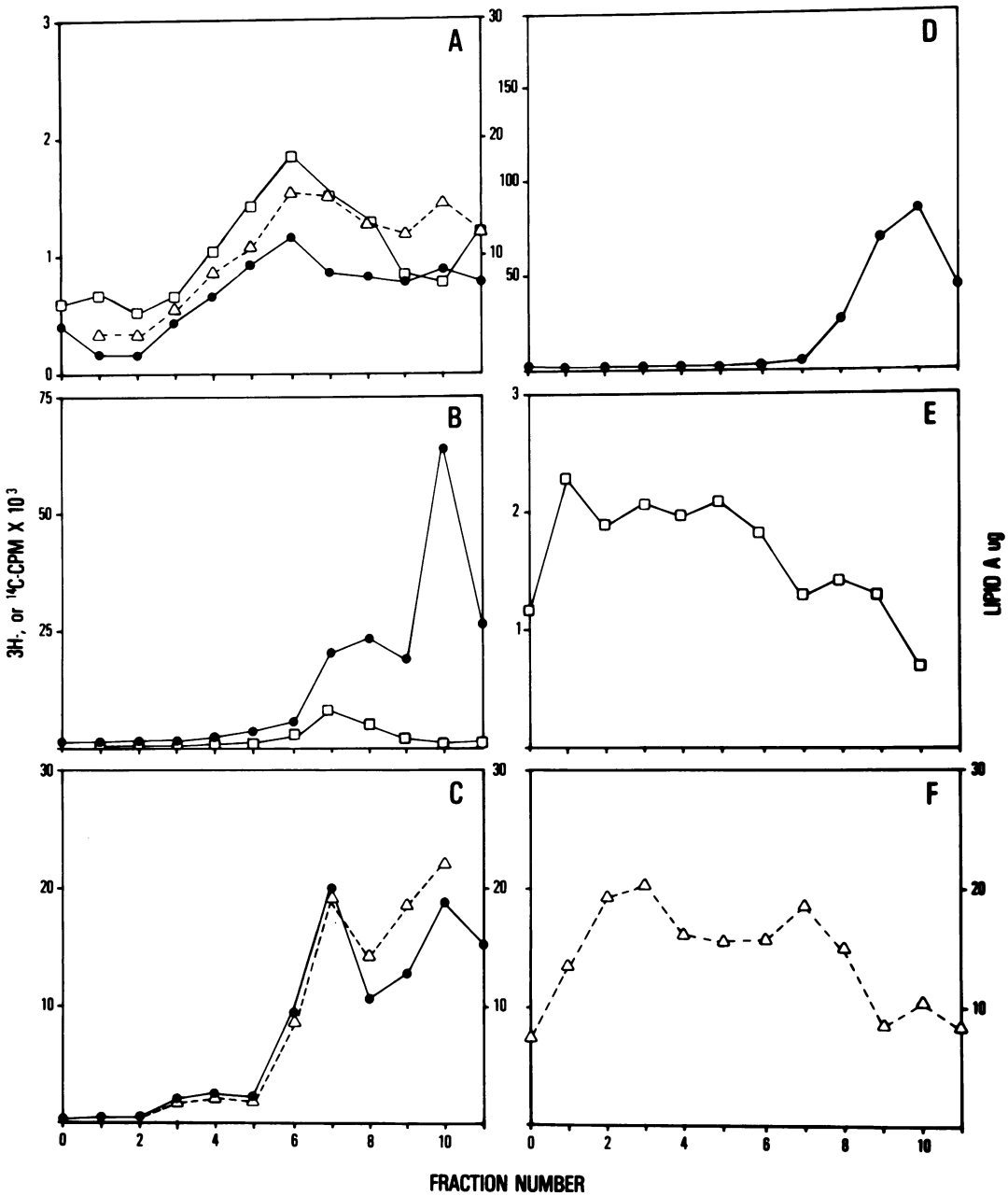

FIG. 2. Sucrose gradient profile of liposome-incorporated HSV-1 antigens, lipid A, and nonincorporated HSV-1 antigens and lipid A. (A) Liposomes containing HSV-1 antigens and lipid A. (B) Liposomes containing HSV-1 antigens. (C) Liposomes containing lipid A. (D) PC/chol liposomes only. (E) Nonincorporated HSV-1 antigens. (F) Nonincorporated lipid A. HSV-1 antigens were labeled with ^{3}H (○); lipids were labeled with [^{14}C]PC (●). Lipid A is expressed in micrograms (△).

somes and free lipid A was added as an adjuvant. This last combination gave a fivefold higher titer than that achieved by the same doses of free viral antigens and lipid A. With 5 μg of HSV-1 antigens incorporated into liposomes and 2 μg of free lipid A, we observed an 80-fold increase in antibody titer over that induced by the same dose of free HSV-1 antigens alone and a 25-fold increase over liposome-incorporated antigens. The maximum immunogenic effect oc-

curred when both HSV-1 antigens and lipid A were incorporated in the same liposomes. Such liposomes were up to fourfold more immunogenic than liposome-incorporated antigens and free lipid A and produced antibody titers 305-fold higher than those induced by free viral proteins. The antibody level induced by 5 μg of antigen and 2 μg of lipid A incorporated in the same liposomes was approximately threefold higher than that achieved by live virus. When higher doses of lipid A were used (>8 μg), there was no additional immunogenic effect noted by incorporating lipid A into the same liposomes as the viral proteins. A pattern of results similar to that depicted in Table 1 was obtained in separate experiments in BALB/c mice and one experiment in C3H/Anf mice. However, lipid A did not enhance the immunogenicity of liposomes in lipid A-unresponsive C3H/HeJ mice (data not shown).

Whereas the liposome antigens induced good antibody responses, no combination of HSV-1 antigens and lipid A induced a cell-mediated immune response detectable by either the DTH reaction or sensitization for secondary in vitro induction of CTL (Table 1). This was true even at doses of viral proteins and lipid A of 100 and 40 μg, respectively (data not shown). Furthermore, injecting the antigens by other routes, such as intramuscularly and subcutaneously, which had little effect on levels of antibody, failed to elicit cell-mediated immunity.

The inability of the protein-liposome preparation to induce a cellular immune response was further examined with liposomes containing viral proteins and plasma membrane antigens syngeneic with the immunized mouse. This was done since in previous studies on the induction of CTL responses in vitro, we showed that liposomes containing HSV-1 antigens and syngeneic H-2 antigens could induce responses, whereas liposomes containing viral antigen alone or viral antigen plus allogeneic H-2 antigen could not (17).

Groups of C3H mice were injected on two occasions at 14-day intervals with either viral protein liposomes (5 to 40 μg) or liposomes with both viral antigens and plasma membrane protein (75 μg) from H-2^k cells (RDM-4). Such liposomes were the same as those shown previously to be immunogenic in vitro (17). Both HSV-1 antigen alone and HSV-1 antigen plus H-2^k liposomes induced a primary and secondary antibody response, with the secondary titers approximating those induced in control groups of mice infected with live virus. Unlike the live virus, the liposomes failed to induce a cell-mediated immune response (data not shown). The incorporation of the H-2^k protein into the viral protein liposome did not enhance levels of antibody production over those induced by viral protein-incorporated liposomes (data not shown).

Others have reported that liposomes containing lipid A at high levels (1.5 mg) can induce anti-PC antibodies and thus could perhaps precipitate some autoimmune complication (26). We assayed sera with high anti-HSV titers from those mice that received lipid A against wells sensitized with PC. Such antibodies were not detectable.

DISCUSSION

Our data show that the immunogenicity of HSV antigens can be improved by incorporating them into liposomes. With such liposome antigens it was possible to induce antibody responses at doses of viral antigens which alone were nonimmunogenic. Incorporating antigens into liposomes has been shown to enhance immunogenicity, at least for antibody production, in numerous systems (9, 21, 31, 33), including viral subunit antigens (11, 16, 20, 24). With hepatitis B antigen, the viral antigen most intensively investigated (11), liposomes could enhance immunogenicity some 750-fold, a much higher level of enhancement than that observed in our studies. However, as indicated by the elegant studies of Kinsky's group using liposomes containing the synthetic hapten mono(azophenylphosphorylcholine)hydroxyphenylpropionyl, many biophysical characteristics of the liposome can markedly influence their immunogenicity (3, 15). In our study, only one type of liposome was used, namely, neutrally charged small oligolamellar liposomes composed of a 7:2 ratio of PC and chol. It therefore remains likely that varying the biophysical properties of the HSV-1-antigen-containing liposome could improve their immunogenicity. Such studies are currently under way in our laboratory.

A notable result of our investigation was the observation that low concentrations of lipid A markedly improved the levels of antibody induced by glycoprotein-enriched HSV-1 antigens. This enhancement was greater with viral protein-incorporated liposomes. Thus the antibody levels in mice receiving viral protein-liposomes plus lipid A were significantly ($P < 0.01$) higher than those which received the same dose of free antigen plus lipid A. The strongest adjuvant effect of lipid A was noted when it was incorporated into the same liposome bilayers as the HSV-1 antigens. Such liposomes induced antibody levels as much as 300-fold higher than those induced by free antigens and about threefold higher than levels induced by viral protein-liposomes plus free lipid A. That lipid A was maximally effective when incorporated into the same liposomal bilayers as the antigen was also

noted by Dancey et al. (8). Indeed, in their studies it was necessary to incorporate lipid A into the same bilayers to obtain an adjuvant effect. This was not our experience with herpesvirus protein antigens, and at higher lipid A concentrations (above 8 μg), the adjuvant effects of free and liposome-incorporated lipid A were similar. Those comparisons were made with doses of HSV-1 antigens that were immunogenic alone. One possible explanation for our findings is that above certain doses lipid A need not have been incorporated into the liposome since close to maximal antibody levels were being induced by both free and liposome-incorporated lipid A at these high levels. Antibody levels produced by the liposome with HSV-1 antigens and lipid A were even higher than those induced by infection with live HSV-1.

It is commonly supposed that immunity to herpesviruses, particularly recovery from infection, involves to a large extent the T-cell immune system (3, 22). Consequently, for subunit herpesvirus antigens to act as effective immunogens, they should be potent stimulators of T-cell immunity. Unfortunately, none of our subunit liposome-incorporated antigens proved immunogenic for T-cells, as judged by two assays of cell-mediated immunity, the DTH responses and stimulation of CTL precursors that could be expanded to become CTL upon secondary restimulation in vitro (18). Infection with live HSV-1 stimulated both cell-mediated immunity parameters (Table 1) although, as reported previously, intact virus inactivated by heat failed to do so (18). Since our assays of antibody production, using protein A to detect binding, mainly detected immunoglobulin G, an antibody class considered thymus dependent (29), the liposome antigens most presumably have stimulated some subsets of T-cells. A failure of liposomal antigens to stimulate detectable cell-mediated responses has been noted in other systems (S. Burakoff and M. Mescher, Cell Surface Rev., in press), but a mechanistic explanation for the failure has not been forthcoming. Certain in vitro studies, including our own with HSV-1 antigens (23, 25), indicate that the poor immunogenicity could lie with a failure to stimulate adequate levels of interleukin 2, a lymphokine produced by helper cells and which is involved in triggering the effector phase of cell-mediated immune responses (30). For example, the poor immunogenicity of liposomal antigens could be overcome by adding extraneous sources of interleukin 2 to the antigen-stimulated cultures (12; Burakoff and Mescher, in press). With HSV-1 antigen preparations, immunogenicity for CTL production can also be improved by the addition of extraneous interleukin 2 (25). Whether or not interleukin 2 production is the limiting step in vivo and the reason that inactivated viral antigens produce little or no cell-mediated immune response is worthy of further investigation.

In conclusion, our studies indicate that herpesvirus protein immunogenicity can be enhanced by presenting antigen in the form of a liposome. Additionally, incorporating an adjuvant, lipid A, leads to still higher levels of antibody production, although the preparations still remain unable to stimulate detectable cell-mediated immunity. Perhaps incorporation in liposomes of potent adjuvants for cellular responses, such as the muramyl dipeptide adjuvants described by Chedid et al. (5), could stimulate adequate levels of cell-mediated immunity.

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