

To the Graduate Council:

I am submitting herewith a dissertation written by Paul Thomas Naylor, entitled "Induction of Immune Responses to Herpes Simplex Virus With Liposomes Containing Viral Antigens". I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

Leaf Huang
Leaf Huang, Major Professor

We have read this dissertation
and recommend its acceptance:

Robert E. Bryant
Joe E. Chalkley
Richard J. Gentry

Accepted for the Council

L. Evans Gosh
Vice-Chancellor
Graduate Studies and Research

INDUCTION OF IMMUNE RESPONSES TO HERPES SIMPLEX VIRUS WITH
LIPOSOMES CONTAINING VIRAL ANTIGENS

A Dissertation

Presented For the
Doctor of Philosophy
Degree

The University of Tennessee, Knoxville

Paul Thomas Naylor

December 1981

3055578

DEDICATION

This manuscript is dedicated to Bernadette King. I would like to thank her for all the help and inspiration she has given me and I will always remember all those late nights we spent in the laboratory. Your kindness and generosity will always be appreciated.

ACKNOWLEDGEMENTS

I would like to thank my committee members, Drs. Leaf Huang, Richard Courtney, Jorge Churchich and Robert Bryant for all their time and helpful discussions. I would like to thank Dr. Barry Rouse for his help and guidance because if it were not for him this dissertation would not be. I would also like to give a special note of appreciation to Leaf Huang for giving me the opportunity to work in his laboratory and for all the guidance he has given me both on scientific and nonscientific matters. Leaf is not only a terrific boss but also a good friend. Last but not least, I would like to express my gratitude to all my lab mates for the comradery and good times we shared. Those will be times that I will never forget and I wish you all luck in your future endeavors.

ABSTRACT

The immunogenicity of detergent extracted herpes simplex virus type 1 (HSV-1) antigens incorporated into liposomes were measured by their ability to induce in vitro secondary anti-HSV-1 specific cytotoxic T lymphocyte (CTL) responses in splenocyte cultures from virus-primed mice or in vivo immune responses in mice.

First, in vitro secondary virus specific CTL responses could be induced provided the liposomes contained viral antigens along with plasma membrane antigens of the same H-2 haplotype as that of the virus primed responder cells. Responses did not occur in cultures stimulated with liposomes containing only viral antigens or with a mixture of liposomes composed respectively of lipid and viral antigens and those composed of lipid and plasma membrane antigens. Thus, it is essential that both the viral and H-2 antigens be incorporated into the same liposome for the induction process to occur.

The ability of these incorporated antigens to induce a CTL response was also dependent on the type of exogenous lipid used to make the liposomes. It was found that a phosphatidylcholine:cholesterol mixture, 7:2 mole ratio, gave the best response. It was also found that once either the viral glycoproteins on the H-2K^k antigen were purified and subsequently incorporated into liposomes with plasma membrane antigens or detergent extracted viral antigens respectively these liposome lost the ability to induce a CTL response. The reason for this loss of induction capability is discussed in the ensuing chapters.

Secondly, liposome incorporated HSV-1 antigens could induce an antibody response in mice whereas free detergent extracted antigens could not. This in vivo response could be further enhanced by incorporating the B-cell adjuvant,

lipid A into the same liposomes as the viral antigens. The induction was also found to be dependent on the dose of lipid A and HSV-I antigens given. Although these liposomes induce a humoral response that is as good or better than that achieved by live virus they failed to induce any cellular responses.

TABLE OF CONTENTS

CHAPTER	PAGE
I. LITERATURE REVIEW	I
A. INTRODUCTION	I
B. HERPESVIRUS	2
C. LIPOSOMES	3
D. LIPOSOMES AND IMMUNOBIOLOGY	5
Humoral Response	5
Cellular Response	7
II. CELL MEDIATED IMMUNITY TO HERPES SIMPLEX VIRUS: INDUCTION OF CYTOTOXIC T LYMPHOCYTE RESPONSES BY VIRAL ANTIGENS INCORPORATED INTO LIPOSOMES	10
A. INTRODUCTION	10
B. MATERIALS AND METHODS	11
Cells and Virus	11
Mice	11
Mouse Immunization and Preparation of Spleen Cell Cultures for <u>In Vitro</u> Immunization with HSV-I or Liposomes	12
<u>In Vitro</u> Immunization of Spleen Cell Cultures with HSV-I or Liposomes	12
Cytotoxicity Assay	13
Plasma Membrane Isolation	14
Plasma Membrane Purity and Detection of Plasma Membrane Antigen	14
Isolation of Solubilized HSV-I Proteins	15
Preparation of Liposomes	16
Liposome Composition	18
Allogeneic Control	19
Fluorescence Depolarization	19
C. RESULTS	20
Plasma Membrane Purification	20
Induction of CTL in C ₃ H Splenocytes from HSV-I Primed Mice	22

CHAPTER	PAGE
II. (Continued)	
Induction of CTL in HSV-I Primed F ₁ Spleen Cells	24
Effect of Lipid Composition on Induction of CTL by Liposomes	26
D. DISCUSSION	26
III. INDUCTION OF CYTOTOXIC TO LYMPHOCYTE RESPONSES BY LIPOSOMES CONTAINING PURIFIED HERPES SIMPLEX VIRUS GLYCOPROTEINS AND H-2K ^k ANTIGEN	31
A. INTRODUCTION	31
B. MATERIALS AND METHODS	32
Cells and Virus	32
Mice	33
Chemicals	33
Mouse Immunization and Preparation of Spleen Cells	33
Allogeneic Control	34
Cytotoxicity Assays	34
Plasma Membrane Isolation	34
Purification of the H-2K ^k Molecule	35
Crude Viral Antigens	36
Purification of HSV-I Glycoproteins	36
Polyacrylamide Gel Electrophoresis and Fluorography	37
Sample Preparation for SDS-PAGE	37
Preparation of Liposomes	38
CTL Induction and Assay	38
Analysis of Liposomes	39
Ficoll Gradient Floatation	39
C. RESULTS	39
Purification of H-2K ^k Antigen	39
Purification of ³ H-HSV-I Glycoproteins	43
Induction of CTL Responses	45
D. DISCUSSION	50
IV. <u>IN VIVO</u> INDUCTION OF ANTI-HERPES SIMPLEX VIRUS IMMUNE RESPONSES BY HSV-I ANTIGENS AND LIPID A INCORPORATED INTO LIPOSOMES	54
A. INTRODUCTION	54

CHAPTER	PAGE
III. (Continued)	
B. MATERIALS AND METHODS	55
Viral Antigens	55
Lipid A	55
KDO Assay	55
Plasma Membrane Isolation	56
Lipids	56
Preparation of Liposomes	56
Analysis of Liposome Composition	57
Immunization and Collection of Sera	59
Antibody Determinations	59
Cytotoxicity Assay	61
Measurement of Delayed-Type Hypersensitivity	61
Electron Microscopy	61
C. RESULTS	61
Incorporation of lipid A and HSV-I Antigens Into Liposomes	61
Induction of Immune Responses	62
D. DISCUSSION	69
V. A MODEL FOR CTL INDUCTION AND SPECULATIONS	72
LIST OF REFERENCES	76
APPENDIX	82
VITA	85

LIST OF TABLES

TABLE	PAGE
1. Induction of Cytotoxicity T Lymphocytes from HSV-1 Primed Mice (C ₃ H-H-2K ^k) by Infectious HSV-1 and by Liposomes of Varying Composition.....	23
2. Induction of Cytotoxic T Lymphocytes from HSV-1 Primed (C ₃ H x BALB/c) F ₁ Mice with Infectious HSV-1 and Liposomes of Varying Composition.....	25
3. Induction of CTL by Liposomes of Various Lipid Composition and Their Apparent Microviscosity.....	27
4. <u>In Vitro</u> Induction of Syngeneic CTL Against HSV-1 by Liposomes Containing Purified HSV-1 and H-2K ^k Antigen	48
5. <u>In Vitro</u> Induction of Allogeneic CTL by Liposomes Containing Purified H-2K ^k Antigen	49
6. Induction of Anti Herpes Virus Immune Responses by HSV-1 Antigens: Dependence on Form of Antigen Presentation and the Adjuvant Lipid A.....	64
7. Induction of Anti Herpes Simplex Virus Immune Responses by Liposomes Containing Viral Antigens and H-2 Antigens	67
8. Injection Site Effects on Induction of Anti Herpes Virus Immune Responses by Liposomes Containing HSV-1 Antigens and Lipid A..	68

LIST OF FIGURES

FIGURE	PAGE
1. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis Profile of ^3H -glucosamine Labelled HSV-I Proteins Derived from the Partially Purified Virions	17
2. Scatchard Plots of ^{131}I -anti H-2K ^k IgG Binding to L Cells (A) and L Cell Plasma Membranes (B)	21
3. Elution Profile of Anti-H-2K ^k IgG Sepharose 4B Affinity Column	40
4. SDS Polyacrylamide Gel and Fluorograph of Purified H-2K ^k Antigen, Crude HSV-I Antigens, and Liposome Incorporated H-2K ^k and Crude HSV-I Antigens	41
5. Elution Profile of ^3H -HSV-I Glycoproteins from a Con A Sepharose 4B Affinity Column	44
6. SDS Polyacrylamide Gel and Fluorograph of Con A Sepharose 4B Purification of HSV-I Glycoproteins	46
7. Electron Micrograph of Phosphatidylcholine:Cholesterol (7:2 mole ratio) Liposomes Containing HSV-I Antigens and Lipid A	58
8. Sucrose Gradient Profiles of Liposome Incorporated HSV-I Antigens and Lipid A	63
9. Model: Working Hypothesis for Induction of Anti Herpes Virus CTL	73

CHAPTER I

LITERATURE REVIEW

A. INTRODUCTION

There has been much recent interest in identifying the component antigens of viruses and bacteria that are responsible for stimulating a protective immune response so that they may be used in the development of subunit vaccines. This is of particular interest with herpes simplex viruses, since they have become a major cause of venereal infections that have reached near epidemic proportions and suspicions have been raised that DNA containing herpesvirus preparations may be oncogenic (1). Therefore any herpesvirus vaccine containing DNA may be ethically unacceptable for use in man (1). A major problem in herpesvirus infections is that after infection, disease persistence of virus is a significant feature and such virus can be reactivated under a variety of circumstances to give rise to recrudescent disease (2). Vaccines that have proven successful must be administered prior to first exposure to virus since once infection has occurred and latency established, disease results from reactivated virus rather than de novo infection (2). Whether or not vaccines could be developed that could prevent disease due to reactivated virus remains a speculative possibility but it has been suggested that recrudescent disease occurs only in those situations where some vital component of immunity is missing, depressed, or the many components of immunity involved in defense against virus and virus infected cells are interacting inappropriately (3,4). Therefore, if this idea is correct then it may be possible to alleviate this

problem by developing immunization protocols that can selectively enhance the effectiveness of the immunoprotective systems. This dissertation analyzes the ability of liposome incorporated herpesvirus antigens to act as vaccines in the induction of a protective immune response directed against the virus.

B. HERPESVIRUSES

The herpesvirus group is characterized as having a linear, double stranded DNA genome that is encapsulated in an icosahedral nucleocapsid exhibiting 162 capsomers on the surface. This nucleocapsid is then covered with a membrane envelope which it acquires as it buds through virus modified patches of nuclear membrane. The virion of HSV contains approximately 33 different polypeptides (5).

The virion polypeptides can be placed in three distinct groups. First, at least six polypeptides are present in the nucleocapsid prior to envelopment. Second, the glycosylated polypeptides are found in the viral envelope, with most projecting out from the envelope as much as 8 nm. Third, a number of polypeptides are associated with the tegument, the filamentous area between the nucleocapsid and the envelope (5).

It appears most likely that the five major glycoproteins of the viral envelope, gC, gA, gE, gB and gD are responsible for the elicitation of immune responses to the herpesviruses. Glycoproteins gC, gB and gA have molecular weights of approximately 120,000-130,000 and gE and gD have molecular weights of approximately 88,000 and 58,000 respectively (5). The purification and subsequent use of these proteins to induce protective immune response to herpesviruses will make available a potentially new way to immunize organisms against these viruses.

The form in which these purified glycoproteins will be presented to the organism is critical in inducing any type of immune response since herpesvirus glycoprotein aggregates have been shown to be non-immunogenic (6). Several researchers have shown that although an aggregated protein or hapten preparation is non-immunogenic it can be rendered immunogenic by reconstituting the protein or hapten into a liposome before testing its' immunogenicity (6, 7, 8, 9, 10, 11). There are many advantages in using liposomes as means of antigen presentation such as, the ability to change the lipid composition, antigen density, surface charge, ease of preparation and their inherent immunogenicity.

C. LIPOSOMES

More than fifteen years ago Bangham .et. al. showed that aqueous dispersions of phospholipids formed closed microscopic structures consisting of one or more concentric lipid bilayers and an equal number of aqueous compartments (12). Since the early work of Bangham there has been a widespread interest in liposomes ranging from their physical chemical characteristics to applications in medicine.

Liposomes can be made with a number of different lipids and by a variety of different techniques. Depending on which lipids are used and by what technique the liposomes are made, liposomes of different size and shape can be produced. Therefore, liposomes have been divided into three major classes based on their size and unilamellar or multilamellar nature as agreed upon informally at the New York Academy of Sciences Meeting on Liposomes and Their Uses in Biology and Medicine (13). These three major classes include the multilamellar large vesicles (MLV), which can range in diameter from a

couple thousand angstroms to fractions of a millimeter (13). Within any single preparation, there is considerable size heterogeneity and variation in the number of lamellae. Those liposomes having few lamellae have been referred to as oligolamellar liposomes. MLV are generally prepared by resuspending dried lipids in an aqueous buffer with mild agitation or by detergent dialysis (13).

Another class of liposomes are the small unilamellar vesicles, (SUV), which range in diameter from 200 to 500 Å and consist of a single bilayer surrounding an aqueous compartment (13). SUV are characterized by osmotic insensitivity, a disproportionate amount (about 70%) of the total lipid located in the outer leaflet of the lipid bilayer and the small radius of curvature which imposes packing strains on the lipid molecules (13). Ultrasonic dispersion is the most widely used method for preparing SUV but they can also be made by injecting an ethanolic solution of phospholipid into a rapidly stirring aqueous solution, injecting an aqueous suspension of phospholipids under high pressure through a small orifice and carefully controlled detergent dialysis.

The third class of liposomes are the large unilamellar vesicles, (LUV), which range in diameter from about 600 Å to several microns (13). These liposomes differ from SUV's in that they do not have small radius of curvature and have equal quantities of lipid in both the inner and outer leaflets. LUV's can be formed by a number of methods including hydrating a thin layer of phospholipids in distilled water, the coalescing of SUV made of phosphatidylserine by calcium followed by calcium removal with ethylenediaminetetracetic acid, injecting an ether solution of lipids into a warm aqueous solution, and a technique called reverse phase evaporation in which LUV are formed from water-in-oil emulsions of phospholipids and buffer in an

excess organic phase, followed by removal of the organic phase under reduced pressure (13).

Liposomes can readily incorporate lipid soluble proteins in their bilayers if such molecules are present during their formation. This can be achieved by mixing lipid soluble proteins and lipids in detergent dialysis or in some cases it is possible to add lipid soluble molecules to pre-made liposomes and achieve incorporation (13).

D. LIPOSOMES AND IMMUNOBIOLOGY

The induction of immune responses by antigen bearing liposomes can be studied in terms of either a humoral or cellular immune response both in vivo and in vitro. Therefore, this review will look at the role liposomes have played in both types of response.

Humoral Response

Kinsky's group has made progress with the synthetic hapten, dinitrophenyl- γ -aminocaproyl-phosphatidyl ethanolamine (DNP-CAP-PE), in determining the effects of liposome composition and hapten density on the induction of a humoral or B-cell response in vivo and in vitro (7, 8, 9, 14). Tadakuma et. al. using DNP-CAP-PE have shown that as the hapten density increases from 0.625% to 10% of the total lipid, the immune response, measured as a function of hemolytic plaque formation, increases monotonically to a maximal response between 5 and 10% hapten density (14). In these experiments all liposomes were prepared with dipalmitoylphosphatidylcholine and dipalmitoylphosphatidic acid in a 10:1 mole ratio along with the appropriate amount of hapten. Also, in all cases tested the total amount of hapten

administered was the same; therefore, the results were only attributable to hapten density and not a dose effect. These same authors also found that the pre-incubation of cells with low hapten density liposomes could prevent those cells from being induced by a subsequent addition of high hapten density liposomes. Finally, they concluded that there has to be a critical number of hapten molecules per unit area on the liposome surface or the induction process does not occur. If these results are indicative of all humoral responses with liposomes, then one must determine the most effective density for each antigen.

Dancey, Yasuda and Kinsky prepared liposomes of different lipid composition with 5% hapten density and tested their relative immunogenicity to analyze the effect of lipid composition on induction of humoral responses by liposomes bearing the synthetic hapten DNP-CAP-PE in vitro and in vivo (8). The liposome composition was changed such that the liposomes had different phase transition temperatures and thus were either "fluid" or "solid" at room temperature. The results from the in vitro study showed that liposomes having a higher phase transition temperature or the more "solid" liposomes were the most immunogenic. The change in phase transition temperature was achieved by using phosphatidylcholines differing in fatty acyl chain length and therefore physical properties. Although the in vitro study indicated that more solid liposomes were most immunogenic, this did not hold completely true for the in vivo studies, because to obtain a maximal response in vivo liposomes also required cholesterol in their bilayers irregardless of their fluidity. It appears as though fluidity is important in induction both in vivo and in vitro but so is the nature of the environment in which the antigen resides.

Another interesting observation of these experiments was the natural adjuvanticity of liposomes. Micelles of the hapten DNP-CAP-PE were not

immunogenic, at any dose tested, but upon reconstitution into liposomes they were. These studies clearly show that to utilize liposomes as a means of presenting an antigen the type of lipids used to make liposomes is a very critical factor in determining the antigen's ability to induce an immune response.

Cellular Response

Studies on the induction of a cell mediated or T-Cell immune response using liposomes as a means of antigen presentation can be divided into two categories: allogeneic responses and syngeneic responses. The allogeneic response is characterized as being an anti non-self response in which the major histocompatibility antigens serve as the antigen source. The earliest experiments done to indicate the major histocompatibility antigens as the proteins responsible for the allogeneic response were done by using purified murine plasma membranes to stimulate a secondary anti H-2 haplotype cytotoxic T lymphocyte response (15) that was H-2 haplotype restricted. The next experiments done to show that this response was attributable to a plasma membrane antigen involved the reconstitution of detergent-solubilized plasma membrane proteins into liposomes (16, 17). These liposomes were then used to stimulate a H-2 haplo-type restricted secondary cytotoxic T lymphocyte response. The final proof that the major histocompatibility antigens could be solely responsible for the allogeneic response was provided by Englehard and co-workers (16). These authors reconstituted purified human major histocompatibility antigens, HLA-A and HLA-B into liposomes and showed that these liposomes could induce a haplotype restricted allogeneic response. Englehard et. al. also found that the maximal CTL response was obtained with liposomes that have a similar HLA antigen density per unit area as that of a

normal cell (18, 19). This last observation could be very important when trying to produce liposomes bearing major histocompatibility antigens and viral antigens for the induction of a syngeneic response.

Research on the induction of syngeneic secondary CTL by liposomes bearing viral and MHC antigens has progressed similarly to the studies of allogeneic responses. The first experiments done to stimulate an in vitro secondary CTL response to virus infected cells were done with liposomes bearing purified viral antigens and plasma membranes components. The first viral systems studied were vesicular stomatitis virus by Loh et. al. (10) and Sendai virus by Finberg and Mescher (11). Both groups found that the induced CTL were H-2 haplotype restricted in killing and that the viral antigens and the MHC antigens had to be incorporated into the same liposome for induction to occur. Hale and co-workers then demonstrated that the induction of a syngeneic response only required viral antigen and purified MHC antigen (20, 21). Hale's group showed that a syngeneic CTL response could be induced in vitro with liposomes bearing the purified G protein from vesicular stomatitis virus and purified H-2 antigens from the H-2k and H-2D regions of the major histocompatibility gene complex. This same group also showed that the HN protein from Sendai virus and purified H-2 antigen could also stimulate a syngeneic response when incorporated into the same liposomes. Another interesting observation in Hale's experiments was that if antibody to either the viral antigen or the MHC antigen was used to precipitate either protein in the presence of detergent both the viral antigen and the MHC antigen co-precipitated (20). This observation gives circumstantial evidence of the idea that the viral antigen and the MHC antigen form a complex in the liposome bilayer and it is probably this complex that the responding cells recognize.

As mentioned previously in this review, many antigens and haptens are nonimmunogenic in an aggregated form but become immunogenic when incorporated into liposomes. This natural adjuvanticity of liposomes has been studied in several laboratories with different antigens (6, 9, 22, 23). Manesis et. al. (22) found that Hepatitis B surface antigens could be rendered immunogenic upon incorporation into liposomes and Rooijen et. al. (23) showed that immune responses to human serum albumin and bovine gamma globulin could be enhanced if the antigens were associated with liposomes. This adjuvant activity of liposomes, however, is not sufficient to evoke the same magnitude response that a live virus inoculum can induce in the viral systems or a hapten derivatized whole cell can induce in the synthetic hapten models. This apparent inability of liposomes to evoke a maximal immune response has been alleviated by the incorporation of additional adjuvants, into liposomes bearing antigen. Dancey et. al. (24) and Yasuda et. al. (9) show that liposomes bearing synthetic haptens or viral antigen can be made to evoke a maximal immune response when lipid A is incorporated into the same liposomes as the antigen. Therefore with careful consideration of lipid composition, antigen density, and the use of adjuvants it should be possible to produce a liposome that will stimulate a maximal immune response to a particular antigen and open the door for the development of subunit vaccines.

CHAPTER II

CELL MEDIATED IMMUNITY TO HERPES SIMPLEX VIRUS: INDUCTION OF CYTOTOXIC T LYMPHOCYTE RESPONSES BY VIRAL ANTIGENS INCORPORATED INTO LIPOSOMES

A. INTRODUCTION

Since immunity to several viruses is primarily cell mediated, it is essential that any subunit vaccine preparation should adequately stimulate this form of immunity. Recently, in vitro culture methods such as cytotoxic T lymphocytes (CTL) were shown to provide a convenient means of analyzing the ability of immunogens to stimulate parameters of cell mediated immunity. In previous studies we showed that infectious HSV-1 could induce potent CTL responses in splenocyte cultures from HSV-1 primed mice (25). Unfortunately, certain forms of inactivated virus were ineffective at inducing CTL thus casting doubt as to the potential value of inactivated subunit herpesvirus preparations as suitable immunogens. However, recent studies with Sendai virus and vesicular stomatitis virus (VSV) have indicated that CTL induction requires the responding cells to be presented with antigens coded by the major histocompatibility complex (MHC) as well as the viral antigen (11, 26, 21, 10). Initially Finberg et. al. (11) and subsequently other groups (10, 21, 26) have shown that in vitro induction of CTL by inactivated viral preparations can regularly be achieved if viral antigens are presented to primed responder cells in the form of liposomes which additionally contain the appropriate H-2 allo-antigens. Whether or not a similar situation holds true in vivo has yet to

be ascertained. The purpose of the present investigation was to explore the effectiveness of herpesvirus antigens incorporated into liposomes to induce anti-*HSV-1* specific CTL in vitro.

B. MATERIALS AND METHODS

Cells and Virus

The mouse cell line, strain L-929 (*H-2^k*) obtained from Flow Laboratories (McLean, VA) was used as the syngeneic target cell in all cytotoxicity assays. A second mouse cell line, A-31 3T3, strain BALB/c, (*H-2^d*) obtained from Dr. Ray Tennant, Biology Division, Oak Ridge National Laboratory, was used as an allogeneic target cell in the cytotoxicity assays. Both the above cell lines were passaged and maintained in McCoy's 5A medium supplemented with 5% donor calf serum (DCS).

The virus used in all studies was herpes simplex virus type 1, strain KOS (*HSV-1*). Virus stocks were grown in human embryonic lung (MRC5) or HEP-2 cells as previously described by Bone and Courtney (27). Virus titers were determined in Vero cells.

Mice

Female C3H (*H-2^k*) and male BALB/c (*H-2^d*) were obtained from Cumberland View Farms, Clinton, Tennessee, at approximately 3 weeks post gestation and injected with *HSV-1* at 6-7 weeks of age. Mice of the (C3H x BALB/c)*F₁* strain were bred at the University of Tennessee animal unit, and were injected intraperitoneally with 10^7 pfu of *HSV-1* when 6 weeks old.

Mouse Immunization and Preparation of Spleen Cell Cultures for In Vitro Immunization with HSV-1 or Liposomes

These procedures have been described in detail elsewhere (25). Briefly, mice (C3H or (C3H x BALB/c) F₁) were injected intraperitoneally with 10^7 pfu of HSV-1 in 0.2 ml of medium. Four weeks or more post infection, spleen cell suspensions were made and resuspended in RPMI 1640, supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol. The cell suspensions were cultured in plastic tissue culture petri dishes at a cell concentration of 2×10^6 /ml and 1×10^6 cells/cm² surface area.

In Vitro Immunization of Spleen Cell Cultures with HSV-1 or Liposomes

The cultures of primed splenocytes were restimulated (in vitro) with 0.1 ml of infectious HSV-1 (10^7 iu pfu/ml) or liposome preparations. For the restimulation using liposome suspensions, the spleen cell suspensions were placed in a small volume of RPMI 1640 medium (5 ml) without serum, and 400 µl of the various liposome suspensions were then added. The cells plus liposomes were left to interact, with occasional agitation, at 37°C for a period of approximately 2 hours. Fresh RPMI 1640 medium containing FCS was then added to give a final cell concentration of 2×10^6 /ml. Both the virus and liposome stimulated spleen cells were incubated at 37°C in a humidified CO₂ (5%) incubator for a period of 5 days prior to measurement of cytotoxicity.

Cytotoxicity Assay

The assay was carried out as previously described (25). Briefly, L cells and 3T3 cells were suspended in RPMI 1640, 5% FCS at a cell concentration of 3 to $5 \times 10^6/\text{ml}$ and infected at 37°C with infectious HSV-1 at a multiplicity of infection of 3. After infection for 60 min, the cells were washed once and resuspended in media and $\text{Na}^{51}\text{CrO}_4$ (NEN, Boston, MA) was added to a concentration of $100\mu\text{Ci}/\text{ml}$. The infected cell targets, along with control uninfected target cells, were labelled for 1 hour at 37°C . Following labelling, the cells were washed 3 times, resuspended in RPMI 1640 plus 5% FCS, enumerated and diluted as necessary. Target cells, 1×10^4 per well, were added to round bottom microtitre plates along with effector cells at varying effector cell to target cell ratios (100:1, 50:1, 25:1). The assays were performed at 37° for 7 hours in a CO_2 humidified incubator. All assays were run in quadruplicate. At the end of the assay, the microtitre plates were lightly centrifuged and 50% of the contents from each well ($100 \mu\text{l}$) were collected for the measurement of radioactivity. Standard errors were invariably less than 4% and results were expressed as the mean percent specific release computed according to the formula below: Percent specific ^{51}Cr release =

$$\frac{\text{Experimental} - \text{medium control}}{\text{Total releasable chromium} - \text{medium control}}$$

Total releasable chromium - medium control

The total releasable chromium was obtained by lysing target cells with 3% Triton X-100. In some experiments aliquots of the effector cells were treated with anti-thy 1.2 serum plus complement as described previously (25) to prove that the CTL were T cell in nature.

Plasma Membrane Isolation

Plasma membranes were isolated from L-929 cells (H-2^k) grown in suspension in a modified Dulbecco's spinner medium supplemented with 10% DCS and gentamycin (500 μ g/ml) or from P815 cells growing in vivo in DBA/2 mice. The cells were harvested by centrifugation (2000 xg, 2 min) and washed three times with phosphate buffered saline solution (PBS) pH 7.4. The washed cells were then resuspended in cold PBS at 5×10^8 cells per 10 ml and kept on ice until used. The washed cells were then placed in a nitrogen cavitation bomb and equilibrated at 750 psi of nitrogen for 20 min at 4°C (28). The equilibrated cell suspension was then released from the bomb, resulting in cell disruption. The disrupted cell suspension was centrifuged for 30 seconds at 1500 xg to remove intact cells and cell nuclei. The supernate was then centrifuged in a linear sucrose gradient (25-50%) for 18 hours at 10,000 xg. The fractions containing plasma membrane (38-42%) were collected and washed twice with PBS by centrifugation. The final membrane pellet was resuspended in PBS containing 1 μ M phenyl methyl sulfonyl fluoride. The total protein concentration was determined by the method of Lowry (40).

Plasma Membrane Purity and Detection of Plasma Membrane Antigen

The purification of L-cell (H-2^k) plasma membrane was assessed by binding of ¹³¹I labeled monoclonal anti H-2K^k IgG to whole cells and to the purified plasma membrane vesicles. The monoclonal anti H-2K^k IgG was purified from 11-4.1 cell supernatants by the procedure of Mescher et. al. (29) and subsequently radiolabeled using chloramine T (30). After labeling, the free

iodine was removed by dialysis against PBS. In addition, plasma membranes were radioiodinated with ^{125}I (30) to assess the recovery of plasma membrane protein. The antibody binding experiment was done by incubating whole cells (5×10^5 cells = 228 μg protein) or plasma membranes vesicles (25 μg protein or 126 cpm/ μg) in PBS with varying amounts of labeled anti H-2K^k IgG (50 μg to 5000 μg) for 1 hr at 37°C. After two washes in PBS (100,000 xg, 60 min) the levels of ^{125}I and ^{131}I in the cell and membrane pellets were measured in a multichannel gamma counter. The final cell or membrane pellets were counted. Protein content of the whole cells and isolated plasma membranes were also determined by the method of Lowry (40).

Isolation of Solubilized HSV-1 Proteins

HEp-2 cells grown in Dulbecco's modification of Eagle's medium supplemented with 10% DCS in roller bottles were infected with HSV-1 at a multiplicity of infection of 5 and virus growth was allowed to continue until the CPE was 3+ to 4+. Tritium-labelled glucosamine (specific activity 38 Ci/mmmole) was added to one of the infected roller bottles 4 hours post infection (10 $\mu\text{Ci/ml}$ of media). The supernatant from infected cultures was harvested following the removal of whole cells and cellular debris by centrifugation at 3000 xg for 15 minutes. The supernatant was further centrifuged for 1 hour at 100,000 xg to pellet whole virus and the pellet was collected and resuspended in 12 mls of reticulocyte standard buffer (RSB) (pH 7.4). Two mls of virus suspension was applied to 13 mls of a linear sucrose gradient (20%-60% sucrose in RSB) and centrifuged for 12 hours at 100,000 xg (SW 27.1 rotor). Following centrifugation, the gradient was collected in 0.5 ml fractions and each fraction was assayed for radioactivity (presence of ^3H glucosamine). The partially

purified HSV-1 was found to band in the 50-55% sucrose fraction. Those fractions containing virus were diluted in RSB and centrifuged for 2 hrs at 100,000 xg, and the resulting virus pellet was harvested and resuspended in a small volume of RSB. Virus purity was analyzed by discontinuous polyacrylamide gel electrophoresis (31). In order to solubilize the membrane glycoproteins, the virus fraction was then treated with 2% deoxycholate (DOC) for 1 hr at 37°C. The solubilized virus was then centrifuged for 1 hour at 100,000 xg to pellet the viral nucleocapsids. The solubilized envelope proteins found in the supernatant were then used for the subsequent studies with the liposomes. The gel profiles of the tritiated glucosamine labelled viral proteins used for liposome preparation are shown in Fig. 1.

Preparation of Liposomes

Phosphatidylcholine (PC) and cholesterol (Chol) or other lipids as indicated dissolved in chloroform at a mole ratio of 7:2 (PC:Chol), were aliquoted into test tubes, evaporated to dryness under N₂ gas and vacuum dessicated for 30 minutes. The dry lipids were suspended by bath sonication in PBS to give a final concentration of 20 mg/ml of lipid. The suspended lipids were subsequently probe sonified to clarity with a Heat Systems sonicator (model W-375) at setting 2.5 to produce small unilamellar liposomes. The PC:Chol liposomes (820 µg) were aliquoted into test tubes and 5% in DOC. The mixture was briefly vortexed to ensure adequate mixing. To the lipid-DOC mixture, isolated L-cell plasma membrane (570 µg protein) and HSV-1 proteins (250 µg protein) were added to give a final lipid to protein ratio of 10:1 (w/w) and a plasma membrane protein to HSV-1 protein ratio of 2.25:1 (w/w). The total volume of each sample was adjusted to 3.0 ml with PBS. The lipid-

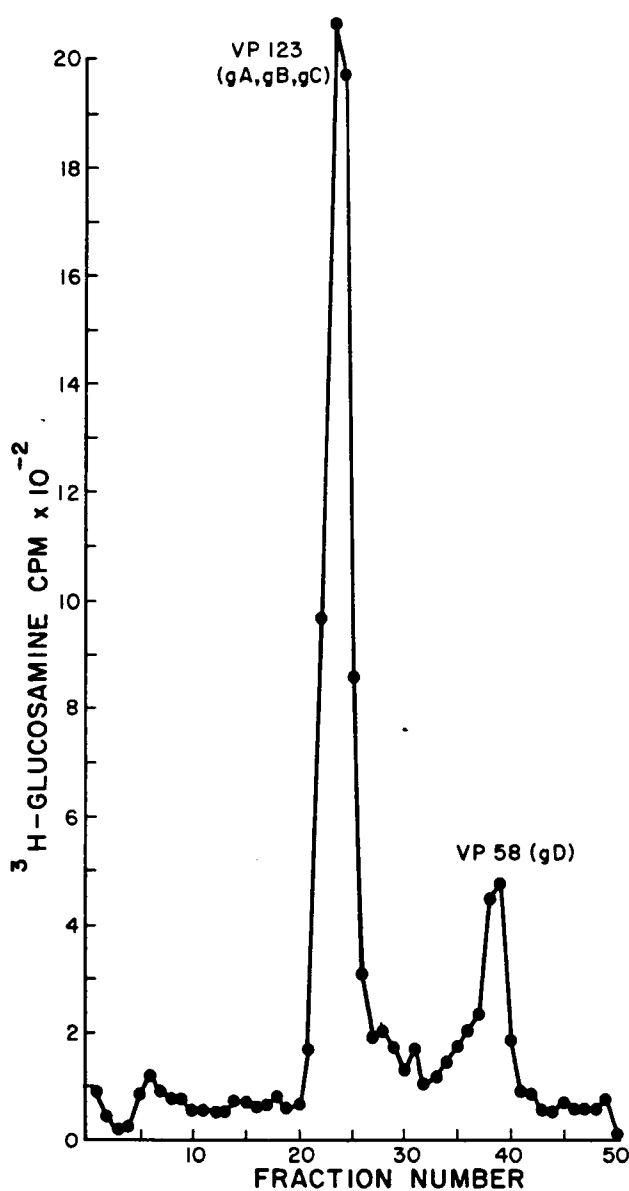


Figure 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis profile of ^3H glucosamine labelled HSV-1 proteins derived from the partially purified virions. These proteins were used in the preparation of virus protein containing liposomes. The glycoproteins gA, gB and gC of the VP123 region and gC of the VP58 region are indicated.

DOC protein samples were then allowed to incubate at room temperature for 10 minutes before being placed in dialysis bags (molecular weight cut-off 15,000 daltons). The mixture was dialyzed for 72 hours against PBS pH 7.4 containing 0.02% sodium azide + 1 μ M PMSF with repeated changes of the dialysis buffer to allow liposome formation and incorporation of the HSV-I and membrane proteins. Seventy two hours were required to lower the DOC concentration to less than 0.03% as monitored by ^3H -DOC. The DOC concentrations were approximately 0.7% and 0.45% at 24 hr and 48 hr, respectively. After dialysis, the liposome suspension was filter sterilized using Acrodisc filters (0.45 μ m Gelman), before use in the induction assays. The presence of viral glycoproteins in the liposomes was determined by SDS-polyacrylamide gel electrophoresis. The gel profile of the ^3H -glucosamine labeled viral proteins of liposomes with HSV-I incorporated was similar to that shown in Figure 1. Further biochemical characterization of these liposomes will be given in a subsequent communication.

Liposome Composition

The composition of the liposome preparation was varied as follows:

- a) liposomes containing both solubilized HSV-I antigens and plasma membrane antigens (LIPID:HSV-I:PM), b) liposomes containing solubilized HSV-I antigens only (LIPID:HSV-I), c) liposomes containing solubilized plasma membrane antigens only (LIPID:PM), d) liposomes composed of exogenous lipid only (LIPID).

Allogeneic Control

The antigenicity of the H-2^k plasma membrane containing liposome preparations was checked by adding the liposomes to spleen cells from BALB/c (H-2^d) mice previously immunized intraperitoneally with 10⁷ viable L cells (H-2^k) and assaying for the generation of specific cytotoxicity against ⁵¹Cr labelled L target cells (H-2^k).

Fluorescence Depolarization

Fluorescence depolarization of the hydrophobic fluorescent probe 1,6-diphenyl, 1,3,5-hexatriene (DPH) as a function of temperature was measured by the method of Lentz, Barenholz and Thompson (36) using a Schoeffel Instruments (Westwood, N.J) spectrofluorometer. DPH was excited at 360 nm and its fluorescence was detected at 430 nm using instrument filter with a cut off for wavelengths below 400 nm. Liposomes were mixed with 2 μ l of DPH in the same manner as described in Lentz, et. al. with exception of the incubation temperature, which in this system was 45°C (36). The general theory of the fluorescence probe depolarization technique as applied to membrane systems has been outlined in detail by Shinitzky et. al. (37). The use of DPH as a fluorescent probe specific for the hydrophobic region of lipid bilayers has been described previously by Shinitzky and Barenholz (38). This technique relies on the determination of the rotational motion of the probe molecule. This rotational motion is characterized by the fluorescence polarization (P):

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

where $I_{||}$ is the fluorescence intensity parallel to the plane of polarization of the excitation beam and $I_{\perp}$ is the fluorescence intensity perpendicular to the excitation beam.

The fluorescence polarization can be related to the apparent microviscosity of the probe environment by the equation:

$$\left(\frac{I}{P} - \frac{1}{3}\right) = \left(\frac{I}{P_0} - \frac{1}{3}\right) \left(1 + \frac{RT}{V\eta} \tau\right)$$

when P is the observed polarization, P_0 is the intrinsic polarization of the fluorescent probe, R is the gas constant, T is absolute temperature, V is the effective molar volume of the probe, τ is the lifetime of the excited state, and η is the apparent microviscosity. In our studies P_0 was calculated to be 0.419 by analyzing the polarization of DPH in glycerol over a range of temperature and plotting the data as $\frac{I}{P} - \frac{1}{3}$ vs $\frac{T}{N}$ yielding a y-intercept value of $2.054 = \frac{I}{P_0} - 0.333$. From this same data we calculated the effective molar volume of DPH to be $294.6 \text{ cm}^3/\text{mole}$. The value of τ used in our calculations was 10.25 ns.

C. RESULTS

Plasma Membrane Purification

To show that the isolation procedure for plasma membrane was effective and that an antigenic source of H-2^k was obtained, the H-2^k antigen sites on whole cells and the isolated plasma membranes were quantitated by an antibody binding approach. The data obtained from these experiments were analyzed by Scatchard plots (Fig. 2). Least-squares fit analyses for the data showed that the dissociation constants (K_D) for L-cells and plasma membranes to be $7.1 \times 10^{-8} \text{ M}$ and $1.1 \times 10^{-7} \text{ M}$, respectively, indicating the H-2^k antigen was unaltered by the isolation procedure. These same analyses also showed the

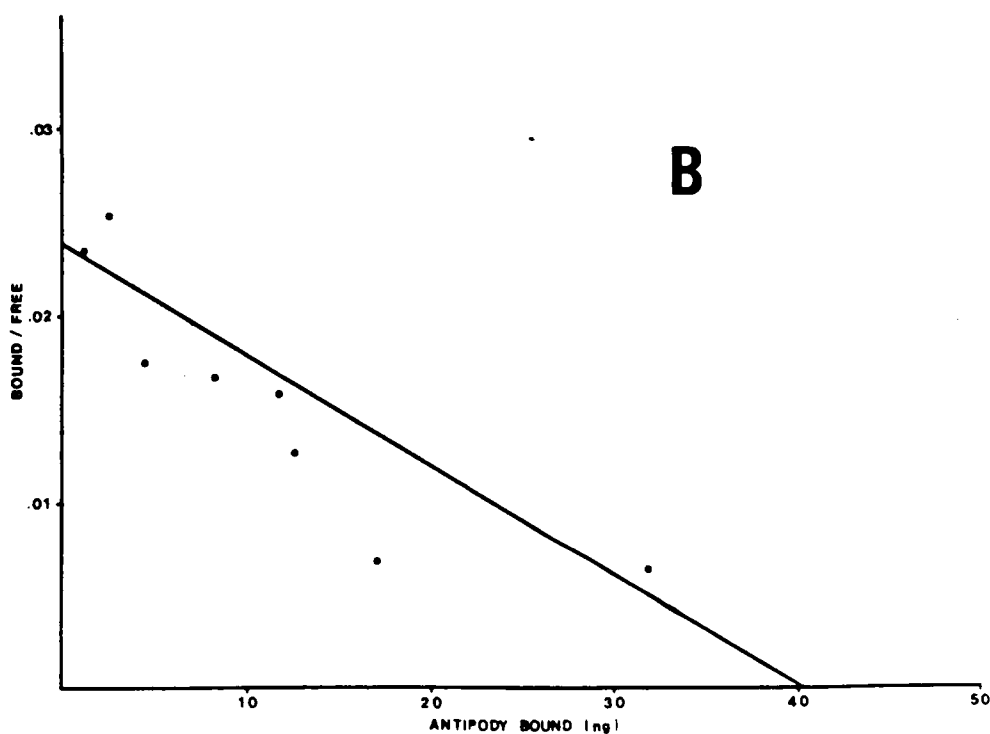
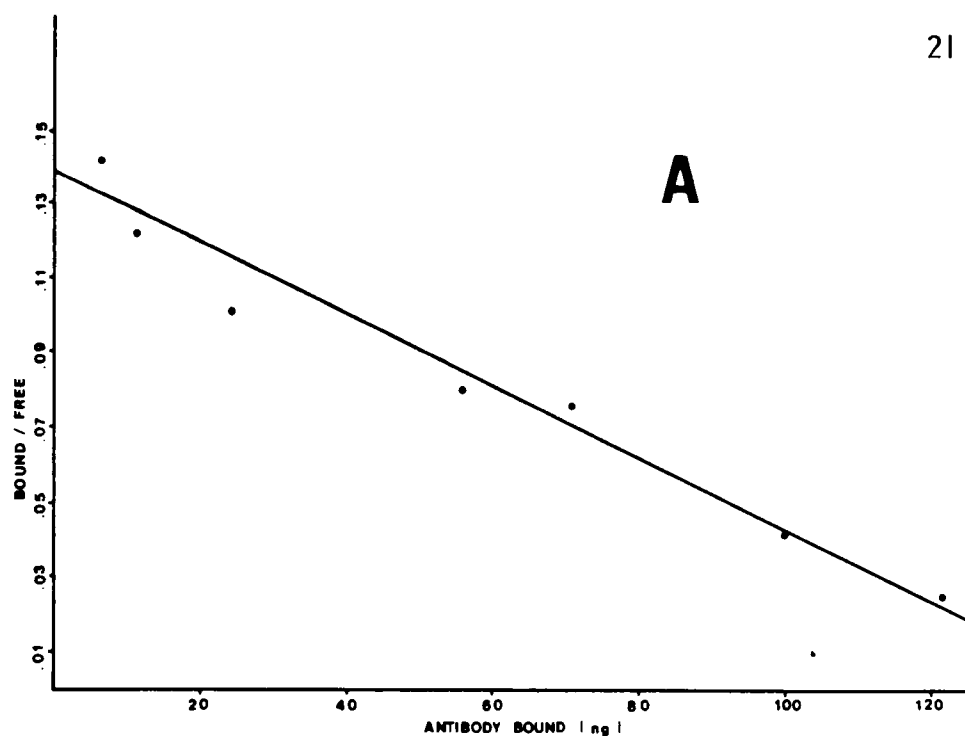


Figure 2. Scatchard plots of ^{131}I -anti H-2K^k IgG binding to L cells (A) and L cell plasma membranes (B). The correlation coefficient of the least square fit is 0.99 for A and 0.76 for B.

amount of antibody bound to be 1.65 ng/ μ g protein for plasma membrane and 0.65 ng/ μ g protein for whole cells. These data indicate that there is approximately a 2.5 fold purification of the plasma membrane. It should be noted that this value is a lower limit because the percentage of the right side out vesicles in the plasma membrane preparation is unknown. The H-2^k antigen binding sites are presumably not available for antibody binding in inside out vesicles since these sites are on the inside of the vesicles.

Induction of CTL in C3H Splenocytes from HSV-I Primed Mice

Previously, we have shown that secondary CTL responses can be generated in vitro following the culture of HSV-I primed spleen cells with infectious HSV-I virus (25). The activity of the CTL was virus specific and H-2 restricted and the effector cells were shown to be T cell in nature (25). Whereas infectious virus elicited CTL induction in vitro, neither heat-inactivated virus nor detergent solubilized virus could do so. However, as shown in Table I, detergent solubilized virus could elicit CTL induction when presented to cultures in the form of liposomes which additionally were composed of plasma membrane proteins extracted from cells of the same H-2 type as the responding cell population (LIPID:HSV-I:PM). Induction was not achieved with liposomes composed of lipid and virus alone (LIPID:HSV-I, Table I) nor by free detergent-extracted virus dialyzed free of DOC (data not shown). The CTL induced by the LIPID:HSV-I:PM liposome were destroyed by treatment with anti-thy-1 serum and complement and thus were T cell in nature.

It is apparent from the data shown in Table I that induction of anti-HSV-I CTL was H-2 restricted and required intimate association between virus and plasma membrane antigens. Thus induction was not achieved in cultures

Table 1. Induction of Cytotoxic T Lymphocytes from HSV-1 Primed Mice (C3H-H-2K) by Infectious HSV-1 and by Liposomes of Varying Composition

STIMULANT	EFFECTOR CELL	PERCENT SPECIFIC LYSIS ^a									
		L-HSV					L cell				
		100	50	25	100	50	25	100	50	25	A31-HSV
Lipid-HSV-1:PM ^b	C3H immune ^c	24.0 ^d	18	12.1	3.6	2.0	1.0	2.9	2.4	1.6	
Lipid-HSV-1	C3H immune	9.3	8.6	8.0	0	0	0	2.7	1.6	0.5	
Lipid-PM	C3H immune	8.1	4.3	3.2	0	0	0	ND ^e	ND	ND	
Lipid	C3H immune	4.0	4.5	4.4	0	0	0	ND	ND	ND	
Lipid-HSV-1 + Lipid-PM	C3H immune	3.4	3.2	2.1	0	0	0	3.1	2.5	1.6	
None	C3H immune	3.6	1.8	1.6	0	0	0	ND	ND	ND	
Lipid-HSV-1:PM ^f	C3H immune	8.7	8.5	6.8	0	0	0	2.0	1.2	0	
Infectious virus (10 ^{8.0} pfu)	C3H immune	78.1	53.6	37.8	9.0	9.3	6.1	12.4	9.6	5.1	
Infectious virus (10 ^{6.0} pfu)	C3H immune	45.0	30.0	15.1	8.4	5.2	4.6	9.7	5.6	3.4	
Lipid-PM	BALB/c L Cell ^g	40.2	30.0	10.0	50.6	32.0	15.1	3.1	2.6	1.6	

^aMean of quadruplicate samples at effector to target cell ratios of 100:1, 50:1 and 25:1. The assays were performed for 7 hr. Standard errors were less than 4%. The target cells were L cells uninfected with HSV-1, L cells and A31 cells infected with HSV-1.

^bLiposome composed of lipid, virus, and plasma membrane antigen from H-2K cells.

^cSpooled spleen cells (4×10^7) from C3H mice, 4 weeks postinfection with HSV-1 (10^8 pfu), were cultured in vitro for 5 days with the different liposomes.

^dThe cytotoxicity was destroyed by treatment of cells with anti-thy 1 serum plus C.

^eND, not done.

^fLiposome composed of lipid, virus, and plasma membrane antigen from H-2K cells.

^gBALB/c (H-2D) mice were inoculated with L cells (H-2K) ($10 \times 10^6/0.2$ ml) i.p. and boosted with further H-2 antigen every 3 weeks before in vitro restimulation with liposomes.

stimulated with LIPID:HSV-1:PM liposomes where the PM component was allogeneic to the HSV-1 primed responder population. Furthermore, cultures stimulated with a 1:1 mixture of LIPID:HSV-1 liposomes and LIPID:PM liposomes failed to generate CTL. The PM component of liposomes was shown to be immunogenic in that all liposomes containing H-2^k PM, could induce secondary anti-H-2^k CTL in spleen cells from BALB/c mice primed with H-2^k alloantigen.

Whereas, CTL induction by the LIPID:HSV-1:PM liposomes could regularly be demonstrated, the levels of cytotoxicity were always less than 35% of those observed in cultures stimulated with infectious virus. The observation that liposome preparations are less effective inducers of CTL than is infectious virus has also been reported in other viral systems (10, 21, 26).

Induction of CTL in HSV-1 Primed F₁ Spleen Cells

As indicated above, induction of anti-HSV-1 CTL by liposome preparations appeared to be H-2 restricted. Further evidence for this notion came from experiments in which HSV-1 primed (C3H x BALB/c)F₁ splenocytes were cultured with various liposomes. As shown in Table 2, whereas free infectious virus induced CTL were able to lyse virus infected H-2^k or H-2^d targets, cultures stimulated with LIPID:HSV-1 PM (H-2^k) liposomes generated only cytotoxicity against infected L target cells that expressed the same H-2 haplotype as was in the liposomes. Similarly F₁ cultures stimulated with LIPID-HSV-1 PM (H-2^d) liposomes generated cytotoxicity only against infected A31 target cells (H-2^d). As in the C3H cultures, F₁ splenocytes only responded to liposomes composed of both virus and PM in the same liposome and not to either component alone, or to mixtures of liposomes containing only virus or PM. The reason infectious virus induced CTL active against both H-2^d and H-

Table 2. Induction of Cytotoxic T Lymphocytes from HSV-1 Primed (C3H x BALB/cF₁) Mice with Infectious HSV-1 and by Liposomes of Varying Composition

STIMULANT	EFFECTOR CELL	PERCENT SPECIFIC LYSIS ^a							
		L-HSV				A31-HSV (H-2 ^d)			
		100	50	25		100	50	25	L cell (H-2 ^k)
Lipid:HSV-1 PM	H-2 ^k x H-2 ^d F ₁	20.0	16.0	5.0		0.9	0	0	3.6
Lipid:HSV-1	H-2 ^k x H-2 ^d F ₁	0	0	3.1		0	0	0	2.4
Lipid:PM	H-2 ^k x H-2 ^d F ₁	4.5	1.0	1.5		0	0.9	0	ND ^c
Lipid	H-2 ^k x H-2 ^d F ₁	0	0.4	0.5		0	0	0	ND
Lipid:HSV-1 + Lipid:PM	H-2 ^k x H-2 ^d F ₁	3.2	1.0	0		1.8	0	0	1.9
Lipid:HSV-1:PM ^e	H-2 ^k x H-2 ^d F ₁	3.7	2.8	0.6		30.4	29.3	18.0	3.3
Infectious Virus	H-2 ^k x H-2 ^d F ₁	66.9	50.0	38.5		52.7	27.1	14.0	7.8
None	H-2 ^k x H-2 ^d F ₁	2.8	1.9	0		1.6	0.9	0	3.5

^a25 x 10⁶ pooled, spleen cells from (C3H x BALB/cF₁) mice 4 weeks after infection with 10⁷ pfu of HSV-1 were cultured in vitro for 5 days with the various stimulants.

^bMean of quadruplicate samples at effector to target cell ratios of 100:1, 50:1, 25:1 during a 7 hour assay. Standard errors were less than 4%.

^cND, not done.

^dLiposomes were composed of lipid, virus and plasma membranes from H-2^k cells.

^eLiposomes were composed of lipid, virus and plasma membranes from H-2^d cells.

2^k targets was presumably because the virus could infect macrophages of the F_1 responder population and thus virus antigens would be presented in both a $H-2^k$ and $H-2^d$ environment. The observation that macrophages are required for the response of primed spleen cells to infectious HSV-1 was reported elsewhere (33).

Effect of Lipid Composition on Induction of CTL by Liposomes

Tables 1 and 2 clearly demonstrate that induction of a virus specific anti HSV-1 CTL response is H-2 restricted and the induction process requires the presence of both viral and MHC antigens within the same liposome. This requirement of both antigens within the same membrane (Table 1) suggests that there may be some essential interaction between viral and MHC antigens before induction can be achieved. If this is so, then the types of lipids used to make the liposomes could have a marked effect on their induction capabilities. The effect of exogenous lipids on the ability of liposomes to induce a CTL response was studied and the data in table 3 indicate that liposomes of different lipid composition differ greatly in their ability to induce a CTL response and in their bilayer microviscosity. The results (Table 3) show that liposomes of too low (PC alone) or too high (SM:Chol:PC and DPPC) bilayer microviscosity were relatively ineffective at inducing CTL. Liposomes of intermediate bilayer microviscosities (PC:Chol, Azolectin, and PC:chol:DCP) were shown to be most effective.

D. DISCUSSION

The experiments described in this manuscript indicate that the induction of secondary CTL responses in vitro with inactivated herpes simplex virus preparations could be readily achieved providing the antigens were presented

Table 3. Induction of CTL by Liposomes of Various Lipid Composition and Their Apparent Microviscosity^a

Lipid Composition ^b	$\frac{\text{L-HSV}}{100:1}$	$\frac{\text{L-cell}}{100:1}$	$\frac{\text{A31-HSV}}{100:1}$	$\frac{\text{Apparent Microviscosity}}{\text{centipoise, } 37^{\circ}\text{C}}$
PC	7.5	1.4	1.2	73.6
PC:Chol (7:2)	24.0	2.6	2.3	79.4
Azolectin	12.4	1.3	1.5	136.5
PC:Chol:DCP (7:2:1)	27.0	1.5	2.1	152.7
SM:Chol:PC (7:2:1)	2.1	1.8	1.6	160.8
DPPC	1.8	1.7	1.6	243.7

^aSpleen Cells of C₃H mice primed with HSV-1 virus were used.

^bLiposomes containing 250 μg HSV-1 antigens, 570 μg plasma membrane proteins and 820 mg lipids of indicated composition.

to primed splenocytes in the form of liposomes. In contrast viral antigens alone failed to induce CTL. Furthermore, the induction of anti-HSV CTL also required that plasma membrane protein of the same H-2 type as that of the responder population be incorporated into the same virus-containing liposome. In fact, even mixtures of liposomes comprised respectively of lipid and virus and lipid and plasma membrane failed to induce CTL in primed cultures. Finally, CTL induced in (H-2^k x H-2^d)F₁ virus-primed splenocytes with Lipid:Virus:H-2^k liposomes expressed their cytotoxicity solely against H-2^k infected target cells. These observations all indicate that induction of HSV-1 specific CTL requires the corecognition of virus plus a plasma membrane protein and extend to HSV similar observations made for CTL induction against Sendai virus (11, 26) and VSV (10, 21). Indeed it appears that the co-presentations of virus and a plasma membrane antigen coded for by the MHC (H-2 restriction phenomenon) is required in order to achieve optimum CTL induction as well as for the effector activity of CTL (33, 34). A satisfactory molecular explanation for the observance of H-2 restriction at the level of induction has not been made and the topic was discussed in recent reviews by Burakoff et. al., (33) and by Zinkernagel and Doherty (34). The liposomes used in this study contained partially purified fractions of plasma membrane proteins so the molecular identity of the proteins that were corecognized along with those of the virus cannot be clearly defined. Most likely, however, the plasma membrane antigens involved were those coded for by the H-2D and H-2K regions of the MHC. Elegant and persuasive evidence for this concept was recently provided by Hale et. al., (21) who showed that liposomes containing highly purified H-2 antigen along with viral antigen were effective inducers of CTL. Circumstantial evidence that H-2K and H-2D region products form

the actual co-immunogens could be made from our data which showed that the PM-containing liposomes induced allogeneic CTL only in alloantigen primed cultures. It is well accepted that the antigens responsible for inducing such responses as well as the targets against which CTL react are products of the H-2D and H-2D regions of the major histocompatibility complex (18, 35).

Although we showed that inactivated viral preparations in liposomes were immunogenic, they were less so than was free infectious virus, an observation similar to that made in other virus systems investigated (10, 11). Possible explanations for the lower immunogenicity could include antigen dose, antigen denaturation during liposome preparation, presence of toxic impurities such as residual detergent and several variables in liposome physical chemistry. Indeed with regard to the latter, we have observed that lipid composition can markedly affect liposome immunogenicity - an affect seemingly related to the fluidity of the lipids used.

In addressing the question as to how different lipids change the microenvironment within the vesicles such that induction of CTL activity is modified we examined the microviscosity of the liposome bilayer by fluorescence depolarization, using the hydrophobic probe, 1,6-diphenyl-1,2,3-hexatriene. This data (Table 3) provided us with an interesting correlation between the liposomes bilayer microviscosity and its ability to induce a CTL response. The data indicate that as the microviscosity within the bilayer increases from a low of 73.6 centipoise, in phosphatidylcholine vesicles, to 152.7 centipoise in phosphatidylcholine:cholesterol:dicetyl phosphate vesicles the antigens are still capable of interacting in a way that is conducive to induction of CTL, but once the microviscosity reached a level of 160.8 centipoise, sphingomgelin:cholesterol:phosphatidylcholine, the bilayer becomes too rigid for

the appropriate interaction between antigens and the subsequent induction of CTL to take place. This dependence on bilayer microviscosity in the liposomes ability to induce a CTL response and information obtained from the induction experiments with spleen cells from preimmune F₁ mice (BALB/c x C³H) (Table 2) indicate that the liposomes are truly presenting the antigens to the spleen cells and not cells that have engulfed liposomes and reexpressed the viral antigens on their cell surfaces.

We therefore propose that the MHC antigens and the viral antigens in liposomes mediate a direct interaction with the induction machinery required to produce cytotoxic T lymphocytes.

Currently, efforts are underway to elucidate the molecular explanation of liposome immunogenicity and to seek ways of optimizing this property for herpesvirus antigens. Our interests also lie in assessing the immunogenicity of liposomes containing more defined herpesvirus glycoproteins. Thus, since the question of the oncogenicity of herpesvirus DNA remains open, future vaccines against HSV must be DNA free as well as being adequately immunogenic (25). Although the viral antigens used in the present work were processed so as to lack the nucleocapsid and were composed principally of glycoproteins, certainly multiple viral and possibly some cellular antigens were also present. The immunogenicity of liposomes of defined biochemical composition is currently under investigation in our laboratory.

CHAPTER III

INDUCTION OF CYTOTOXIC T LYMPHOCYTE RESPONSES
BY LIPOSOMES CONTAINING PURIFIED HERPES
SIMPLEX VIRUS GLYCOPROTEINS
AND H-2K^k ANTIGEN

A. INTRODUCTION

Recently, liposomes containing viral antigens and components of the major histocompatibility complex (MHC) have been used in in vitro culture methods to stimulate cytotoxic T lymphocytes (CTL) (10,11,26,39). Interest in this system stems from the observation of Zinkernagel and Doherty that CTL from virus immune mice lysed only H-2 compatible virally infected cells (34) and that CTL recognize many foreign antigens in the context of self MHC gene products (10,11,26,39). Therefore using a liposome model to induce CTL, it should be possible to elucidate the minimum molecular components necessary to induce CTL. This finding will have great significance on the development of subunit vaccines especially for viruses such as herpes simplex that have been reported to be oncogenic (1).

The earliest attempts at inducing CTL with liposomes containing viral antigens and MHC components were done with Sendai virus and vesicular stomatitis virus (VSV) antigens and plasma membrane reconstituted together in liposomes (10,11). These early experiments showed that it was essential for the MHC antigens and the viral antigens to be in the same liposomes for induction to occur, but gave little insight to what plasma membrane components were essential for induction. Hale and coworkers were the first group to

reconstitute purified viral antigens and purified H-2K^k antigens into the same liposome and report induction of a CTL response (20,21). These reports provide evidence that only a viral antigen and a MHC gene product are essential to induce a CTL response. This present study examines what minimum molecular requirements are necessary to induce a CTL response to herpes simplex virus.

B. MATERIALS AND METHODS

Cells and Virus

The mouse cell line, strain L-929 (H-2^k) obtained from Flow Laboratories (McLean, VA) was used as the syngeneic target cell in all cytotoxicity assays. A second mouse cell line, A-31 3T3, strain BALB/c, (H-2^d) obtained from Dr. Ray Tennant, Biology Division, Oak Ridge National Laboratory, was used as an allogeneic target cell in the cytotoxicity assays. The mouse lymphoma, RDM-4 (H-2^k) obtained from Dr. Matther Mescher, Department of Pathology, Harvard Medical School, was used to isolate plasma membranes and as the source of the H-2K^k molecule. All the above cell lines were passaged and maintained in McCoy's 5A medium supplemented with 5% donor calf serum (DCS).

The virus used in all studies was herpes simplex type 1, strain KOS (HSV-1). Virus stocks were grown in human embryonic lung (MRC5) or HEP-2 cells as previously described by Bone and Courtney (27). Virus titers were determined in Vero cells.

Mice

Female C3H (H-2^k) and male BALB/c (H-2^d) were obtained from Cumberland View Farms, Clinton, Tennessee, at approximately 3 weeks post gestation and injected with HSV-1 at 6-7 weeks of age.

Chemicals

Phosphatidylcholine (PC) used in this study was purified from egg yolks (45). Cholesterol (Chol) was purchased from Sigma. EN³HANCE, used in fluorography was purchased from New England Nuclear. Con A-Sepharose 4-B and CNBr-activated Sepharose 4B were purchased from Pharmacia Fine Chemicals.

Mouse Immunization and Preparation of Spleen Cells

Young C3H mice (H-2^k) injected intraperitoneally with 0.2 ml of inocula containing 10^8 pfu per ml of HSV-1 and at least 4 weeks post infection were killed by cervical dislocation and their spleens were removed aseptically. Single cell suspensions were prepared by teasing the cells through 80 mesh sieves into Hank's balanced salt solution containing 5% FCS (HBSS). After one wash in HBSS, erythrocytes were lysed by exposure to 0.84% NH₄Cl. Cells were then washed twice more in HBSS. The cell aggregates were removed and the viable cells were enumerated. To measure cytotoxicity, spleen cells were adjusted to 2×10^6 cells/ml in RPMI 1640 containing 10% FCS, 2mM glutamine, penicillin (100 U/ml), streptomycin (100μg/ml), and 5×10^{-5} M 2-mercaptoethanol and added to 100mm plastic tissue culture dishes with approximately 40×10^6 cells/dish. The spleen cell cultures were incubated in a humidified CO₂ (5%) incubator for 5 days in the presence of liposomes (400 μl/plate) or 10^7 PFU of HSV-1, after which cells were harvested and washed twice in medium and viable

cells were adjusted to desired concentration for cytotoxicity assay in RPMI-HEPES medium.

Allogeneic Control

The antigenicity of the H-2K^k plasma membrane containing liposomes and the H-2K^k molecule containing liposome preparations were checked by adding the liposomes to spleen cells from BALB/c (H-2^d) mice previously immunized intraperitoneally with 10⁷ viable RDM-4 cells (H-2^k) and assaying for the generation of specific cytotoxicity against ⁵¹Cr labelled L target cells (H-2^k).

Cytotoxicity Assays

Cytotoxicity assays were conducted the same as that reported in Chapter II.

Plasma Membrane Isolation

Plasma membranes were isolated from RDM-4 cells grown in McCoy's 5A medium plus 10% DCS. The cells were harvested by centrifugation at 2,000 xg for 2 min. The cells were then washed 3 times in PBS and finally resuspended in cold 1/10 PBS to allow osmotic swelling, and incubated on ice for 10 min. The swollen cells were then disrupted by Dounce homogenization in a 15 ml Dounce homogenizer using 15 strokes, monitoring disruption under the microscope. The disrupted cells were then centrifuged for 30 seconds at 2,000 xg to remove whole cells, nuclei and large debris. The resulting supernatant was then layered over a linear 20-60% sucrose gradient and centrifuged at 100,000 xg for 14 hr at 4°C. Those fractions containing plasma membrane (38-

42% sucrose) were collected and the membranes were washed twice with PBS by centrifugation. The final membrane pellet was resuspended in PBS containing 1 μ M phenyl methyl sulfonyl fluoride (PMSF) at 1-3 mg protein/ml and stored frozen less than a month. The protein concentration was determined by the method of Lowry (40).

Purification of the H-2K^k Molecule

The intact H-2K^k antigen was detergent solubilized and purified using an immunoabsorbent column as described in detail by Herrmann and Mescher (29). In brief, 1×10^9 RDM-4 cells were harvested from culture media and washed twice in PBS pH 7.4. Then 5×10^7 RDM-4 cells were suspended in 2 ml PBS and subsequently radioiodinated by a modification of Hubbard and Cohn's method (41). This involved adding 10 units of lactoperoxidase activity to the cells followed by 1 mCi of ^{125} I and finally enough H_2O_2 to make its final concentration 20 μ M. The cells were incubated 20 min at room temperature followed by repeated washes in PBS to remove free iodine. The iodinated cells, used as a marker, were mixed with the unlabelled cells ($1-2 \times 10^7$ cpm for 5×10^7 cells) and together they were detergent solubilized with 0.5% NP-40 in 15 mM Na phosphate buffer pH 7.3. The solubilized cells were centrifuged for 10 min at 2,000 xg to remove large cellular debris and nuclei. The resulting supernatant was centrifuged for 45 min at 100,000 xg in a 60 Ti rotor to remove any detergent insoluble material. The resulting supernatant was applied to the immunoabsorbent column, prepared by coupling the monoclonal antibody that specifically binds the H-2K^k antigen, produced by 11-4.1 cells as described by Oi et al. (42), to Sepharose 4B. The column was washed with 15 column volumes of 0.5% NP-40 buffer pH 8.0 to remove any unbound proteins. Finally the H-

2×10^6 molecules were eluted by washing the column with 0.5% DOC in 10 mM Tris, 140 mM NaCl pH 8.0. Fractions were monitored for radioactivity and peak fractions were pooled and concentrated. Concentrated samples were tested for protein by the method of Lowry and stored frozen until used (40).

Crude Viral Antigens

Crude viral antigens were prepared following the procedure outlined in Chapter II.

Purification of HSV-1 Glycoproteins

HSV-1 glycoproteins labeled with ^3H -glucosamine were purified on a Con A-Sepharose 4B column from crude HSV-1 antigens prepared as described above. First, 15 ml of swollen Con A-Sepharose 4B (10 mg Con A/ml gel) was suspended in 0.1 M acetate buffer pH 6.0 containing 1 M NaCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 1 mM MnCl_2 and 0.1% merthiolate and used to pack a 1 x 12 cm column. The column was washed with 100 ml of PBM buffer (0.01 M Na phosphate, 1 mM MgCl_2 pH 7.2) followed by 30 ml of PBM buffer containing 1% DOC. Crude ^3H -HSV-1 antigens were then applied to the column followed by 75 ml of PBM buffer containing 1% DOC to wash through any unbound material. Finally, the ^3H glycoproteins were eluted by washing the column with PBM buffer containing 1% DOC and 0.2 M α -methyl-D-mannoside. Fractions (0.5 ml) were monitored for radioactivity and peak fractions were pooled and concentrated in an Amicon concentrator using a PM-30 filter. Concentrated samples were then dialyzed against 2 changes of 1000 volumes of PBS pH 7.4 to remove α -methylmannoside. Dialyzed glycoproteins were next analyzed for protein by the method of Lowry

and stored frozen until used (40). Purity of the purified samples was checked by polyacrylamide gel electrophoresis followed by fluorography.

Polyacrylamide Gel Electrophoresis and Fluorography

Samples of purified ^3H -HSV-I glycoproteins, purified H-2K^k antigen, crude ^3H -glucosamine labeled HSV-I antigens and liposome incorporated viral and H-2K^k antigens were all subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the method described by Laemmli (44). Following electrophoresis, the gels were stained and prepared for fluorography by impregnating the gel with EN ^{3} HANE, a commercially available autoradiograph enhancer, for 1 hr at room temperature. Following incubation the excess EN ^{3} HANE was removed and the gel was rinsed in cold tap water to precipitate the fluorescent compounds in the gel. The gel was subsequently dried and placed in direct contact with Kodak X-OMAT X-Ray film. One week later the film was developed.

Sample Preparation for SDS-PAGE

Due to the low concentration of antigens to be analyzed by SDS-PAGE, and the presence of detergent in these samples it was necessary to concentrate these samples by acid precipitation. First, lysozyme was added to the antigen, which served as a carrier molecule, and then the antigen-lysozyme mixture was precipitated with ice cold 10% trichloroacetic acid (TCA). Next, the protein-detergent precipitant formed by the cold TCA was pelleted by centrifuging at 2,000 xg for 10 min. Pelleted protein-detergent precipitant was then resuspended in ice cold acetone to remove detergent from the protein. The remaining precipitated protein was pelleted by centrifugation at 2,000 xg for 10 min. The

resulting protein pellet was then processed for SDS-PAGE as described by Laemmli (44).

Preparation of Liposomes

PC and Chol dissolved in CHCl_3 at a mole ratio of 7:2 (PC:Chol) were aliquoted into test tubes, evaporated to dryness under N_2 gas, and vacuum dessicated for 30 min. The dried lipids were resuspended in PBS by bath sonication to a final concentration of 20 mg/ml of lipid. The resuspended lipids were subsequently probe sonified to clarity with a Heat Systems sonicator (model W-375) at setting 2.5. The PC:Chol liposomes were aliquoted into test tubes along with plasma membrane, purified H-2K^k antigen, HSV-I antigens, Con A purified HSV-I glycoproteins or combinations of these proteins as indicated in the results. This mixture then received PBS and enough 5% DOC so that the final DOC concentration was 1.0%. In each sample the lipid to total protein ratio was 5:1 by weight. The lipid, protein and detergent mixtures were then placed in dialysis bags (m.w. cut-off 15,000 daltons) and dialyzed for 72 hr against PBS pH 7.4 containing 2 μM PMSF and 0.02% sodium azide.

CTL Induction and Assay

All spleen cells receiving H-2K^k containing liposomes received 15 μg of H-2K^k antigen per 4.0×10^7 cells. In the case of plasma membranes they received 150 μg protein for the same number of cells. Spleen cells receiving liposomes containing crude ^3H -HSV I antigens and Con A purified glycoproteins received 250 μg and 100 μg of protein per 4.0×10^7 cells respectively. After 5 day culture, CTL assays were done as described in the previous chapter.

Analysis of Liposomes

To determine if the viral and H-2K^k antigens had successfully been reconstituted into liposomes, the reconstituted liposomes were pelleted by centrifugation at 100,000 xg for 1 hr or floated on Ficoll gradients and subsequently analyzed by SDS polyacrylamide gel electrophoresis and fluorography for reconstituted proteins.

Ficoll Gradient Floatation

Liposome incorporated material was separated from non-incorporated material by first mixing 1ml of reconstituted liposomes with 1ml of 25% Ficoll-400 in PBS pH 7.4. Then 2 ml of 10% Ficoll-400 in PBS pH 7.4 was carefully layered on top of the liposome-Ficoll mixture. Then 1ml of PBS was layered on top of the 10% Ficoll solution. This Ficoll gradient was then centrifuged for 30 min at 2,000 xg in a Sorvall GLC-3 centrifuge. Following centrifugation the liposomes banding at the PBS-10% Ficoll interface were collected and analyzed for protein content by SDS polyacrylamide gel electrophoresis and fluorography.

C. RESULTS

Purification of H-2K^k Antigen

2×10^9 RDM-4 cells were detergent solubilized and the major histocompatibility antigen, H-2K^k was purified from this solubilized preparation using an immunoabsorbent column. Fig. 3 shows the elution profile for this column. After a period of washing the H-2K^k antigen was eluted off the column with a DOC-salt solution. The eluted fractions (165 to 200) were pooled, concentrated, and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4).

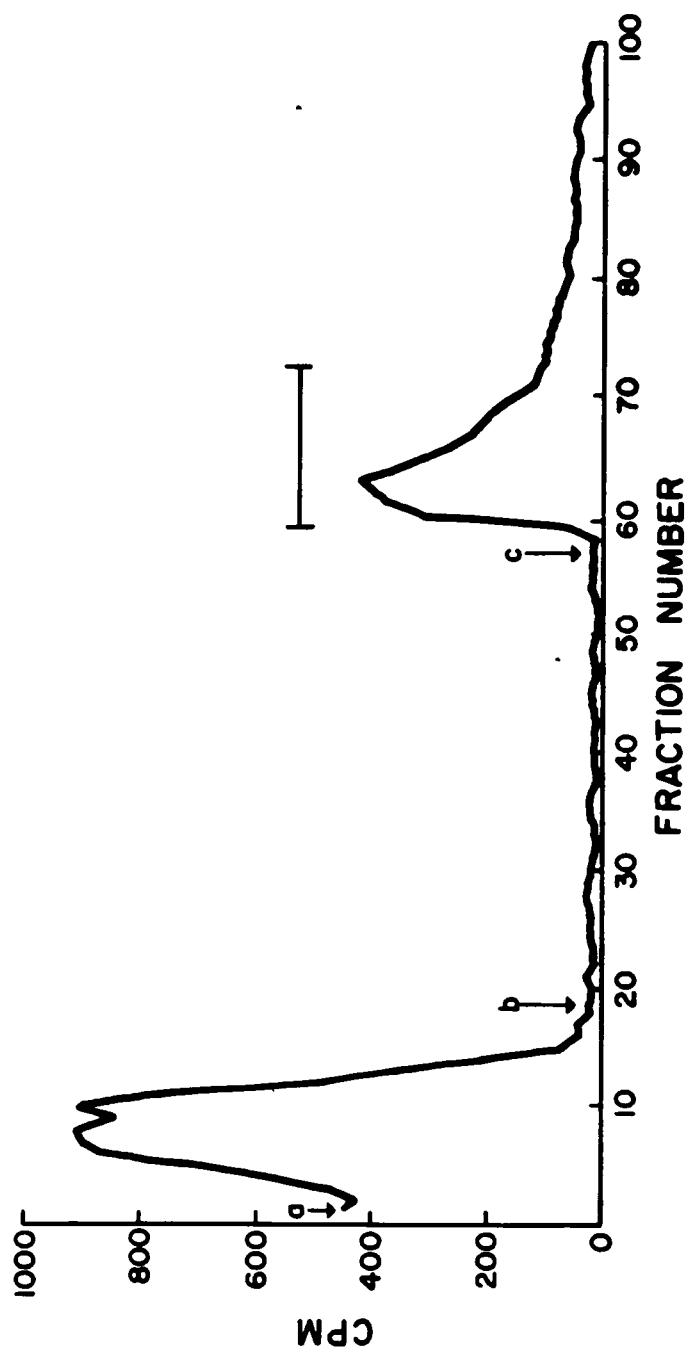


Figure 3. Elution profile of Anti H-2K^k IgG on a Sepharose 4B affinity column. Whole cell extract was loaded on column and at point a column was washed with 75 ml of 0.5% NP-40, 10 mM Tris pH 8.0. At point b H-2K^k antigen was eluted from the column by washing with 0.5% DCC, 140 mM NaCl, 10 mM Tris pH 8.0. Fractions under the bar were pooled and concentrated.

Figure 4. SDS polyacrylamide gel and fluorograph of purified H-2K^k antigen, crude HSV-1 antigens and liposome incorporated H-2K^k and crude HSV-1 antigens. Panel A is the coomassie blue stained gel and panel B is the corresponding fluorograph. Lane 1 is the low molecular weight standards, 94,000, 68,000, 45,000, 30,000, 21,000, and 14,000 daltons respectively. Lane 2 is purified H-2K^k antigen, Lane 3 is crude HSV-1 antigens. Lane 4 is liposomes containing HSV-1 and H-2K^k antigens. Lane 5 is liposomes containing HSV-1 antigens. Lane 6 is high molecular weight standards 200,000, 120,000, 94,000, 68,000, and 45,000 daltons respectively. Lanes 4 and 5 show representative profiles of liposomes that were obtained by centrifuging the reconstituted liposome preparations for one hour at 100,000 xg or by floatation on Ficoll gradients.

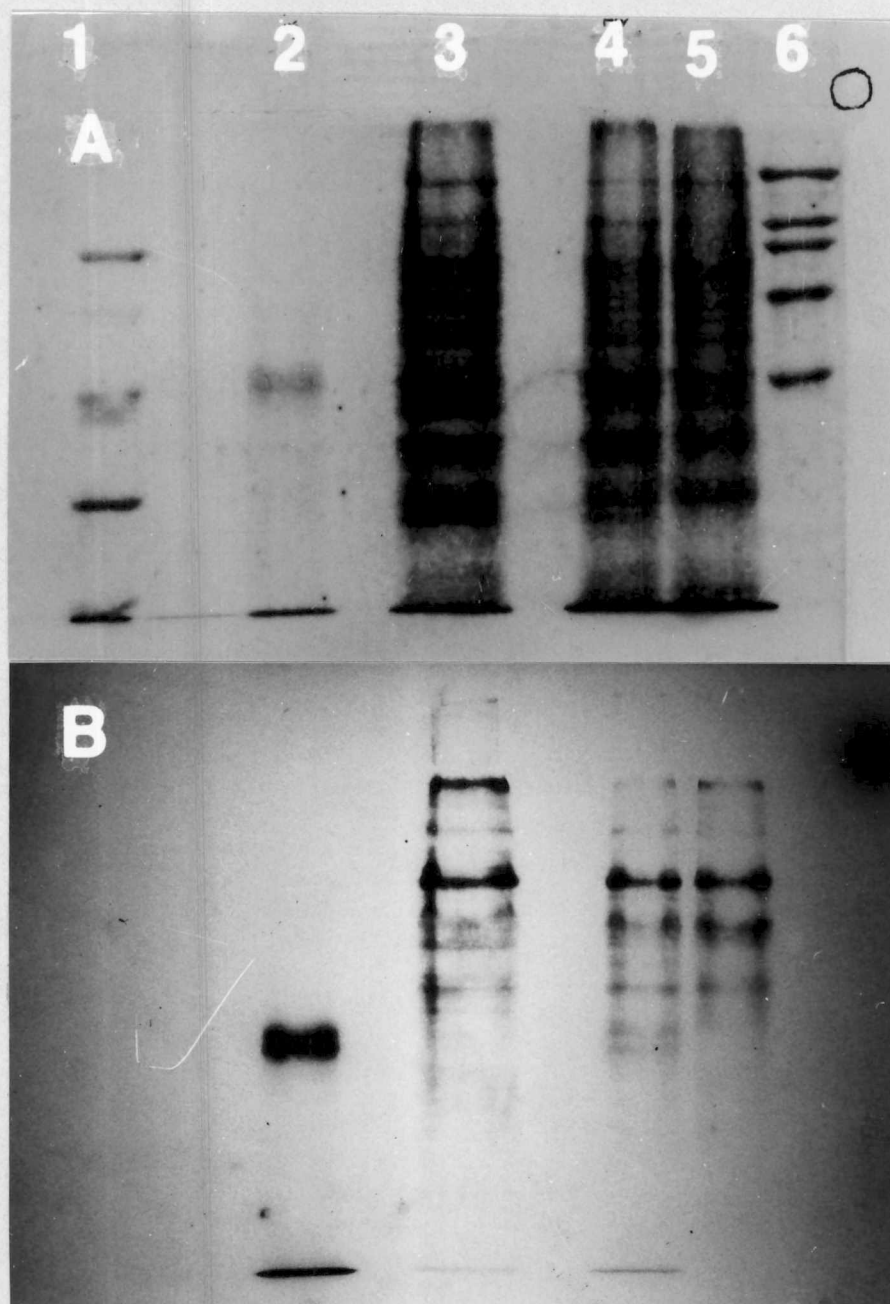


Figure 4.

The proteins from this pooled peak exhibited three protein bands by gel analysis, having molecular weights of approximately 45,000 to 50,000 daltons for the two heavy bands and about 12,000 for the lighter band. These molecular weights correspond with the heavy and light chain molecular weights of the H-2^k antigen purified by Hermann and Mescher (29). They reported that the heavy and light chains of H-2K^k had molecular weights of approximately 47,000 and 13,000 respectively. The only difference in the molecule we isolated as compared to that isolated by Hermann and Mescher was the presence of two heavy chains in our sample as opposed to one in their sample. We attribute this difference to their overexposure of the autoradiogram thereby masking the appearance of two similar bands. The two H-2K^k molecules probably differ in their degree of glycosylation since it has been reported that H-2K^k molecules have two asparagine linked carbohydrate chains of about 3,300 daltons each, on its heavy chain (29). Each purification on this scale yields approximately 100 - 200 μ g of H-2K^k antigen.

Purification of ³H-HSV-I Glycoproteins

To determine if the HSV-I glycoproteins were responsible for the viruses ability to stimulate a cytotoxic T cell response we purified those glycoproteins utilizing their ability to bind to Con A. The crude HSV-I antigens (11.23 mg), were applied to a Con A-sepharose 4B column and subsequently glycoproteins were eluted from the column with buffers containing 1% DOC - 0.2 M α -methyl-mannoside. The elution profile for this column (Fig. 5) shows that the glycoproteins were eluted as a sharp peak. This peak contained 1.4 mg of

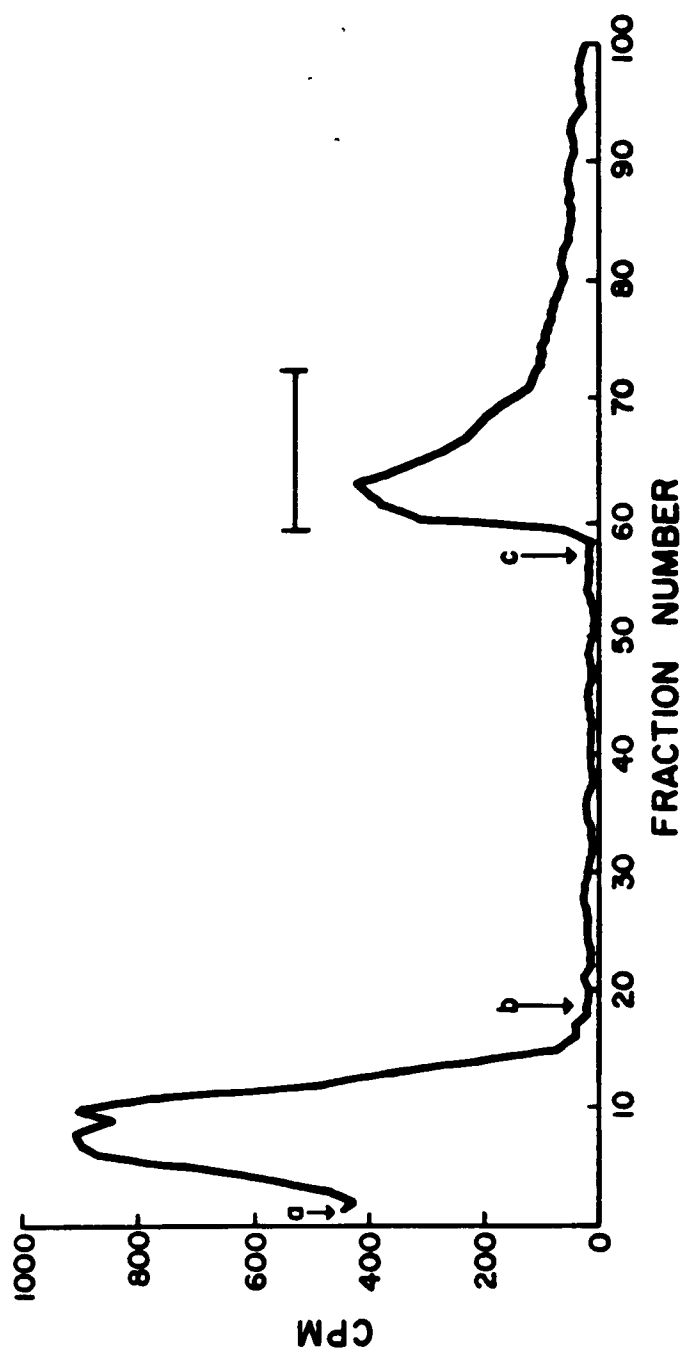


Figure 5. Elution profile of ^3H -HSV-1 glycoproteins from a Con A Sepharose 4B affinity column. Crude ^3H -HSV-1 antigens were loaded on the column at point a and nonadsorbed material was washed from the column with 1% DOC in PBM buffer starting at point b. ^3H -HSV-1 glycoproteins were eluted from the column with 1% DOC 0.2 M α -methylmannoside in PBM buffer starting at point c. Fractions under the bar were pooled, concentrated and analyzed for protein by SDS polyacrylamide gel electrophoresis.

protein or 12.5% of the loaded protein and 30.6% of the loaded cpm (^3H glucosamine). After pooling the fractions in this peak (Fractions 59-75) they were concentrated to 2 ml and subjected to SDS-polyacrylamide gel electrophoresis (Fig. 6). The fluorograph shows the purified glycoproteins to contain the same glycoproteins as the crude antigens and the Coomassie Blue stained gel shows the corresponding protein bands plus a large protein band at the bottom of the gel. This large band is due to carrier protein lysozyme, added to the glycoproteins to aid in TCA precipitation, which was used as a means of getting the glycoproteins out of the detergent.

Induction of CTL Responses

Previously, we have shown secondary CTL responses can be generated in vitro after the culture of HSV-1 primed spleen cells with liposomes containing crude HSV-1 antigens and plasma membrane components (43) or infectious HSV-1 virus (25) with the latter giving the best response. The activity of the CTL was virus specific and H-2 restricted and the effector cells were shown to be T cell in nature (25). In this present study, we attempt to further elucidate which molecules are responsible for the induction of CTL directed against herpesvirus infected cells. The data in Table 4 showed that while liposomes containing crude HSV-1 antigens and plasma membrane components are capable of inducing a CTL response, liposomes containing crude HSV-1 antigens and purified H-2^k antigen are not. The inability of these liposomes to stimulate a CTL response is not due to a defective H-2K^k antigen because this same antigen can induce an allogeneic response (Table 5). This apparent lack of induction capability is not attributable to the antigen's inability to incorporate into

Figure 6. SDS polyacrylamide gel and fluorograph of Con A sepharose 4B purification of HSV-1 glycoproteins. Panel A is the coomassie blue stained gel and panel B is the corresponding fluorograph. Lane 1 is high molecular weight standards, 200,000, 120,000, 94,000, 68,000 and 45,000 daltons respectively. Lane 2 is material that washed through the column. Lane 3 is eluted HSV-1 glycoproteins. Lane 4 is the HSV-1 antigens loaded on the column. Lane 5 is eluted HSV-1 glycoproteins. Lane 6 is high molecular weight standards.

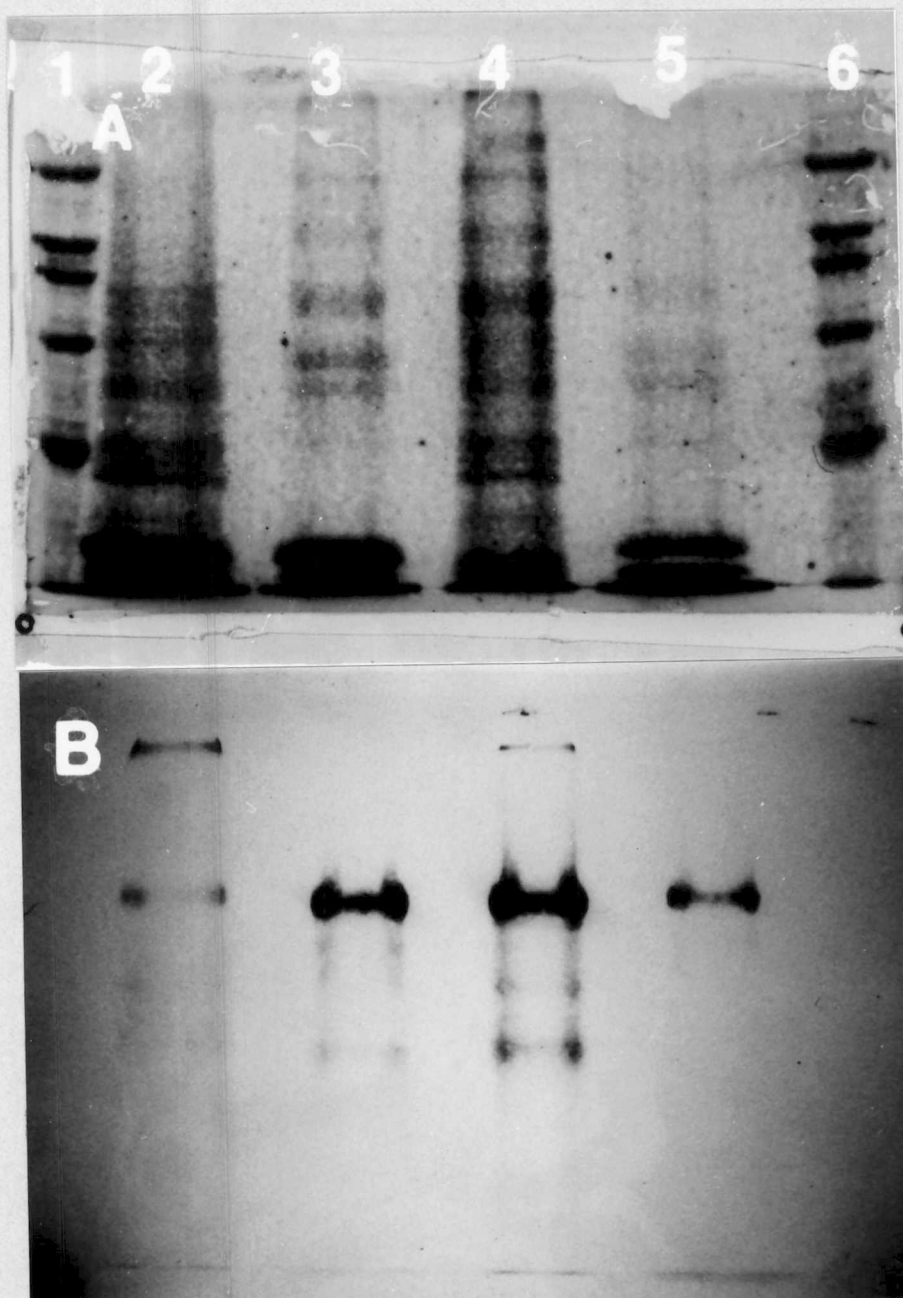


Figure 6.

Table 4. In Vitro Induction of Syngeneic CTL Against HSV-1 by Liposomes Containing Purified HSV-1 and H-2K^k Antigens^a

Stimulant ^c	%Specific Lysis ^b											
	L-cell-HSV			L-cell			A-31 cell-HSV			A-31 cell		
	100	50	25	100	50	25	100	50	25	100	50	25
PC:Chol ^d -H-2K ^k -HSV	2.8	1.7	1.1	2.9	2.6	1.2	3.1	2.7	2.4	3.0	3.1	2.9
PC:Chol-PM-HSV ConA	3.3	2.4	1.4	3.1	3.0	2.9	4.1	3.7	2.1	3.7	3.8	2.1
PC:Chol-PM-HSV	14.1	7.9	6.8	0.2	0.2	0.3	2.3	1.7	0.1	1.9	2.0	2.5
PC:Chol-PM	1.0	2.4	1.4	4.5	4.1	3.5	4.4	3.2	2.1	2.7	2.6	2.9
PC:Chol-H-2K ^k	1.4	1.1	0.6	1.9	1.7	0.2	2.7	2.1	1.7	2.6	2.7	1.6
PC:Chol-HSV	2.1	1.2	0.7	0.8	0.2	0.4	1.6	0.7	0.2	23	1.9	2.0
PC:Chol-HSV ConA	1.9	1.4	1.1	1.5	0.7	0.2	3.9	4.1	3.4	4.8	3.9	2.6
10 ⁷ PFU HSV-1	90.1	87.2	70.5	20.3	16.0	9.7	29.5	28.4	16.8	20.2	14.9	13.1

^aHSV-1 antigens are designated "H-HSV" if they are a detergent extract of purified verions or "H-HSV ConA" if the viral glycoproteins have been purified by ConA column chromatography. MHC antigens are designated "PM" if they are derived from plasma membrane or "H-2K^k" if they represent the purified molecule.

^bMean of triplicate samples at effector to target ratios of 100:1, 50:1, and 25:1. Standard errors were less than 4%. Target cells were L-cells and A-31 cells both uninfected and infected with HSV-1. These data reflect the numbers obtained from at least six separate experiments.

^cPooled spleen cells (4×10^7) from C3H mice, 4 weeks postinfection with 10⁷ PFU HSV-1, were cultured in vitro for 5 days with the different liposomes, then assayed for CTL.

^dPC:Chol, Phosphatidylcholine:cholesterol 7:2 mole ratio.

Table 5. In Vitro Induction of Allogeneic CTL by Liposomes Containing Purified H-2K^k Antigen^a

Stimulant ^c	%Specific Lysis ^b											
	<u>L-cell-HSV</u>			<u>L-cell</u>			<u>A-31 cell-HSV</u>			<u>A-31 cell</u>		
	100	50	25	100	50	25	100	50	25	100	50	25
PC:Chol ^c -H-2K ^k	51.7	33.9	16.7	32.8	20.2	10.9	4.1	2.3	3.1	3.1	2.5	0.7
10 ⁶ C ₃ H cells	87.7	85.1	81.9	70.3	74.1	54.6	15.6	9.6	5.7	11.5	6.7	3.8
NONE	16.0	15.4	6.7	2.9	28	1.3	16.5	8.3	5.4	13.5	9.1	6.8

^aMean of triplicate samples at effector to target ratios of 100:1, 50:1 and 25:1. Standard errors were less than 4%. The target cells were L-cells and A-31 cells and L-cells and A-31 cells infected with HSV-1.

^bLiposomes or C₃H cells were incubated for 5 days in culture with 4 x 10⁷ spleen cells from BALB/c mice that had received 10⁷ RDM-4 cells intraperitoneally 4 weeks prior to experiments.

^cPC:Chol, Phosphatidylcholine:Cholesterol 7:2 mole ratio.

liposomes because Fig. 4 shows that both the H-2K^k antigen and the crude HSV-I glycoproteins are reconstituted into these liposomes as analyzed by ficoll gradient floatation and by centrifugation.

Table 4 also shows that liposomes containing purified HSV-I glycoproteins and plasma membrane components also fail to induce a CTL response. The inability of these liposomes to induce a CTL response is somewhat surprising in light of the fact that several other purified viral antigens have been shown to be active in inducing a secondary CTL response (20, 21). It must be concluded that the herpesvirus model is more complex than those above mentioned systems because even in the case of unpurified antigens and plasma membranes containing liposomes we can not evoke responses comparable to that evoked by other viral systems. The possibility does remain, however, that even though these purified glycoproteins exhibit the same gel pattern as unpurified glycoproteins and they incorporate into liposomes they may have been denatured.

D. DISCUSSION

The data presented in this manuscript indicate that while crude HSV-I antigens and plasma membrane components reconstituted together in liposomes can stimulate a secondary CTL response this activity is abrogated if either component is further purified. This is not surprising with regard to the purified H-2K^k antigen because Herrmann and Mescher have recently reported that upon purification and subsequent reconstitution into liposomes this antigen loses its antigenicity (46). These same authors also report that detergent solubilized plasma membranes lose their ability to evoke an allogeneic T cell response if the preparation is first centrifuged at 100,000 xg for 45 min to remove detergent

insoluble material (46). They also report both purified H-2K^k antigen and detergent solubilized plasma membranes can be rendered as effective as native plasma membranes if some detergent insoluble material, which they define as matrix proteins, is added to the preparation during the reconstitution step (46). Herrmann and Mescher describe this matrix material as being similar to the spectrin matrix of erythrocytes and being composed mostly of actin and proteins with molecular weights of 70,000, 69,000, 38,000 and 36,000 (46). Therefore, it is possible that purified H-2K^k and crude virus antigen-containing liposomes could induce a CTL response if some matrix material is added to these liposomes during reconstitution. To date, only Hale and coworkers have reported induction of secondary CTL with liposomes containing purified viral and H-2K^k antigens (20, 21). This group has reported induction with both purified VSV antigens and Sendai virus antigens incorporated into liposomes along with purified H-2K^k antigen and show that the responses achieved by purified viral and H-2K^k antigens are of the same magnitude as those achieved by liposomes containing viral antigens and plasma membrane components (20,21). The success of Hale's group and failure of this study to induce CTL with liposomes containing purified viral and H-2K^k antigen may be attributable to several factors. First, the type of liposomes used in this study and in Hale's study were decidedly different. We made our liposomes with PC:Chol (7:2 mole ratio) while Hale's group uses dimyristoylphosphatidylcholine only. We also used DOC as a solubilizing detergent and Hale's group uses cholic acid. We employed a lipid to protein ratio of 5:1 (w/w) while Hale's group uses 1:1 (w/w). The type of resulting liposomes were different in that we produced large oligolamellar liposomes and

Hale's group produces large unilamellar liposomes (20,21). Second, the type of virus may have some bearing on our results as no preparation of herpesvirus antigens and plasma membrane components have ever induced the level of CTL activity elicited by live virus. Usually this response is 20% of that achieved by live virus, while liposomes containing Sendai virus or VSV antigens and plasma membrane components routinely elicit responses similar to that achieved by live virus (10, 11, 20, 21, 39).

We were somewhat surprised that liposomes composed of purified HSV-1 glycoproteins and plasma membranes failed to induce a secondary CTL response since several laboratories have demonstrated that liposomes containing different purified viral antigens and plasma membranes could induce CTL responses (10, 11). It is unlikely that the glycoproteins of herpes simplex virus are not the antigens responsible for the induction of CTL since these are the major proteins found on the viral envelope (5).

The herpesvirus glycoproteins have also been shown to be involved in the recognition and lysis of virally infected cells by CTL using the drugs tunicamycin and 2-deoxy-D-glucose which inhibit glycosylation of proteins (47, 58). Cells infected with HSV-1 and treated with such drugs failed to be lysed by CTL. Also temperature sensitive mutants of HSV-1, which at nonpermissive temperatures exhibit diminished glycoprotein synthesis, when grown in cells at nonpermissive temperatures failed to be lysed by CTL while cells infected with these mutants at permissive temperatures were lysed (47, 58).

Another potential problem with inducing CTL with liposomes containing purified HSV-1 antigens may have been the dose of antigen the spleen cell population received. Rough calculations based on there being about 1000 copies

of the 120,000 glycoprotein per virus particle and 100 virion particles for every plaque forming unit detected in a plaque assay. We calculated that the quantity of HSV-I antigens given to the spleen cells by 10^7 PFU of HSV-I was the same or higher than that introduced by liposomes containing 250 μg of crude viral antigens or 10 μg of purified HSV-I glycoproteins. Since the virus has the ability to infect cells and replicate and liposomes don't we may need to increase the quantity of liposomes given to these spleen cell populations to evoke a CTL response.

CHAPTER IV

IN VIVO INDUCTION OF ANTI-HERPES SIMPLEX VIRUS IMMUNE RESPONSES BY HSV-I ANTIGENS AND LIPID A INCORPORATED INTO LIPOSOMES

A. INTRODUCTION

The trend in vaccine development is to identify the component antigens of viruses and bacteria that are responsible for stimulating a protective immune response. 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 (5). By *in vitro* approaches we have shown that HSV glycoproteins are poor immunogens but their immunogenicity can be markedly improved by incorporation into liposomes (43). Furthermore, studies with hepatitis B viral components and several nonviral antigens (7, 8, 22, 23) have shown that incorporating these antigens into liposomes leads to an enhanced immune response. It has also been shown that additional incorporation of an adjuvant such as lipid A into the antigen-containing liposomes further enhances the immune response against the antigen (9, 24). In this communication, we evaluate the use of liposomes to improve the in vivo immune response against HSV glycoproteins.

B. MATERIALS AND METHODS

Viral Antigens

Viral antigens were prepared following the procedure outlined in Chapter II.

Lipid A

The lipid A used in this study was a generous gift from David Morrison, Emory University, Atlanta, GA. It was extracted from the rough mutant (Re) of Salmonella minnesota R595 as described by Galanos et. al. (48) with modifications of Skelley et. al. (49) 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 (49). The lipid A was suspended in PBS pH 7.4 at 800 $\mu\text{g/ml}$ and stored at -20°C until use. Lipid A was quantitated by the thiobarbituric acid used to detect 2-keto-3-deoxyoctanoic acid (KDO assay) (50).

KDO Assay

Samples of lipid A or liposomes containing lipid A, approximately 200 μl , were first treated with 10 μl of 0.4N H_2SO_4 and heated for 8 min. at 100°C . The treated samples were allowed to cool to room temperature before adding 200 μl of 25 mM sodium periodate in 0.125N H_2SO_4 . The samples were incubated for 20 min at room temperature followed by addition of 400 μl of 2% sodium arsonite in 0.5N HCl with rapid mixing. Subsequently 1.6 ml of 0.3% 2-Thiobarbituric acid was added and the sample heated for 12 minutes at 100°C . Quantitation was achieved by monitoring samples for absorbance at 550 nm and producing a standard curve for lipid A to compare the liposome-lipid A

preparations with. This standard curve gave an absorbance₅₅₀ of 0.009 per mg lipid A.

Plasma Membrane Isolation

Plasma membranes were isolated from RDM-4 cells grown in McCoy's 5A plus 10% DCS. The cells were then washed 3 times in PBS and finally resuspended in cold 1/10 PBS to allow osmotic swelling, and incubated on ice for 10 min. The swollen cells were then disrupted by Dounce homogenization in a 15 ml Dounce homogenizer using 15 strokes, monitoring disruption under the microscope. The disrupted cells were then centrifuged for 30 seconds at 2,000 xg to remove whole cells, nuclei and large debris. The resulting supernatant was then layered over a linear 20-60% sucrose gradient and centrifuged at 100,000 xg for 14 hr at 4°C. Those fractions containing plasma membrane (38-42% sucrose) were collected and the membranes were washed twice with PBS by centrifugation. The final membrane pellet was resuspended in PBS containing 1 μ M phenyl methyl sulfonyl fluoride (PMSF) at 1-3 mg protein/ml and stored frozen less than a month. The protein concentration was determined by the method of Lowry (40).

Lipids

Phosphatidylcholine (PC) used in this study was purified from egg yolks (45) ¹⁴C-PC was purchased from Amersham at 114 mCi/mmol. Cholesterol (Chol) was purchased from Sigma.

Preparation of Liposomes

PC and Chol, dissolved in CHCl₃ at a mole ratio of 7:2 (PC:Chol), were aliquoted into test tubes, evaporated to dryness under N₂ gas, and vacuum dessicated for 30 min. The dried lipids were resuspended in PBS by bath

dessicated for 30 min. The dried lipids were resuspended in PBS by bath sonication to a final concentration of 20 mg/ml of lipid. The resuspended lipids were subsequently probe sonified to clarity with a Heat Systems sonicator (model W-375) at setting 2.5. Liposomes used in assessing HSV-I glycoproteins and lipid A incorporation were prepared with tracer ^{14}C -PC (126 $\mu\text{Ci}/\text{mmole}$) added to the original lipid mixture before drying to serve as the lipid marker. The sonicated lipids were then mixed with appropriate quantities of HSV-I glycoproteins in 2% DOC, lipid A, 5% DOC, and PBS to give a final solution of lipid, HSV-I glycoproteins and lipid A at lipid to protein ratio of 5:1 (w/w) and a final DOC concentration of 1%. The lipid, HSV-I glycoproteins, lipid A and detergent mixture was then placed in dialysis bags (m.w. cut-off 15,000 daltons) and dialysed for 72 hr against PBS (pH 7.4) containing 2.0 μM PMSF and 0.02% sodium azide, yielding oligolamellar liposomes containing lipid A and the HSV-I glycoproteins as seen in Fig. 7. Liposomes containing HSV-I glycoproteins alone, lipid A alone, or containing RDM-4 plasma membranes were also prepared in the same manner.

Analysis of Liposome Composition

To show that the lipid A and HSV-I glycoproteins were incorporated into the liposomes, sucrose density gradient centrifugation of liposomes was carried out.

Briefly, 500 μl of liposomes or liposomal components were placed on a 5 ml linear 5 to 20% sucrose gradient and centrifuged for 5 hr at 200,000 $\times g$ in a Beckman SW 50.1 rotor. The gradients were fractionated and all fractions were monitored for ^3H -HSV-I glycoproteins, ^{14}C -PC, and lipid A.

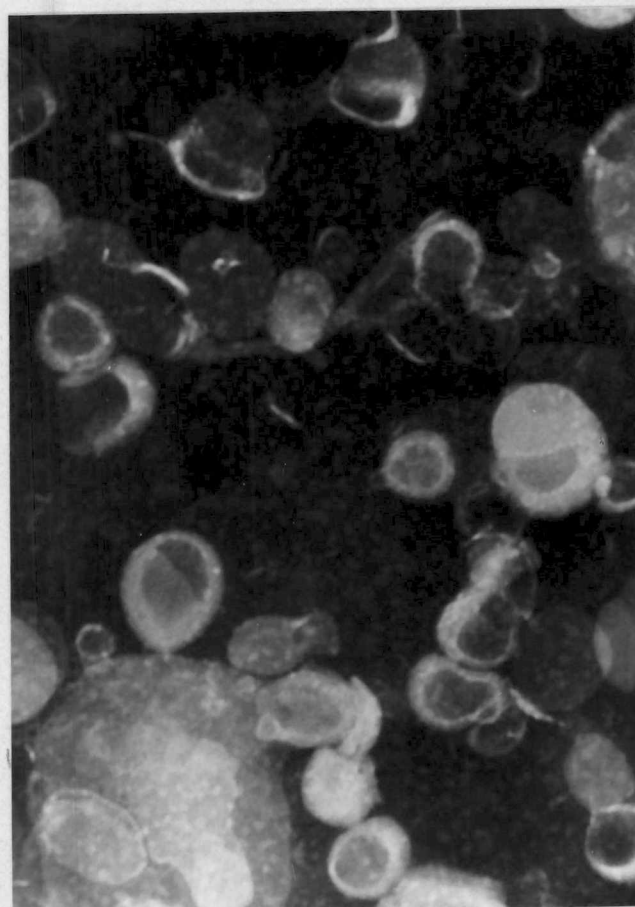


Figure 7. Electron micrograph of phosphatidylcholine:cholesterol (7:2 mole ratio) liposomes containing HSV 1 antigens and Lipid A Scale: 1cm = 770 Å.

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, Tennessee. Mice were injected intraperitoneally, unless otherwise indicated, with appropriate inoculums on day 1. In all cases where the mice were to receive two types of inoculum 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^o) sera and it was tested for antibody activity as described under antibody determinations. The bled mice were subsequently reinoculated with appropriate antigens the same day. On day 20, the mice were bled again via the other retro-orbital sinus. The resulting sera yielded an antibody response we refer to as a secondary response (2^o). 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 (25). The generation of secondary (2^o) CTL was tested for via the cytotoxicity assay following a five day in vitro culture period during which the spleen cells were incubated with 10⁷ PFU of HSV-1 to induce the secondary CTL.

Antibody Determinations

A solid-phase radioimmunoassay (RIA) was employed for measuring antibodies that was a modification of the assay described in detail by Zollinger et. al. (51). In brief, HSV-1 viral stocks, grown in HEp-2 cells, were diluted to 10⁷ plaque-forming units (PFU) per ml using distilled water. The diluted virus

stock was used to coat the wells of a plastic microtiter plate (Cooke Engineering Co., Alexander, 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 hr at 37°C to allow virus adsorption to the plate. Following the incubation period the unbound virus was washed from the wells with five washes of phosphate buffered saline containing 2% Tween-20, (PBS-Tween) with 1 mg/ml bovine serum albumin added. Sera to be tested for anti HSV-1 antibodies were serially diluted with PBS-Tween and 0.1 ml aliquots of each dilution were placed in different wells of HSV-1 bound microtiter plates. The plates were then incubated for 2 hr at 4°C. Following the incubation period the plates were washed five times with PBS-Tween to remove any unbound antibody. The antigen-antibody complexes were then determined by adding protein A (Zymed labs) to each well that was first radiolabeled by the chloramine T method (30) with ^{125}I (5-9 mCi/mg) and incubating for an additional 2 hr at 40°C, followed by 5 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 cpm vs. antiserum dilution. The midpoint of the descending linear portion of the curve was identified. RIA units were calculated by multiplying the cpm by the dilution factor at this midpoint (56).

An identical protocol was used to test sera for anti-lipid antibodies and anti HEp-2 cell antibodies except in the anti-lipid antibody test the microtiter plates were allowed to adsorb 50 μg of phosphatidylcholine per well instead of HSV-1 virus. The anti-HEp-2 cell antibody test was done by first adsorbing detergent solubilized whole cell extracts to the microtiter plates.

Cytotoxicity Assay

The cytotoxicity assays were carried out as described in Chapter II.

Measurement of Delayed-Type Hypersensitivity

The protocol employed for the measurement of delayed-type hypersensitivity was a modification of the method reported by Crowle (52). In brief, mice were inoculated with liposomes or virus as previously mentioned and following their last bleeding were challenged in the right hind footpad with 0.025ml HSV-1 (1×10^7 PFU) and in the left hind footpad with HBSS. Control mice received HBSS in both footpads. The thickness of the footpads was measured with calipers 24 hr after challenge. The DTH response was expressed as a percentage of footpad increase at 24 hr. Significance was determined using the student's t-test at 95% confidence limits.

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. A drop of 1% phosphotungstic acid solution pH 7.6 was immediately 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.

C. RESULTS

Incorporation of lipid A and HSV-1 Antigens Into Liposomes

To ascertain whether the HSV-1 glycoproteins 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. 8, panel A, indicate that there were two types of liposome populations in this preparation and that the HSV-1 antigens and lipid A appeared in the faster migrating peak, approximately 80-85% of the initial material. The appearance of these two liposome peaks reflects the heterogeneity of this preparation, as seen in Fig. 7. The preparation contains both oligolamellar and unilamellar liposomes, with the oligolamellar ones being greater in number. The remaining panels in Fig. 8 show that both HSV-1, (panel B) and lipid A, (panel C) incorporate into liposomes by themselves with a slightly slower migrating profile than the liposomes containing both components. The panels D, E, and F also show that the individual liposomal components band quite differently by themselves as opposed to the banding observed when they are incorporated into liposomes. In panels E (HSV-1) and panel F (lipid A) both liposomal components smear across the bottom of the gradient, most likely as a function of aggregate size. Panel D 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 mice injected with varying 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 6.

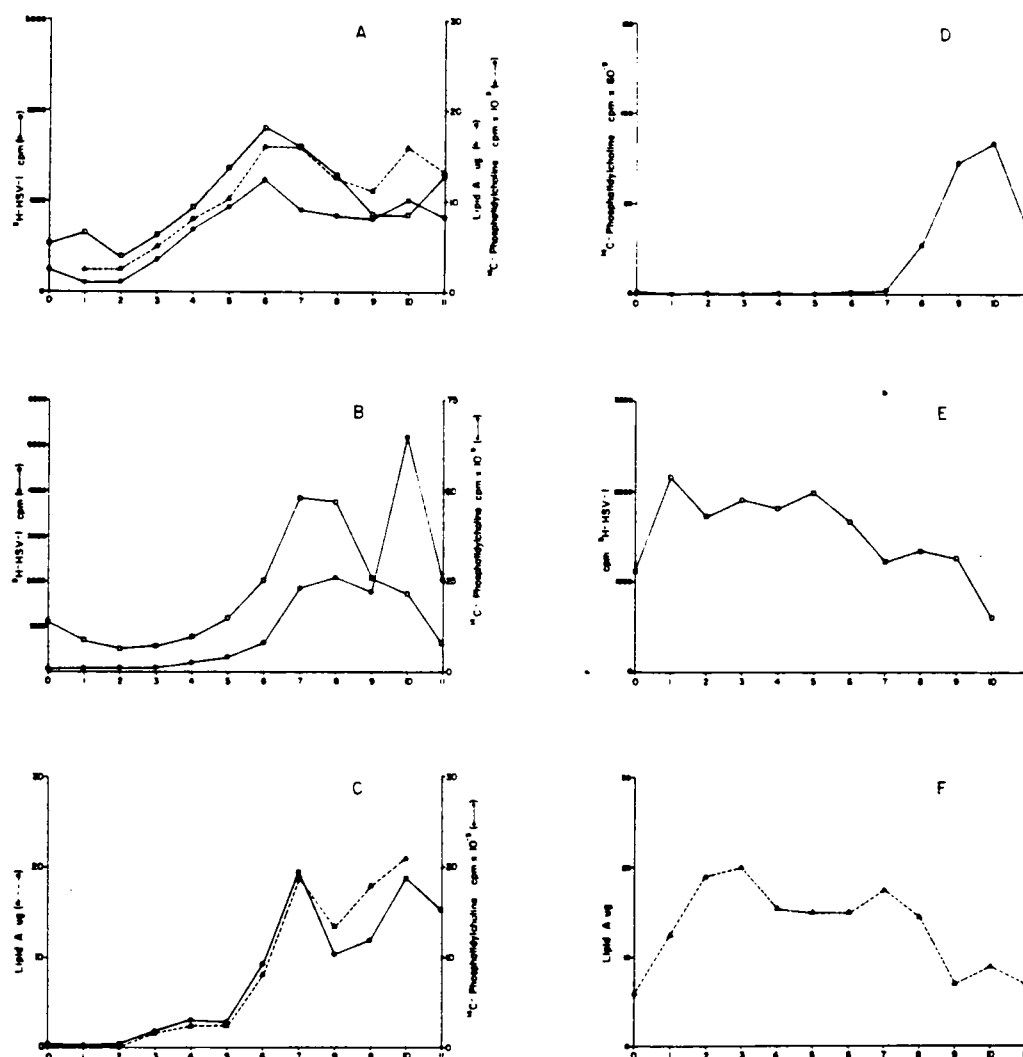


Figure 8. Sucrose gradient profiles of liposome incorporated HSV-I antigens and lipid A. Panel A is liposomes containing HSV-I antigens and lipid A. Panel B is liposomes containing HSV-I antigens. Panel C is liposomes containing lipid A. Panel D is PC:Chol liposomes only. Panel E is nonincorporated HSV-I antigens. Panel F is nonincorporated lipid A.

Table 6. Induction of Anti Herpes Virus Immune Responses by HSV-1 Antigens: Dependence on Form of Antigen Presentation and the Adjuvant lipid A

Inoculum ^a	Antibody Titer (RIA units $\times 10^{-3}$) 10 ²⁰	DTH	²⁰ CTL % Cr51 Release ^b
HSV ^c gp 5 μ g	0	-	0
HSV gp 1 μ g	0	-	0
HSV gp 0.2 μ g	0	-	0
PC:Chol ^d -HSV gp 5 μ g	0	-	0
PC:Chol-HSV gp 1 μ g	0	-	0
PC:Chol-HSV gp 0.2 μ g	0	-	0
HSV gp 5 μ g + Lipid A 2 μ g	0	-	0
HSV gp 1 μ g + Lipid A 2 μ g	0	-	0
HSV gp 0.2 μ g + Lipid A 2 μ g	0	-	0
PC:Chol-HSV gp 5 μ g + Lipid A 2 μ g	6.0	-	0
PC:Chol-HSV gp 1 μ g + Lipid A 2 μ g	4.7	-	0
PC:Chol-HSV gp 0.2 μ g + Lipid A 2 μ g	0	-	0
PC:Chol-HSV gp 5 μ g-Lipid A 2 μ g	7.1	-	0
PC:Chol-HSV gp 1 μ g-Lipid A 2 μ g	4.2	-	0
PC:Chol-HSV gp 0.2 μ g-Lipid A 2 μ g	0	-	0
10 ⁷ PFU HSV-2 HI ^e	6.1	-	0
10 ⁷ PFU HSV-1	33.6	+++	70

^aBALB/c mice were injected intraperitoneally with inoculum on day 1, bled 10 days later and reinoculated, bled 10 days later and tested for antibody titer, DTH and CTL activity.

^bSecondary CTL directed against HSV-1 virus were tested for using HSV-1 infected L-cells and A-31 cells and 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.

^cDetergent extracted HSV-1 virus was dialyzed to remove detergent, forming aggregates of antigen.

^dPC:Chol, Phosphatidylcholines:Cholesterol 7:2 mole ratio.

^eVirus was heat inactivated by heating for 1 hour, at 56°C.

This table shows that while free HSV-1 glycoprotein aggregates are not very immunogenic by themselves their immunogenicity can be enhanced at least 3 fold by incorporating the antigens into liposomal bilayers. It can also be seen that addition of the adjuvant, lipid A, to the free glycoproteins can stimulate an even more dramatic increase in antibody titer. Five μg of free glycoprotein plus 2 μg of lipid A gives approximately a 16 fold higher antibody titer than free glycoprotein alone and a 5 fold higher response than the liposome incorporated HSV-1 antigens. This same table also shows that if the HSV-1 antigens are incorporated into liposomes and free lipid A is added one finds an even greater enhancement in antibody titer as opposed to the free glycoproteins and free lipid A. By incorporating the HSV-1 antigen into a liposome one finds a five fold higher titer than that achieved by the free glycoproteins and the same dose of lipid A. With 5 μg of HSV-1 antigens incorporated into a liposome and 2 μg of lipid A added there is a 80 fold increase in antibody titer over that achieved by free glycoprotein alone and a 25 fold increase over liposome incorporated glycoproteins. The most dramatic results were obtained when both HSV-1 antigens and lipid A were incorporated in the same liposomes. When 5 μg of HSV-1 antigens and 2 μg of lipid A were incorporated into the same liposomes these liposomes induced an antibody titer that was about 4 fold higher than that induced by liposome incorporated HSV-1 antigens and free lipid A at the same dose. This response was a remarkable 305 fold increase in antibody titer over that achieved by the same dose of free viral antigens. The level of antibody titer obtained by 5 μg of antigen and 2 μg of lipid A incorporated in the same liposomes was close to 3 fold higher than that achieved by live virus.

Table 6 also demonstrates that no combination of HSV-1 antigens and lipid A could induce a cellular response. The inability of these liposomes to induce a cellular response was further examined using liposomes containing the mouse major histocompatibility antigens along with the HSV-1 antigens since we previously showed that liposomes containing HSV-1 antigens and H-2 antigens could induce a secondary cytotoxic T-cell response in vitro (43). Groups of mice were inoculated twice with liposomes containing HSV-1 and H-2 antigens, at doses similar to those used in the in vitro studies. The mice were then tested for both humoral and cellular immune responses and the data in Table 7 shows that no measurable cellular response could be detected and the antibody response was minimal, thereby eliminating H-2 antigens as a necessary component in inducing an in vivo response to viral antigens.

To be certain that intraperitoneal injections were the optimal route to administer the liposomes we tested the effect of injection site on the induction of immune responses. Table 8 shows that the site of injection had little effect on the liposomes' ability to induce a humoral response and no route gave a cellular response.

We were also concerned about the ability of lipid A to induce anti-lipid antibodies and subsequent autoimmune complications because it has been reported that high levels of lipid A, 1.5 mg, can induce anti PC antibodies when incorporated into liposomes (53). We attempted to detect any anti PC:Chol antibodies in the sera collected during these experiments and were unable to detect any antibody activities (data not shown). This is presumably due to the low levels of lipid A we use. We also tested the sera for anti HEP-

Table 7. Induction of Anti-Herpes Simplex Virus Immune Responses by Liposomes Containing Viral Antigens and H-2 Antigens

Inoculum ^a	Antibody Titer (RIA units $\times 10^{-3}$)	DTH	²⁰ CTL % Cr51 Release ^b
PC:Chol ^b -HSV gp 25 μ g-H-2K 75 μ g	0	-	0
PC:Chol-HSV gp 5 μ g-H-2K 75 μ g	13.3	-	0
PC:Chol-HSV gp 25 μ g + PC:Chol-H-2K 75 μ g	3.8	-	0
PC:Chol-HSV gp 5 μ g + PC:Chol-H-2K 75 μ g	3.0	-	0
PC:Chol-HSV gp 25 μ g	2.2	-	0
PC:Chol-HSV gp 5 μ g	8.8	-	0
10 ⁷ PFU HSV-1 (C ₃ H)	44.6	+++	76
10 ⁷ PFU HSV-1 (BALB/c)	92.9	+++	71

^aC₃H/HeJ mice, (3 per group), were injected intraperitoneally with inoculum on day 1, bled 10 days later and reinoculated, bled 10 days later and tested for antibody titer, DTH, and CTL activity.

^bPC:Chol, Phosphatidylcholine:cholesterol, 7:2 mole ratio.

^cAnimals were sacrificed and spleens were removed and tested for the presence of anti-HSV cytotoxic T lymphocytes.

^dSecondary CTL directed against HSV-1 virus were tested for using HSV-1 infected L-cells and A-31 cells and noninfected L-cells and A-31 cells as targets. Only the results for the HSV-1 infected A-31 cells are reported as all other control target cells were not killed under any conditions tested.

Table 8. Injection Site Effects on Induction of Anti-Herpes Virus Immune Responses by Liposomes Containing HSV-1 Antigens and Lipid A

Site ^a	Antibody Titer (RIA units $\times 10^{-3}$)		DTH	²⁰ CTL %Cr ⁵¹ Release ^b
	1 ^o	2 ^o		
Intraperi- toneally	19	98	-	0
Intravenous	6	90	-	0
Subcutaneously	17	100	-	0
Intramuscular	16	120	-	0

^aBALB/c mice (3 per group) were injected with liposomes containing 8 μ g HSV-1 antigens and 2.5 μ g lipid A.

^bSecondary CTL directed against HSV-1 virus were tested for using HSV-1 infected L-cells and A-31 cells and 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 conditions tested.

2 cell antibodies because our HSV-1 glycoproteins were extracted from virus grown in HEp-2 cells. This test also proved negative (data not shown).

D. DISCUSSION

The data presented in this manuscript clearly show that detergent extracted HSV-1 antigens can invoke an in vivo humoral response if the antigens are presented in the form of liposome incorporated antigens. This is in contrast to free viral antigens which fail to induce any immune response, except at high doses. This apparent adjuvant effect of liposomes has been observed previously in many different systems (7, 8, 9, 22, 23). Yasuda et. al. (7, 8, 9) have reported in their studies that while liposomes act as adjuvants the lipid composition and the density of the synthetic hapten DNP-CAP-PE in liposomes is critical in achieving a maximal immune response. Throughout our study we have used a fixed lipid composition and antigen density; therefore it is possible that the adjuvant activity of these liposomes could be even higher if these parameters are varied.

Another interesting observation in this study is the ability of endotoxin to enhance the immunogenicity of both free and liposome incorporated HSV-1 antigens. This enhancement in antibody production by lipid A was also found to be synergistic with the ability of liposomes to enhance antibody production. Table 6 clearly shows that a mixture of lipid A and free glycoproteins induces an antibody titer higher than that achieved by free glycoproteins alone. This table shows the synergistic effect of lipid A on the adjuvanticity of liposomes since when lipid A is administered with liposome incorporated HSV-1 antigens the resulting antibody titer is significantly higher than that obtained by the

same dose of free glycoproteins and lipid A. The synergism between lipid A and liposome incorporated HSV-I antigens is further enhanced when the two are incorporated into the same liposomes. It was these liposomes that gave the highest antibody titers, as much as 300 fold higher response than free glycoproteins and 3 fold higher response than 10^7 PFU live virus. Dancey et. al. (24) reported that enhancement of liposomal immunogenicity by lipid A requires its presence in the same liposome as the hapten DNP-CAP-PE. Dancey's finding is similar to ours in that we also find the maximal responses when both the lipid A and antigens are incorporated in the same liposome but we also found free lipid A was capable of enhancing the ability of liposome bound antigens to invoke a high antibody titer. We therefore conclude that lipid A can enhance antibody production to antigens when administered either free or incorporated in the same liposomes as the antigen, with the latter giving the highest antibody titers under carefully controlled conditions.

It was also noted in the results that all attempts at inducing a cellular response to HSV-I virus with liposome incorporated or free virus antigens proved to be fruitless. This was a bit distressing because many lines of evidence indicate that in the recovery from several viral infections, including herpesvirus, T lymphocytes play a major role (3, 54). Therefore, it is essential that any antiviral vaccine should stimulate a T cell response. The inability of the subunit vaccines employed in this study, to induce a T-cell response may be attributed to the inability of liposomes to properly interact with or stimulate all the subsets of T-cells required to invoke a T-cell response. In vitro work done in Burakoff's laboratory (55) has provided evidence that liposome associated antigens may fail to adequately stimulate all components involved in CTL induction. His

laboratory has shown that alloantigen-containing liposomes could be made at least 20 fold more immunogenic by first feeding the liposomes to the Ia positive syngeneic macrophages and then adding these macrophages to a syngeneic spleen cell population along with more liposomes or by eliminating the macrophage step and adding an extraneous source of interleukin-2, the T-helper cell product (55). It is perhaps not surprising that lipid A containing liposomes failed to stimulate a T cell response, since lipid A is a known B cell mitogen and would not be expected to stimulate the appropriate T cells (57). We are presently preparing liposomes containing HSV-I antigens and a T cell mitogen, a lipid soluble derivative of muramyl dipeptide. We are also examining the effect exogenous interleukin 2 has on the ability of these liposomes to induce a T cell response.

CHAPTER V

A MODEL FOR CTL INDUCTION AND SPECULATIONS

This chapter is intended to bring together the conclusions and possibilities discussed in earlier chapters concerning the induction of secondary CTL responses in mice. This will be accomplished with the aid of a proposed model that explains the nature of the secondary CTL induction process.

The induction of secondary allogeneic and syngeneic CTL responses by whole cells, liposomes containing viral and/or MHC antigens and viruses is a well documented phenomenon, but experiments aimed at elucidating the sequence of events surrounding this process have just recently been undertaken. Therefore, with all the available data considered, I present the model in Figure 9 as a working hypothesis for the induction of secondary CTL. This model involves the interactions of 3 different cell types, 2 soluble protein factors and antigen. First, the cell types involved are, macrophages (M ϕ), T helper cells (TH), which express the cell surface antigen Lyt-1, and cytotoxic lymphocytes (CTL) which express the cell surface antigens Lyt 2 and 3. Second, the soluble protein factors are interleukin:1 (IL-1) which is a M ϕ derived protein that stimulates the maturation of pre T-helper cells into mature T helper cells and interleukin 2 (IL-2) which is a mature T helper cell derived protein that stimulates the development of CTL. The third component of this model is antigen which can have many forms including soluble antigens, whole cells, viruses, liposomes and others. In discussing the model, I will use liposomes as the antigen since liposomes are the antigens of interest in this dissertation.

In inducing a secondary CTL response in a spleen cell population taken from preimmune mice the induction process begins with the addition of liposomes

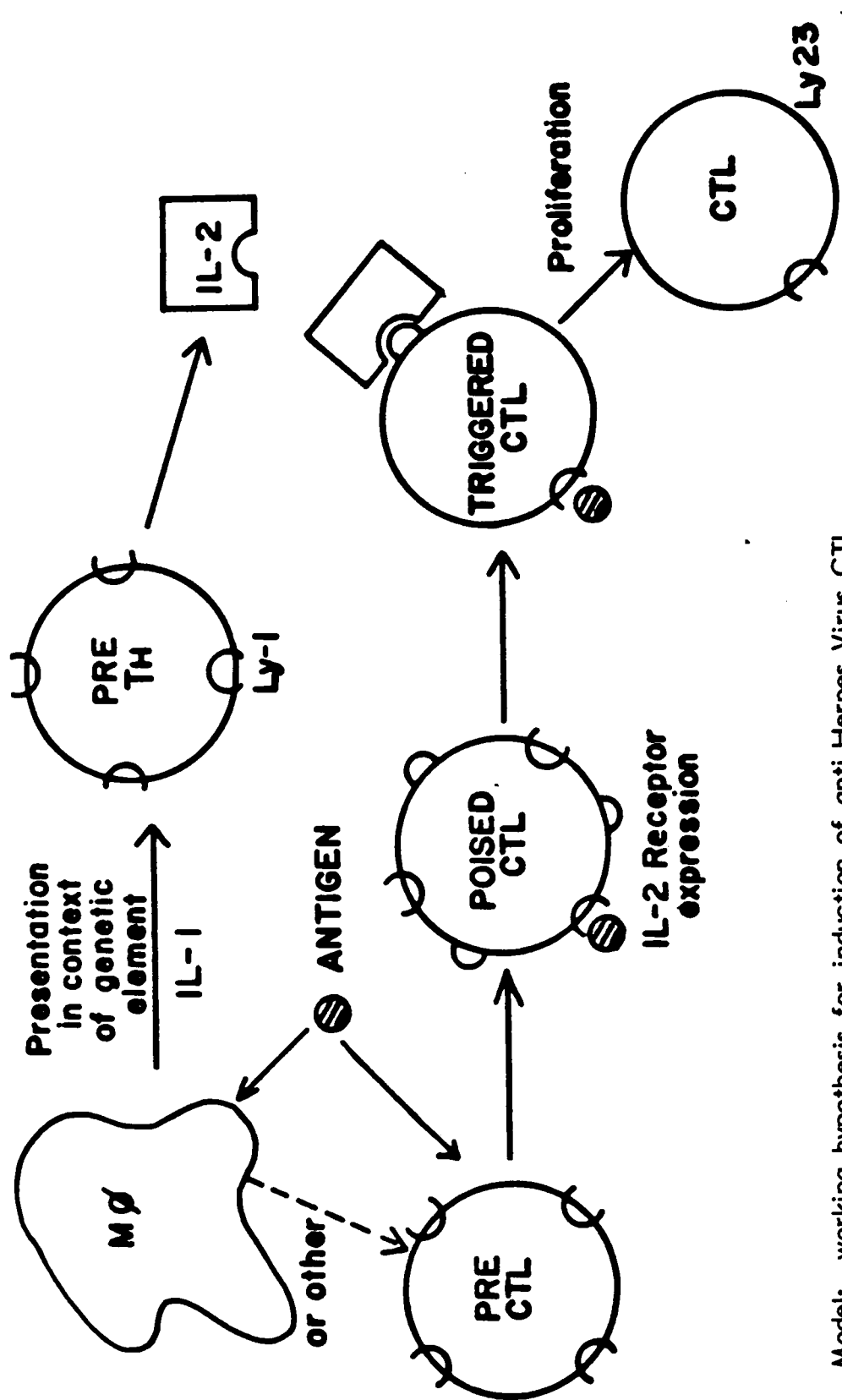


Figure 9. Model: working hypothesis for induction of anti-Herpes Virus CTL.

or antigen to the population. As depicted by the model the liposomes react with both $M\phi$ and CTL's in varying states of development. First, the interaction of liposomes with $M\phi$ results in their uptake and subsequent processing such that the liposomal antigens are now expressed on the macrophage cell surface in context with the MHC Ia antigens. This $M\phi$ also begins to liberate IL-1 which in turn stimulates the development of T helper cells but this maturation process is not complete until the $M\phi$ presents the processed antigen to the T helper cell. After the T helper cell interacts with both IL-1 and $M\phi$ it now matures and produces IL-2 which is necessary for development of CTL. The second branch of this pathway involves the liposomes interaction with pre-CTL and poised CTL. The interaction of liposomes with the CTL may not be the only way these cells can be induced as it is possible that the $M\phi$ processed liposomes may also serve as inducers. The next step in the CTL maturation process in terms of timing is not well understood but we propose that pre CTL interact with liposomes and become poised CTL which become further developed upon interaction with IL-2. Once the naive CTL interact appropriately with both liposomes and IL-2 it now develops into a mature CTL and proliferates. Evidence for this model has been provided by work done by Weinberger (59, 60), Burakoff (61) and Herrmann (46). First, Weinberger et. al. showed that induction of an allogeneic secondary CTL response with liposomes containing H-2K^k antigen required the presence of Ia antigen bearing $M\phi$ in the cultured spleen cell population (59). Weinberger also showed that ¹²⁵I labelled H-2K^k antigen incorporated in liposomes was found to be $M\phi$ associated after several hours of incubation. He also showed that by incubating Ia positive $M\phi$ with H-2K^k containing liposomes prior to adding spleen cells to this preparation and subsequently adding additional liposomes, the pre-incubation of $M\phi$ with liposomes

reduced the quantity of liposomes needed to induce the same response in spleen cell populations not treated with liposome associated M ϕ by 20 fold (59). Also incubation of the spleen cell population with anti Ia serum inhibited the induction of CTL (59). Further evidence by this author showed that if M ϕ were inactivated by ultraviolet irradiation, induction was abrogated but if M ϕ were allowed to react with liposomes for at least 4 hrs before U.V. irradiation and IL-1 was added to the preparation induction could still occur (59). This evidence clearly links M ϕ to IL-1 production and possible processing of antigens. Both Weinberger (60) and Burakoff (61) show that T-helper cells produce IL-2 and Burakoff (61) also showed that 5×10^4 cell equivalents of H-2K^k antigen incorporated into liposomes was ineffective in inducing a CTL response but with the addition of IL-2 a maximal CTL response was achieved. This data indicated that low levels of antigen were ineffective at inducing the production of IL-2 but still had the capacity to interact with CTL's. This inability of low levels of antigen to stimulate the production of IL-2 may not only be due to a dose effect because Herrmann has reported that low levels of antigen can be made more immunogenic with the addition of matrix proteins (46). Therefore, with regard to the experiments conducted in this dissertation, it is quite possible that in inducing anti herpes simplex virus CTL, we are failing adequately to stimulate the production of IL-2 due to low antigen dose or lack of matrix proteins. In our in vivo studies we may find it very advantageous to add exogenous IL-2 to the mice since it appears this part of the induction pathway is most sensitive to dose and requires the interaction of large numbers of M ϕ with the antigens.

LIST OF REFERENCES

LIST OF REFERENCES

1. Hilleman, M.R. 1976. Herpes Simplex Virus Vaccines. *Cancer Res.* 36:857.
2. Nahmias, A. and B. Roizman. 1973. Infections with Herpes-Simplex Viruses 1 and 2. *New Eng. J. Med.* 289:667.
3. Rouse, B.T. and Babiuk. 1978. Mechanisms of Recovery from Herpes Virus Infections *Canad. J. Comp. Med.* 42:414.
4. Shore, S.L. and A.J. Nahmias. 1981. Immunology of Human Infections in *Comprehensive Virology* Fraenkel-Conrat and Eager (ed.), Plenum Press. In Press.
5. Spear, P.G. and B. Roizman, 1980. Herpes Simplex Viruses, In *Molecular Biology of Tumor Viruses*, 2nd edition J. Tooze (ed.), Cold Spring Harbor Laboratory p. 615.
6. Naylor, P.T., H.S. Larsen, L. Huang, and B.T. Rouse, 1981. In Vivo Induction of Anti Herpes Simplex Virus Immune Responses by HSV-T Antigens and Lipid A Incorporated Into Liposomes. *J. Immunol.* Submitted for publication.
7. Yasuda, T., G.F. Dancey, and S.C. Kinsky. 1977. Immunogenic Properties of Liposomal Model Membranes. *J. Immunol.* 119: 1863.
8. Dancey, G.F., T. Yasuda, and S.C. Kinsky, 1978. Effect of Liposomal Model Membranes Composition on Immunogenicity. *J. Immunol.* 120: 1109.
9. Yasuda, T., G.F. Dancey, and S.C. Kinsky, 1977. Immunogenicity of Liposomal Model Membranes in Mice: Dependence on Phospholipid Composition. *proc. Natl. Acad. Sci.* 74: 1234.
10. Loh, D., H.R. Alonzo, A.H. Hale, D. Baltimore and H.N. Eisen, 1979. Synthetic Phospholipid Vesicles Containing A Purified Viral Antigen and Cell Membrane Proteins Stimulate the Development of Cytotoxic T Lymphocytes. *J. Exp. Med.* 150: 1067.
11. Finberg, R., M. Mescher and S.F. Burakoff, 1978. The Induction of Virus Specific Cytotoxic T Lymphocytes With Solubilized Viral and Membrane Proteins, *J. Exp. Med.* 148: 1620.
12. Bangham, A.D., M.M. Standish and J.C. Watkins, 1965. Diffusion of Univalent Ions Across the Lamellae of Swollen Phospholipids. *J. Mol. Biol.* 13: 238.
13. Pagano, R.E. and J.N. Weinstein, 1978. Interactions of Liposomes With Mammalian Cells. *Ann. Rev. Biophys. Bioeng.* 7: 435.

14. Tadakuma, T., T. Yasuda, S.C. Kinsky, and C.W. Pierce, 1980. The Effect of Epitope Density on the In Vitro Immunogenicity of Hapten-Sensitized Liposomal Model Membranes. J. Immunol. 124: 2175.
15. Lemonnier, F., M. Mescher, L. Sherman and S. Burakoff, 1978. The Induction of Cytotoxic T Lymphocytes With Purified Plasma Membranes. J. Immunol. 120: 1114.
16. Mescher, M., L. Sherman, F. Lemonnier and S. Burakoff, 1978. The Induction of Secondary Cytotoxic T Lymphocytes by Solubilized Membrane Proteins. J. Exp. Med. 147: 946.
17. Fast, L.D. and D.P. Fan, 1978. Dissociated and Reconstituted Subcellular Alloantigen Capable of Stimulating Mouse Cytotoxic T Lymphocytes In Vitro. J. Immunol. 120: 1092.
18. Engelhard, V.H., J.L. Strominger, M. Mescher and S. Burakoff, 1978. Induction of Secondary Cytotoxic T Lymphocytes by Purified HLA-A and HLA-B Antigens Reconstituted Into Phospholipid Vesicles. Proc. Natl. Acad. Sci. 75: 56818.
19. Engelhard, V.H., J.F. Kaufman, J.L. Strominger and S. Burakoff, 1980. Specificity of Mouse Cytotoxic T Lymphocytes Stimulated With Either HLA-A or HLA-B or HLA-DR Antigens Reconstituted Into Phospholipids Vesicles. J. Exp. Med. 152: 54_s.
20. Hale, A.H., M.J. Ruebush and D.T. Harris, 1980. Elicitation of Anti-Vesicular Stomatitis Virus Cytotoxic T Lymphocytes With Purified G Protein and H-2 Antigens Incorporated Into Phospholipids Vesicles. J. Immunol. In Press
21. Hale, A.H., M.J. Ruebush and D.T. Harris, 1980. Elicitation of Anti-Viral Cytotoxic T Lymphocytes With Purified Viral and H-2 Antigens. J. Immunol. 125: 428.
22. Manesis, E.K., C.H. Cameron and G. Gregoriadis, 1979. Hepatitis B Surface Antigen-Containing Liposomes Enhance Humoral and Cell-Mediated Immunity to the Antigen. FEBS Letters. 102: 107.
23. Rooijen, N.V. and R.V. Nieuwmegen, 1980. Liposomes in Immunology: Multilamellar Phosphatidylcholine Liposomes as a Simple, Biodegradable and Harmless Adjuvant Without Any Immunogenic Activity of It's Own. Immunol. Comm. 9: 243.
24. Dancey, G.F., T. Yasuda and S.C. Kinsky, 1977. Enhancement of Liposomal Model Membrane Immunogenicity by Incorporation of Lipid A. J. Immunol. 119: 1868.
25. Lawman, M.J.P., B.T. Rouse, R.J. Courtney and R.D. Walker. (1980). Cell Mediated Immunity Against Herpes Simplex: Induction of Cytotoxic T Lymphocytes. Infect. Immun. 27: 133.

26. Hale, A.H., M. Ruebush and D.T. Harris. (1980). Elucidation of Antiviral Cytotoxic T Lymphocytes with Purified Viral and H-2 antigens. *J. Immunol.* In press.
27. Bone, D.R. and R.J. Courtney. (1974). A Temperature Sensitive Mutant of Herpes Simplex Virus Type I Defective in the Synthesis of the Major Capsid Polypeptide. *J. Gen. Virol.* 24: 17.
28. Wallach, D.F.H. and V.B. Kamat. (1966). Preparation of Plasma Membrane Fragments from Mouse Ascites Tumor Cells. *Methods in Enzymology* 8: 164.
29. Hermann, S.H. and M.F. Mescher. 1979. Purification of the H-2K^k Molecules of the Murine Major Histocompatibility Complex. *J. Biol. Chem.* 254: 8713.
30. Hunter, N.M. and F.C. Greenwood. (1962). Preparation of ¹³¹Iodine Labelled Human Growth Hormone at High Specific Activity. *Nature* 194: 495.
31. Courtney, R.J., R.M. McCombs and M. Benyesh-Melnick. (1971). Antigens Specified by Herpes Virus. II Effect of Arginine Deprivation in the Synthesis of Cytoplasmic and Nuclear Proteins. *Virol.* 43: 356.
32. Rouse, B.T. and M.J.P. Lawman. (1980). Induction of Cytotoxic T Lymphocytes Against Herpes Simplex Virus Type I: Role of Accessory Cells and Amplifying Factor. *J. Immunol.* 124:2341.
33. Burakoff, S.J., C.S. Reiss, R. Finberg and M.F. Mescher. (1980). Cell Mediated Immunity to Viral Glycoproteins. *Rev. Inf. Dis.* 2: 62.
34. Zinkernagel, R.M. and P.C. Doherty. (1979). MHC Restricted Cytotoxic T Cells: Studies on the Biological Role of Polymorphic Major Transplantation Antigens Determining T Cell Restriction Specificity, Function and Responsiveness. *Adv. Immunol.* 27: 51.
35. Cerottini, J.C. and K.T. Brunner. (1976). Cell Mediated Cytotoxicity, Allograft Rejection and Tumor Immunity. *Adv. Immunol.* 18: 67.
36. Lentz, B.R., Y. Barenholz and T.E. Thompson. (1976). Fluorescence Depolarization Studies of Phase Transitions and Fluidity in Phospholipid Bilayers. I. Single Component Phosphatidylcholine Liposomes. *Biochem.* 20:4521.
37. Shinitzky, M., A.C. Dianoux, C. Gitler and G. Weber. (1971). Microviscosity and Order in the Hydrocarbon Region of Micelles and Membranes Determined with Fluorescent Probes. I. Synthetic Micelles. *Biochem.* 10:2106.

38. Shinitzky, M., and Y. Barenholz. (1974). Dynamics of the Hydrocarbon Layer in Liposomes of Lecithin and Sphingomyelin Containing Dicetylphosphate. *J. Bio. Chem.* 249:2652.
39. Hale, A.M., and M.J. Ruebush. 1980. Minimal Molecular and Cellular Requirements for Elicitation of Secondary Anti-Vesicular Stomatitis Virus Cytotoxic T lymphocytes. *J. Immunol.* 125:1569.
40. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1955. Protein Measurement with the Folin Phenol Reagent. *J. Bio. Chem.* 193:265.
41. Hubbard, A.L. and Z.A. Cohn. 1975. Externally Disposed Plasma Membrane Proteins I Enzymatic Iodination of Mouse L cells. *J. Cell. Biol.* 64:438.
42. Oi, V.T., P.P. Jones, J.W. Goding, L.A. Herzenberg and L.A. Herzenberg. 1978. Hybridoma Antibody Production In Current Topics in Microbiology and Immunology. Melchers, Potter, and Warner Eds. Springer-Verlag p. 115.
43. Lawman, M.J.P., P.T. Naylor, L. Huang, R.J. Courtney and B.T. Rouse. 1981. Cell-Mediated Immunity to Herpes Simplex Virus: Induction of Cytotoxic T Lymphocyte Responses by Viral Antigens Incorporated Into Liposomes. *J. Immunol.* 126:304.
44. Laemmli, U.K. 1970. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature.* 227:680.
45. Huang, L., and R.E. Pagano. 1975. Interaction of Phospholipid Vesicles with Cultured Mammalian Cells. *J. Cell Bio.* 67:38.
46. Herrmann, S.H. and M.F. Mescher. 1981. Secondary Cytotoxic T lymphocyte Stimulation By Purified H-2K^k In Liposomes. *Proc. Natl. Acad. Sci.* 78:2488.
47. Carter, V.C., P.A. Schaffer and S.S. Tevethia. 1981. The Involvement of Herpes Simplex Virus Type I Glycoproteins In Cell-Mediated Immunity *J. Immunol.* 126:1655.
48. Galanos, C., O. Luderitz, and O. Westphal. 1969. A New Method for the Extraction of R Lipopolysaccharides. *Eur. J. Biochem.* 9:245.
49. Skelly, R.R., P. Munkenbeck and D.C. Morrison. 1979. Stimulation of T-Independent Antibody Responses by Hapten-Lipopolysaccharides without Repeating Polymeric Structure. *Infect. Immun.* 23:287.
50. Cynkin, M.A., and G. Ashwell. 1960. Estimation of 2-deoxy Sugars by Means of the Malonaldehyde-Thiobarbituric acid reaction. *Nature (London)* 186:155.

51. Zollinger, W.D., J.M. Dalrymple and M.S. Artenstein. 1976. Analysis of Parameters Affecting the Solid Phase Radioimmunoassay Quantitation of Antibody to Meningococcal Antigens. *J. Immun.* 117:1788.
52. Crowle, A.J. 1975. Delayed Hypersensitivity in the Mouse. *Adv. Immunol.* 20:197.
53. Schuster, B.G., M. Neidig, B.M. Alving and C.R. Alving. 1979. Production of Antibodies Against Phosphocholine, Phosphatidylcholine, Sphingomyelin, and lipid A by Injection of Liposomes Containing Lipid A. *J. Immunol.* 122:900.
54. Bloom, B.R. and B.R. Zisman. 1975. Cell Mediated Immunity in Viral Infections. In *Viral Immunology and Immunopathology*. A.L. Notkins, ed. Academic Press, New York. p. 113.
55. Burakoff, S., and M. Mescher. 1981. Reconstituted Membranes and Liposomes in the Study of Lymphocyte Interactions. *Cell Surface Reviews*. In Press.
56. Alving, C.R., B. Banerji, J.D. Clements and R.L. Richards. 1980. Adjuvanticity of Lipid A and Lipid A fractions in Liposomes. In *Liposomes and Immunobiology*. B. Tom and H. Six, Ed. Elsevier. p 67.
57. Berry, L. J. 1977. Bacterial Toxins. In *CRC Critical Reviews in Toxicology*. p. 239.
58. Lawman, M.J.P., R.J. Courtney, R. Eberle, P.A. Schaffer, M.K. O'Hara and B.T. Rouse. 1980. Cell-Mediated Immunity to Herpes Simplex Virus: Specificity of Cytotoxic T Cells. *Infect. Immun.* 30:451.
59. Weinberger, O., S.H. Herrmann, M.F. Mescher, B. Benacerraf and S.J. Burakoff. 1980. Cellular Interactions in the Generation of Cytotoxic T Lymphocyte Responses: Role of Ia positive Splenic Adherent Cells In Presentation of H-2 Antigen. *Proc. Natl. Acad. Sci.* 77:6091.
60. Weinberger, O., S.H. Herrmann, S.H. Mescher, B. Benacerraf and S.J. Burakoff. 1981. Cellular Interactions in the Generation of Cytotoxic T Lymphocyte Responses. Analysis of the Helper T Cell Pathway. *Eur. J. Immunol.* In press.
61. Burakoff, S.J., O. Weinberger, S.H. Herrmann and M.F. Mesher. 1981. Cellular Interactions in the Regulation of the Cytotoxic T lymphocyte Response: The Effect of Suboptimal Antigen Dose. *Transplant. Proc.* In press.

APPENDIX

ABBREVIATION LIST

HSV-I	Herpes Simplex Virus Type I
CTL	Cytotoxic T Lymphocytes
DNA	Deoxyribonucleic Acid
MLV	Multilamellar Vesicles
SUV	Small Unilamellar Vesicles
LUV	Large Unilamellar Vesicles
DNP-CAP-PE	Dinitrophenyl-E-Aminocaproyl-Phosphatidylethanolamine
MHC	Major Histocompatibility
VSV	Vesicular Stomatitis Virus
PFU	Plaque Forming Unit
FCS	Fetal Calf Serum
DCS	Donor Calf Serum
PBS	Phosphate Buffered Saline Solution
PSI	Pounds Per Square Inch
RSB	Reticulocyte Standard Buffer
DOC	Deoxycholate
PC	Phosphatidylcholine
Chol	Cholesterol
PMSF	Phenyl Methyl Sulfanyl Fluoride
SDS	Sodium Doecyl Sulphate
PM	Plasma Membrane
DPH	1,6-Diphenyl-1,3,5-Mexatriene
K_D	Dissociation Constant
DCP	Dicetyl Phosphate

SM	Sphingomyelin
DPPC	Dipalmitoyl Phosphatidylcholine
HBSS	Hank's Balanced Salt Solution
NP-40	Nonidet P40
PBM	Phosphate Buffered Magnesium
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
KDO	2 -Keto-3-Deoxyoctanoic Acid
RIA	Radioimmunoassay
DTH	Delayed Type Hypersensitivity
M ϕ	Macrophages
TH	T Helper Cells
IL-1	Interlukin 1
IL-2	Interlukin 2

VITA

Paul Thomas Naylor was born October 12, 1952 in Plainfield, New Jersey. He attended primary and secondary school in New Jersey and upon graduating from Hunterdon Central High School in June 1971, he moved to Maryville, Tennessee to attend Maryville College. Paul graduated from Maryville College in June 1975 with a B.A. degree. He then enrolled in the Department of Biochemistry at The University of Tennessee, Knoxville to obtain a Ph.D. degree. After receiving a Ph.D. this summer, Paul will be moving to Memphis, Tennessee to enroll in the UTCHS College of Medicine to pursue a M.D. degree.