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Influence of peptide acylation, liposome incorporation and synthetic immunomodulators on the immunogenicity of herpes simplex virus glycoprotein D peptides : implications for subunit vaccines

Ketil Brynestad

To the Graduate Council:

I am submitting herewith a thesis written by Ketil Brynestad entitled "Influence of peptide acylation, liposome incorporation and synthetic immunomodulators on the immunogenicity of herpes simplex virus glycoprotein D peptides : implications for subunit vaccines." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Barry T. Rouse, Major Professor

We have read this thesis and recommend its acceptance:

Robert Moore, Alex Osmand

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Ketil Brynestad
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Abstract

The goal for the development of a synthetic vaccine for herpes simplex viruses is a chemically defined, safe, and effective formulation containing relevant B and T cell epitopes without a macromolecular carrier. Such a synthetic vaccine would eliminate the irrelevant determinants of the virus that may cause undesirable side effects. In this study, a peptide corresponding to amino acid residues 1-23 of glycoprotein D of herpes simplex virus type 1 was chemically synthesized and coupled to a fatty acid carrier by standard Merrifield synthesis procedures. The resulting peptide-palmitic acid conjugate (acylpeptide) exhibited enhanced immunogenicity in mice as compared to that exhibited by the free form of the peptide. When the the acylpeptide was incorporated into liposomes the immunogenicity of the peptide was further increased. Inclusion of the immunomodulators muramyl tripeptide phosphatidlyethanolamine and monophosphoryl lipid A into the same liposome stimulated the strongest immune response. The humoral immune responses induced by the acylpeptide-liposome construct were greater than those induced by peptide in Freund complete adjuvant, and cellular responses were equivalent. The acylpeptide-immunomodulator-liposome formulation also induced significant levels of protective immunity, although the immunity was less than that induced by herpes simplex virus infection. Acylated peptides, especially in liposomes, were taken up more effectively by draining lymph nodes, which possibly explains in part the enhanced immunogenicity of the peptides. Since the acylpeptide-immunoliposome formulation used was nontoxic, it represents a useful way to enhance immunogenicity of subunit peptides used for vaccine purposes in humans and animals.

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Chapter 1

Introduction

A. Herpes simplex virus

1. Pathogenesis of infection

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are responsible for a variety of clinical infections ranging in severity from relatively mild recurrent mucocutaneous lesions of the oral and genital regions to life-threatening encephalitis. These viruses are often responsible for disseminated infections in immunocompromised hosts. Oral infections are usually associated with HSV-1, whereas HSV-2 primarily causes genital infections. Ocular infections frequently lead to scaring of the cornea which impaires vision and is a leading cause of blindness in developed countries. The incidence of neonatal HSV infection, a devastating illness with a high mortality rate, is on the rise. Infections caused by HSV have been recognized since the ancient Greek ages and are among the most prevalent of all encountered by humans. HSV has a complex structure consisting of a linear, double stranded DNA genome in an icosahedral nucleocapsid surrounded by an amorphous layer known as the tegument, and a trilaminar membrane envelope derived from the host cell. After a primary acute infection of the epithelium with HSV, the virus infiltrates sensory nerves innervating the surface epithelial cells at the portal of entry. This results in the establishment of a latent infection during which the virus is present in a nonreplicating

state in the dorsal root ganglia of nerves associated with the site of the original lesion (1). This latent infection is maintained for the life of the individual. After latency is established, certain common triggering factors, including ultra-violet irradiation, immunosuppression, and trauma of the skin or infected nerves, will cause the virus to reactivate (2). Reactivation of the virus in the infected ganglion allows the virus to replicate and move along the sensory nerve to the skin. These periodical recrudescences result in secondary cutaneous herpetic lesions which contain infectious virus (3). The development of clinically apparent lesions is probably influenced by the amount and nature of the infecting virus as well as the reaction time and degree of the host immune response (4). Because infection is rarely fatal and these viruses have the capacity to become latent, it is estimated that over one-third of the world's population has recurrent HSV infections and, therefore, can transmit HSV during episodes of productive infection.

The potential of HSV to cause both acute infection and persist in a latent state poses significant problems for vaccine development. Ideally a vaccine against HSV would stimulate long term protection from acute disease and eliminate or decrease the frequency and severity of recurrent infections. A more complete understanding of the pathogenesis of HSV infection is vital to the development of strategies for preventing not only primary HSV infections but also recurrent infections caused by reactivation of latent virus.

2. Immune response to herpes simplex virus

Although substantial progress has been made in understanding the molecular biology of HSV, the exact mechanisms operating during recovery and protection from HSV infection have not been fully explained with respect

to the role of the varied components of adaptive and inate immunity. Aspects of both humoral and cellular immunity appear to be required for recovery (5-10), though the relative role played by each is unclear. Clearance of the initail site of infection is mediated predominantly by T cells and B cells producing antibody. Ultimate recovery is dependent on the magnitude of these responses. Optimal responses of both types seem to require exposure to live virus. In addition, the immune response to HSV during the acute phase of primary infection involves elements of the host's innate immunity. This antiherpetic activity, which does not require prior exposure to the virus, is induced as a first line of defense, and involves interaction between macrophages, natural killer (NK) cells, interferon (IFN), and other cytokines (4,11-13). Although these non-specific defense mechanisms have been shown to help control HSV infection, their relation to any putative vaccine remains obscure since ultimate recovery from infection is dependent on aquired immune mechanisms.

3. The role of T lymphocytes

The pivotal role played by T lymphocytes in the recovery from primary HSV infections has been clearly demonstrated. The observations that depleting either CD4+ or CD8+ T cell subsets in vivo with specific monoclonal antibodies increases susceptibility to subsequent infection (14), and adoptive transfer of either CD4+ or CD8+ T cells confers protection against either local or systemic infection (15,16), suggests that T cells play an important role in controling HSV-1. $CD4^+$ T cells (MHC class II restricted, T helper lymphocytes and delayed hypersensivity lymphocytes) are required to clear HSV-1 from the periphery, while $CD8^+$ T cells (MHC class I restricted, cytotoxic T lymphocytes

(CTL)) appear to play a role in controlling nervous system infections (17). CTL have also been shown to mediate protective immunity against HSV by killing virus-infected target cells (6,16,18). A majority of CTL have been found to be glycoprotein specific, several of these glycoproteins elicit good CTL responses in immunized animals (19-21). However, Martin et al. (22) showed that approximately one third of the total CTL response specific for HSV-1 was directed against the immediate early protein ICP4. This indicates the importance of nonstructural HSV proteins in generating protective immunity. HSV glycoproteins can also elicit delayed-type-hypersensitivity (DTH) T lymphocytes that are believed to play a role in rapid early clearance of the virus (7,8). T helper lymphocytes are important in clearance of HSV-1 since they are responsible for generating antiviral IgG. Moreover, by providing specific growth factors, T helper lymphocytes are responsible for augmenting the activity of HSV-specific B cells, CTL, NK cells, and macrophages.

4. The role of B lymphocytes

Since neutralizing antibody (nAb) responses do not appear until after virus has been cleared from primary infection sites, humoral immunity does not appear to play a significant role in protecting the host from primary infection. Virus colonizes the local sensory ganglia by 24 to 48 hours postinfection (23), long before antibody responses can be detected. Yet, pre-existing antibody could influence the frequency and severity of recrudescence (24,25), and is unquestionably important in helping decrease the severity of primary infection since it has been demonstrated that mice depleted of B cells develop a more severe infection (26), and exhibit a much more extensive nervous system infection (27). In B cell-suppressed mice,

infected with low doses of virus, T cell mediated immunity alone was able to prevent infection, while high doses caused zosteriform rashes which isindicative of virus spread between neurons. This suggests that antibody plays a supportive role in restricting virus spread (27). Therefore, although the normal antibody response is too late to prevent ganglionic infection, it may occur early enough to reduce the subsquent spread of virus between neurons. Antiserum administered early in the course of infection has also been shown to reduce its severity and limit the spread of virus to the sensory ganglia (7,28), possibly by inhibiting of the transfer of virus from the epithelium to the nervous system (29). This suggests that viral antibody plays an important role in promoting recovery from HSV infections. Although the mechanisms by which antibodies specific for HSV mediate protection in vivo remains uncertain, antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and extracellular neutralization of virions are all thought to play a role in clearing virus from established sites of infection, restricting viral spread, and modifying the appearance and severity of neurological illness induced by virus (28,30). Consequently, it is important to understand how best to induce humoral as well as T cell immunity if any form of vaccine is to be optimally effective.

Animal models can approximate imitation of HSV latency and pathogenesis in humans. The murine model used in this study has been particularly useful in clarifying many of these aspects. Although they cannot serve as a true analog for human HSV disease, mice have been shown to mimic many of the features of HSV infection in man (14,31). Additionally, mice are attractive models due to the wide variety of inbred strains available and the many reagents developed for depleting the various murine lymphocyte subpopulations. For these reasons, mice are an attractive and informative experimental tool for both investigating the role of the complex

immunological responses in human herpetic disease and for designing a successful vaccine which is able to prevent or lessen the severity of HSV infection.

B. HSV Glycoproteins

The antigens encoded by HSV which play a role in mediating protective immunity against viral infection have not yet been completely identified. The identification of candidate antigens for use in vaccines against HSV is complicated in that at least 75 open reading frames have been identified by computer analysis of the HSV genome, and these must be used to choose candidate antigens such as proteins or peptides. Although the function of many of these genes in viral replication or pathogenicity is not known, the major viral glycoproteins have been implicated as playing a prominent role and, thus, are the best candidates for subunit vaccines. Currently, eight antigenically distinct glycoprotein species have been identified. These glycoproteins, designated gB, gC, gD, gE, gG, gH, gI, and gJ, are the major components specific to HSV found on the herpes simplex virion envelope and in membranes of cells infected with virus (9,32-37), and appear to all be involved in the immune response to HSV (38,39). Of these, gC, gE, gG, gI, and gJ are dispensable for replication in most cells in culture (34,40,41), while the other three glycoproteins, gB, gD, and gH, are indispensible and perform all functions related to entry, maturation, egress, cell to cell spread, and resistance to superinfection (42-44). While a precise function has yet to be determined for many of the dispensible glycoproteins, there is evidence that these components may perform functions similar to the nondispensible glycoproteins (45).

The immune response to a primary HSV infection is complex, with a broad range of antibody responses generated against both structural (core and envelope) and nonstructural (regulatory) proteins observed in both mice and humans. The envelope proteins, however, appear to be the main antibody targets, particularly gB, gC, and gD (46). One of these, (gD), has been shown by numerous studies to play a major role in these responses (24,47-49) and therefore represents a logical choice for a subunit vaccine against HSV infections. Moreover, there is evidence that gD plays an important role during the initial phases of the infectious process. Studies with anti-gD monoclonal antibodies have implicated gD in virus adsorption (50), penetration (51,52), cell-to-cell fusion (53,54), and resistance to superinfection (55,56). Recently, HSV virions lacking the gD gene have been used to demonstrate that gD is required for virus penetration but not for the adsorption of the virus to cells (42). Ever since it was first discovered that gD elicits neutralizing antibody more efficiently than any other virus component (58), a great deal of effort has been devoted to studying the immunogenicity of purified recombinant gD, vaccinia vectors expressing gD, and synthetic peptides to this glycoprotein. In a variety of animal systems, these antigens were shown to be an important target of the host humoral immune responses since gD stimulated high titers of complement-independent neutralizing antibody and strong ADCC and CDC activity (47,51,59,60). In addition, cellular immune responses including T cell proliferation and DTH were induced and protective immunity was conferred, albeit, of varying duration (49,61-69). In the mouse gD does not appear to stimulate a CTL response (21,68,70), but in man it has been shown to be part of the CTL repertoire (71). For these reasons gD peptides are the HSV antigens used in this study.

C. HSV-1 gD Peptides

The genes encoding gD of HSV 1 and 2 have been localized and sequenced (72-74). The polypeptide and carbohydrate structure appears to be the same in different strains of HSV (47), and is highly conserved between HSV-1 and HSV-2 (75,76). The binding properties of anti-gD monoclonal antibodies have been studied by several investigative groups and were used to construct a detailed antigenic map of gD (51,77-79). This panel of 17 monoclonal antibodies, which recognize distinct type-common and type-specific sites on HSV 1 and 2 gD, have been arranged into eight separate groups according to their reactivity with gD. Of the groups, four (I, III, IV and VI) recognize discontinuous epitopes which are dependent on the native conformation of the protein for binding (53,60,80). The four other groups (II, V, VII and XI) recognize continuous epitopes which bind both the denatured and native forms of gD (77,81). One of these epitopes, which reacts with group VII monoclonal antibodies, is type common in nature and has been localized to the amino terminal end (residues 1 - 23) of the mature form of gD (residues 26 - 48 of the predicted sequence) by the use of proteolytic digests and overlapping synthetic peptides (59,82). Similar studies showed that groups II, XI and V react within regions 268 - 287, 284 - 301 and 340 - 356, respectively (77,81). Numerous groups have investigated synthetic peptides specific to HSV for possible vaccine use, with the first 23 amino terminal amino acids of HSV gD receiving the most attention (5,10,59,83-90). Synthetic peptides which mimic residues 1 - 23 and 8 - 23 stimulated the production of antibodies that neutralized HSV type 1 and 2 infectivity in vitro (59,83,850), and elicited specific T-cell immune responses (10,88,90). Mice immunized with these peptides conjugated to a protein carrier and emulsified in complete Freunds

adjuvant (CFA) were protected against a lethal challenge with HSV-2 (5,10,88). This epitope contains a region of high hydrophilicity and has two tight ß-turns which are present in solution and centered on residues 14-15 and 18-19, and may be the source of the immunogenic properties of this peptide (88). Several groups have analyzed the 1-23 peptide in an attempt to map the actual B cell epitope. Weijer, et al. (89) showed that antibodies generated to peptides 1-13, 9-21, and 18-30 reacted with the parent molecule gD and neutralized HSV with anti 9-21 exhibiting the highest titers. The antisera to these peptides did not cross-react, indicating that 1-23 contains more than one B cell epitope. Immunization with peptide 9 - 21 and multiple repeats of this peptide stimulate the production of antibodies which neutralize the virus (86,89) and elicits immune responses mediated by T cells (87), indicating that both a B and a T cell epitope are contained within this peptide. In another study, a predictive algorithm, Surfaceplot (84), was used to predict 15 linear segments of gD which have a high probability of surface exposure. Though all the peptides selected produced positive immune responses as measured by ELISA, the highest HSV-1 neutralizing antibody titers were elicited by peptides composed of amino acid residues 2 - 21 and 12 - 21 of HSV-1 gD (83). The group II epitope was identified by Eisenberg et al. (77) using an anti-gD monoclonal antibody with neutralizing activity. However when peptide 268 - 287 was conjugated to keyhole limpet haemocyanin (KLH) via a C-terminal cysteine and used to immunize rabbits, the antipeptide antibodies produced exhibited no neutralizing activity or binding to gD. In contrast, when Strynadka et al. (83) immunized rabbits with a peptide within this region (267 - 276) and coupled it to an N-terminal spacer group, strong neutralizing antibody responses were elicited. These results suggest that the length of a peptide may influence its conformation or that the choice of carrier protein and the point and method of attachment is critical to the design of immunogenic peptide vaccines. In a

recent study, Geerligs et al. (85) used overlapping peptides which covered the entire amino acid sequence of gD to demonstrate that only peptide 10 - 24 elicited antibodies that were able to neutralize HSV-1 infectivity. The group II peptides used in this study, which were composed of residues 260 - 274, 270 - 284 and 260 - 284, did not elicit antibodies which neutralized virus . The reason for this difference may be that additional amino acids at the C terminus or N terminus can affect the conformation of these peptides resulting in varying antibody responses. A further complication in interpreting the immune response elicited by the group II epitope was reported by Weijer et al. (89). Though the group II peptide 267 - 281 conjugated to BSA did not elicit neutralizing antibodies when given alone, increased virus-neutralizing activity was observed when this peptide was injected with the 9 - 21 peptide. This indicates that peptides 9 - 21 and 267 - 281 may constitute continuous parts of a discontinuous epitope. The amino terminal end of gD has also been shown to be recognized by a majority of T cells from human peripheral blood (91), suggesting that the 1-23 peptide may be an important epitope for inducing anti-HSV immunity and a promising candidate for an HSV-directed vaccine in humans.

D. Protein antigenic determinants

1. Major histocompatibiliy complex restriction

In humans as well as experimental animals there are often genetically controlled variations in immune reponses to peptides. Accordingly, one of the major limitations of peptide vaccines is the substantial influence major histcopatibiliy complex (MHC) genes have on the recognition of these peptides

by the immune system (92). A peptide will only be recognized by lymphocytes if it possesses the appropriate epitope, which determines T cell receptor (TcR) binding capability , as well as the appropriate agretope, which permits binding with class II MHC molecules on the surface of antigen presenting cells (APC) (93). Thus, not all individuals will respond to any one antigenic determinant. In addition, antibody responses to many antigenic determinants will not occur unless concommitant T cell recognition also occurs. This T-B synergism, termed the helper effect, is required to generate memory IgG responses and neutralizing antibodies which provides long term humoral immunity. Consequently, an efficacious peptide vaccine will have to embody numerous B and T cell epitopes as well as the relevant agretopes if it is to stimulate an immune response in the majority of individuals of an outbred population. The classical method of identifying B and T cell recognition sites in a protein has been to synthesize a panel of overlapping peptides covering the complete protein sequence and screen them for antigenic and immunogenic activity or recognition by anti-protein antibodies and memory T cells. This is a prohibitively expensive and time consuming approach, but the information obtained can be utilized to formulate predictive schemes based on various shared structural properties by the peptides recognized by the immune system.

2. Epitope prediction

The first step in developing a synthetic peptide vaccine is to identify the regions of a protein that will be recognized by the immune system and to determine the amino acid sequence of these antigenic determinants. The later is achieved by deduction from the nucleic acid sequence of the gene encoding

the protein. The former, and most difficult to accomplish, is to identify the relevant antigenic determinants. In addition to intrinsic factors related to antigenic regions of a protein, many extrinsic factors related to the repertoire of the immune system, such as the MHC and TcR binding capacity of peptides and proteolytic cleavage of proteins by APC makes the task of identifying, or even predicting, potentially applicable peptide vaccines exceedingly complicated and difficult. However, some progress has been made in defining potential B and T cell epitopes based on certain prediction schemes.

3. B cell epitopes

Antigenic B cell determinants are categorized into two types, continuous or conformational (94), depending on the contribution of the primary amino acid sequence or the three dimensional protein conformation to antibody binding. In the majority of cases, antibodies are made against sites found on the native conformation (also termed discontinous or topographical sites) when the native protein is used as the immunogen. Thus, in order to predict with any certainty the location of most of the B cell epitopes in a protein, the three dimensional structure of the protein must be known. Since the three dimensional structure of the majority of proteins have not been elucidated, numerous other methods for predicting surface exposure have been proposed based on structural properties such as accessibility to antibody binding, protruding regions of proteins, hydrophilicity, atomic mobility, segmental mobility of amino acids or linear peptide sequences, occurrence of ß-turns, and occurrence of specific amino acids in the sequence (84,93-96). These intrinsic properties are generally highly correlated to one another. The

tendency of hydrophilic sites is to be exposed to an aqueous environment and, therefore, these sites are usually found on the outside of proteins (95). For this reason, regions containing hydrophilic amino acids such as arginine, aspartic acid, glutamic acid, and lysine are likely to be immunogenic. The likelihood of such regions possessing antigenicity is enhanced if they also contained a ß-turn. This rule, however, is imprecise and can at best predict a limited number of B cell antigenic sites because some of these amino acids may interact with other proteins or nucleic acids, and a large number of surface residues of proteins are nonpolar (96). Though these prediction methods have been partially successful in locating regions of proteins that can be used to produce antibodies that bind to the native, none of the methods have achieved a high level of correct prediction. Moreover, few studies have addressed the more complex issue of predicting or mimicking conformational B cell epitopes. Thus, the prediction of conformational B cell epitopes on protein antigens is proving to be exceptionally difficult, and all of the synthetic peptides found to elicit antibodies which recognize the corresponding proteins have been either continuous determinants or a continuous portion of a conformational site .

4. T cell epitopes

The ability to accurately predict T cell epitopes will have powerful applications in terms of synthetic peptide vaccine development since T cell immune responses are essential not only for cell mediated immune responses but also for memory antibody responses. In contrast to antibodies which usually recognize native conformatiomal antigenic sites free in solution, T cell responses are directed to determinants located in the denatured protein. T

cells recognize segments of continuous (also termed sequential or linear) antigenic sites only after they have been processed and displayed on the surface of APC in association with gene products encoded by the I-region of the MHC (93,97). This property may account for the genetic differences in the immune response to different epitopes. It also avoids the technical pitfalls of epitope conformation and greatly simplifies the difficult task of predicting the multifarious structural properties required for recognition by T cells since only the primary sequence of the protein needs to be known. This information can be obtained from DNA sequence data and eliminates the time and expense needed to obtain large quantities of pure protein. Recently, progress has been made in the delineation of antigenic determinants that are recognized by T cells. A role for peptide amphipathicity in recognition by T cells has been proposed by Delisi and Berzofsky (98). Initial experiments using sperm whale myoglobin led to the observation that T cells from different inbred strains of mice responded to only a limited number of immunodominant antigenic sites (99). When the structure of these sites were investigated, most of the epitopes were found to be amphipathic alpha helices. That is, their hydrophobic (T cell binding) residues were lined up on one side of the polypeptide and their hydrophilic (MHC binding) residues on the other. A small number of different sites were shown to be immunodominant in strains of mice that differed only in their MHC class II. This implies that only a limited number of T sites within a particular protein will be needed for recognition by most of the MHC haplotypes in an outbred population such as humans. A predictive algorithm of amphipathicity was developed based on statistical analysis of the primary amino acid sequence of proteins (98). This algorithm predicts the location of potential immunodominant T cell antigenic sites for vaccine design. Unfortunately, not all immunodominant T cell sites follow these predictive rules as was found for HSV gD where the

immunodominant peptide is not an amphipathic helix (103). This suggests that this algorithm will fail to predict potentailly important epitopes. Another predictive scheme asserts that a typical motif can be found in the majority of T cell determinants and is represented by a four or five residue peptide sequence in which a charged residue or glycine is followed by two or three hydrophobic residues and ends with a polar residue (104). Unfortunately, this predictive algorithm contains a major limitation: this motif occurs with high frequency in all proteins. Both of these proposals have been used successfully to predict previously undefined T helper epitopes in influenza virus (100), the AIDS envelope protein (101), and the malaria circumsprozoite protein (102); and have been substantiated by statistical analysis of known T cell epitopes. However, a theoretical problem is that neither of these algorithms take into account the constraints placed on T cell antigen recognition by MHC restriction and TcR binding specificity. In addition, the prediction schemes do not apply to B cell epitopes which do not require processing by antigen presenting cells for immune recognition, and may not apply to class-I-restricted CTL epitopes which undergo antigen processing by different mechanisms than class-II-restricted epitopes.

Clearly, the field of epitope prediction is still in its infancy and a great deal of data needs to be gathered before is it possible to accurately predict the location of B and T cell recognition sites of proteins. This information is needed to allow researchers to rationally design synthetic peptide vaccines which will stimulate protective immune responses in all individuals of an outbred population.

E. Immunological Enhancement

1. Background

Live attenuated viral vaccines, including HSV, are known to be superior to inactivated or subunit vaccine preparations in their ability to stimulate protective levels of immunity. This is due to their ability to replicate to a limited extent in the host, though the mechanisms responsible for this superiority remains unclear (105). The addition of an immunological adjuvant to live vaccine preparations is not required since the virus multiplies in suseptible host cells and elicits an immune response similar to wild type infection. However, many of these conventional antiviral vaccines are expenisve to prepare, unstable, and require special handling which makes distribution in underdeveloped areas very difficult. Although live attenuated HSV-1 vaccines generate potent anti-viral immunity, similar vaccines will probably be unacceptable due to lingering uncertainty concerning the oncogenic potential of HSV (106). In addition, since the attenuated virus is a living organism, and therefore genetically changeable, the possibility exists that such a replicating virus can revert to a virulent form and cause disease, especially in immunologically compromised individuals. Other disadvatages include the need for refrigerator temperatures for storage and transport and a limited shelf life.

Whereas inactivated whole virus vaccines are unable to replicate, they still have immunogenic properties due to their particulate nature which enhances antigen presentation (107). Even after extensive purification there is the probability that killed vaccines, as well as attenuated vaccines, can contain contaminating substances, notably herpes virus DNA and other

viruses. Moreover, these conventional vaccines, in their purest form, still contain contaminating materials which can cause unpredictable side effects, and may interfere with the stimulation of protective immunity by the immunologically active antigenic determinants. Despite the ability of inactivated vaccines to stimulate the production of high levels of antibody, they are poor inducers of T cell-mediated immunity.

Subunit vaccines usually consist of antigen preparations which are incompletely purified. The use of these preparations results in toxicity problems due to retained contaminants and, like vaccines made of killed virus, induce suboptimal immune reponses due to multiple irrelevent antigens which can lead to antigenic competition (108). In addition, the inclusion of strong adjuvants is required for the induction of T cell-mediated immune responses.

Clearly, there is a need for a new generation of safe and effective vaccines in which the structure can be defined at the molecular level. At present there is considerable interest in producing a safe vaccine free of DNA for the preventing HSV infections or modifying recurrent episodes suffered by infected individuals. Vaccines made of synthetic peptides consisting of immunologically relevant epitopes of the pathogen and free of any irrelevent or potentially harmful epitopes possess obvious advantages as compared to attenuated, killed, or other subunit vaccines; and is a promising avenue of investigation. The major problems to be overcome are; (1) unreliable prediction methods for locating the antigenic determinants of a protein which stimulates an immune response in the majority of individauls, (2) the relatively non-immunogenic nature of peptides, (3) the lack of safe and efficacious adjuvants acceptlable for human use.

2. Adjuvants for peptide vaccines

Recent technological advances in biochemical and biosynthetic techniques have made it possible to routinly synthesize virtually any desired peptide, including those sequences of proteins involved in inducing immune responses against a pathogen (109). The potential attractions of using such peptide vaccines are clear since they would eliminate the need for a source of biological material, for handling virulent microorganisms, and for using genetically engineered viruses and bacteria. Because peptide vaccines involve no infectious agent during any stage of production, they are expected to offer significant safety advantages over vaccines derived from viruses. Peptide vaccines are also superior to purified subunit vaccines produced by recombinant techniques. Subunit vaccines are macromolecules which carry a large number of antigenic determinants. Very few of these determinants are important in stimulating protective immunity. In fact, the unimportant or irrelevant determinants may induce immune suppression which may be detrimental to the hosts' defense mechanisms since the outcome of the immune response may depend upon the balance between T helper and T suppressor cell activation. Importantly, such determinants have been found to be present in HSV gD (90). A defined synthetic immunogen could be constructed to exclude suppressor determinants and, therefore, may be more effective in eliciting immune responses against a viral protein than the purified protein itself. This could be the reason that in many cases a peptide was found to be a superior immunogen as compared to the native protein (110). There is also the chance that some B and T cell determinants could elicit an allergic or autoimmune response in some vaccine recipients; these harmful determinants could also be selectively deleted from the vaccine. Thus, peptide

vaccines have the capacity to stimulate highly specific immune responses free from competing or detrimental components and can easily be altered to accommodate any viral mutations that may occur. In addition, peptide vaccines may also offer advantages in terms of lot-to-lot consistency, purity, cost, and long term storage. As is usually the case with peptides, however, the gD peptides tested to date are weakly immunogenic and potent unacceptable adjuvants have usually been employed to achieve immunity (85-90,111,112). Moreover, it is necessary to couple the peptide to a carrier molecule, often by chemically ill-defined techniques. This procedure is proving to be unacceptable for practical use since amino acids which are essential to the antigenic determinant can be masked or altered during conjugation (113). There is also concern that the use of carriers such as tetanus toxoid may result in carrier-induced suppression of antipeptide immunity (114). Thus, it is apparent that a safe, efficacious, and widely applicable adjuvant formulation is desperately needed to increase the potency of peptide vaccines for inducing and maintaining protective immune responses. Before an adjuvant can be considered ideal for use in human vaccines several requirements will have to be fulfilled, including a nontoxic, biodegradable, non-immunogenic nature, and the ability to elicit both humoral and cell-mediated immune responses to associated antigens. In addition, cost, stability during storage, and ease of production must also be taken into consideration.

3. Aluminum hydroxide based adjuvant

The only adjuvants currently licensed by the United States Food and Drug Administration for use in human vaccines are aluminum salts (115). These vaccines, however, result primarily in humoral immunity with little or no

augmentation of cell-mediated immune responses, and is dependant on the immunogenicity of the antigen (116,117). Humoral responses provide sufficient protection against some bacterial and viral diseases. However, alternate adjuvant formulations will be required for HSV and other viruses in which a vigorous cell-mediated immune response is required for eliciting protective immunity.

4. Freund complete adjuvant

Freund complete adjuvant (CFA), first developed by Freund in 1937, is one of the most powerful adjuvants for inducing HI and CMI and has been employed successfully with many different antigen preparations in experimental animals. CFA is composed of killed mycobacteria such as *Mycobacterium tuberculosis* suspended in a water-in oil emulsion (118). Unfortunately, CFA is not suitable for use as an adjuvant in human or veterinary vaccines because of toxicity problems associated with the mycobacterial component. Administration of CFA causes numerous side-effects including severe granulomatous reactions, pain, fever, arthitis, and anterior uveitis (119). Incomplete Freund adjuvant (IFA) lacks the bacterial component used in CFA and has been successfully employed as vehicles for a number of veterinary vaccines such as hoof-and-mouth disease (118). Although not approved for human use in the United States, IFA has been used extensively in humans in the military to induce a higher antibody response to influenza and poliomyelitis vaccines (120,121). However the complete adjuvant is required for full protection against a lethal HSV challenge using a glycoprotein D vaccine in guinea pigs (122). These water-in-oil emulsions, however, produce granulomas and sterile abscesses at injection sites (108).

The question of carcinogenicity has also been raised by the development of tumors in mice given different mineral oil adjuvants (123). These observations, and the fact that peptides and peptide-protein conjugates are poorly immunogenic, demands that alternative adjuvant formulations, which have an efficacy comparable to CFA and can be authorized for use in humans, be developed.

5. Muramyl di-peptides

Biochemical analysis attempting to identify the active component in the mycobacterium resulted in the isolation of N-acetylmuramyl-L-alanyl-D-isoglutamine, called muramyl dipeptide (MDP) for convenience. The immunomodulator MDP represents the minimal structure that possesses the immuno-adjuvant activity which can be isolated from bacterial cell walls and is capable of replacing whole mycobacterium in FCA (124). The mechanisms of action responsible to the adjuvant activity of MDP are not completely understood but are thought to be varied and complex. An important property is the ability of MDP to activate macrophages as measured by increased chemotaxis and phagocytosis, and secretion of prostaglandins and cytokines (170). When administered in saline along with the antigen, MDP has been shown to augment humoral immune responses to a wide variety of viral antigens and synthetic peptides (125). In addition, MDP was also found to be capable of enhancing non-specific resistance to many viral infections including vaccinia virus, Sendai virus, Semliki Forest virus, influenza virus, and HSV (126-129). MDP administered in liposomes has been shown to induce CMI as well as humoral immunity (130,131). A number of shortcomings, however, have limited or prevented the clinical use of MDP: it requires a

specified vehicle such as water-in-oil emulsion to exert its full adjuvant activity (132); is retained poorly within liposomes; is rapidly eliminated from the body; and has only moderate effects on cell-mediated immunity to many viral and bacterial pathogens (133). Moreover, it possesses some of the same unacceptable immunopharmacological properties as FCA. For example, MDP is pyrogenic and has the capacity to induce generalized inflammatory reactions, uveitis, and arthritis (134). These shortcoming motivated the development of a wide range of MDP analogs and derivatives in an effort to select effective compounds which possess the adjuvant activity of the parent molecule without the side effects. These derivatives have been shown to exhibit a wide range of adjuvant potency and side effects (117,125,130,131,135,136). Some hydrophilic MDP derivatives have exhibited the best immunostimulant effects with the least toxic effects so far obtained (137). Unfortunately, these hydrophilic MDPs induce mostly humoral responses since the induction of CMI appears to depend upon the hydrophobicity of the antigen and/or adjuvant (117). The more toxic hydrophobic MDP derivatives have been shown to replace CFA for the induction of CMI (117,135).

6. Muramyl tri-peptide

The lipophilic MDP derivative N-acetyl-muramyl-L-alanyl-D-iso-glutaminyl-L-alanine-2-(1',2'-dipalmitoyl-sn-glycero-3'-hyroxy-phosphoryl-oxy)-ethylamide-monosodium salt, called muramyl-tripeptide (MTP-PE), is one of the best candidates for use as an adjuvant since it is a synthetic product of high purity and is available in large quantity, exhibits low levels of toxicity, prolonged plasma half-life, is slowly released from lipophilic binding sites and compartments, and is effective in stimulating both humoral and cellular

immune responses against HSV-gD and other vaccine antigens (131,136-139). In addition, MTP-PE, in contrast to hydrosoluble MDP, is particularly well-suited for being incorporated stably into liposomes due to its two lipophilic tails, and its antiviral properties and adjuvanticity are considerably more pronounced than those of the watersoluble parent compounds (138-140). The adjuvant activity of MTP-PE appears to be mediated, at least in part, through its ability to activate macrophage, as measured by release of superoxide anion, membrane IL-1 expression, and Ia expression (141). These specific responses of the macrophage to MTP-PE result in enhanced T cell proliferation that is specific to the associated antigen and, importantly, it does this without producing any detectable endogenous pyrogen activity which is associated with systemic administration of MDP(136).

6. Lipid A as an adjuvant

Although the endotoxin, lipopolysaccharide (LPS), isolated from the outer membrane of gram-negative bacteria is extremely toxic to humans, it also possesses the capacity to stimulate beneficial immune responses. After the chemical structure of lipid A, a complicated glycophospholipid that constitutes the lipid moiety of LPS, was established as the structural component responsible for the biological activities of LPS (142), numerous studies on its immunoadjuvant properties were performed. Lipid A associated with liposome has served as an adjuvant for augmenting the immune responses against a variety of protein antigens, including HSV, Epstein-Barr virus, and malaria sporozoite (143-146). Unfortunately, this immunostimulant can cause severe side effects including endotoxicity and pyrogenicity that precludes its use in human beings, though these effects are reduced strongly by incorporating

lipid A into liposomes (146). Therefore, several approches have been utilized in an attempt to develop synthetic lipid A analogues that possess the beneficial biological activities of the parent molecule with minimal harmful effects (145,147,148). One form of lipid A, mono-phosphoryl lipid A (MPL), in which the phosphate group at the C-1 potion of the toxic diphosphoryl lipid A was removed, was found recently to possess many of the beneficial properties, such as adjuvant activity, the ability to activate macrophages, and stimulate interferon gamma activity while exhibiting little or no toxicity in animals (147). For these reasons, and the fact that it can be easily and stability incorporated into liposomes, MPL is one of the more promising synthetic adjuvants currently available for stimulating immunity to liposome-associated peptide antigens. In this study, the capacity of MPL to stimulated immune responses to HSV gD 1-23 was examined alone and in combination with MTP-PE.

F. Immunobiology of Liposomes

1. Background

The liposome is one vehicle of immune potentiation which has few, if any, of the toxicity problems associated with conventional adjuvants. Liposomes are spherical membranous vesicles of various sizes composed of one (unilamellar) or several (multilamellar) concentric lipid bilayers surrounding an internal aqueous space or spaces, and are formed by hydration of a dried lipid film (149). Naturally occuring phosophlipids are the most commonly used class of lipid for liposome formation, though glycerophosphatides, cholestrol, and other types of lipids in combination with phospholipids are also used, depending on the final physiochemical properties

desired (150). For example, incorporation of cholesterol or high melting temperature phospholipids into the lipid bilayer provides rigidity, helps stabilize the bilayer in vivo, and results in liposomes that mimic biological membranes. These liposomes become resistant to attack by high density lipoproteins which would otherwise remove phospholipids from the bilayer resulting in leakage of entrapped materials into the blood (151). The number of parameters which can be varied in the production of liposomes is practically unlimited and allows the investigator to tailor the properties to suit the needs of the particular system under investigation. Because liposomes form closed structures and are impermeable to aqueous solutes, they are ideally suited for delivering vaccine antigens and immunomodulatory drugs. In addition their hydrophobicity make them good adjuvants since it has been shown that lipid moieties that are directly conjugated to protein antigens augment CMI in proportion to their hydrophobicity (152). Thus, it appears that the immunogenicity of nearly any protein can be enhanced by associating it with liposomes (153). Furthermore, liposomes also are attractive because these versatile structures are made of natural constituents which are nontoxic, biodegradable, and, in contrast to protein carrier molecules, non-immunogenic or weakly immunogenic depending on the lipid composition (154). Thus, liposomes are readily acceptable for use in humans. In addition, immuno-modulatory molecules like MPL and MTP-PE can be simultaneously delivered in liposomes, thus further potentiating the immune response.

The immunoadjuvant action of liposomes, initially established by Allison and Gregoriadis in 1974 with diphtheria toxoid (155), has been confirmed and extended by numerous research groups. Adjuvant effects of varying magnitude have been achieved for many medically relevant liposome associated proteins, including foot and mouth disease, influenza, EBV, HSV, and hepatitis viral antigens (144,156-162). Many different aspects of the immune

response were stimulated by these liposomes, including production of antibodies, proliferation and cytotoxicity of T cells, and production of cytokine.

2. Mechanism of action

Despite the significant amount of work done on the delivery of liposomal vaccines, the mechanisms responsible for their immunoadjuvant action is still uncertain, though it is probable that such action is influenced by the rate of antigen release and the mode of interaction with APC. Antigen specific cellular and humoral immune responses are largely controlled by T cells which recognize antigen along with the MHC-encoded molecules on the surface of antigen presenting cells (APC) after the antigen has been taken up and processed (163). After systemic injection, liposomes are readily cleared by the phagocytic APC of the reticuloendothelial system. This property of liposomes makes them an ideal vehicle for delivery of vaccine antigens to these APC, which are known to play an essential role in the induction of immune responses. APC are involved in both intracellular processing and MHC-restricted antigen presentation, and help regulate the immune system (164). The effectiveness of liposomes as antigen carriers is probably due, at least in part, to the fact that the vast majority of systemically administered liposomes are engulfed by cells of the mononuclear phagocyte system (165,166). Recent studies by DelMonte and Szoka indicate that the macrophage is the primary cell type involved in immune responses to liposome associated antigen (167). Hence, it appears that the versatile structure of the liposome allows targeting of associated antigen to the macrophage for processing and initiation of the immune response (168). Evidence that macrophages participate in the adjuvant action of liposomes has been shown since the

immune response is lacking in macrophage depleted animals (169). As with other adjuvants, there are probably several mechanisms of action. Clearance of radiolabeled antigen trapped in multilamellar liposomes is markedly prolonged, suggesting that a depot for antigen release is one of the mechanisms by which they enhance immunity (170). Methods for the preparing and characterizingf liposomes have been discussed at length in several comprehensive reviews (150,171). Multilamellar vesicles (MLVs) are the most commonly used liposomes for carriers of immunogens (171). MLV preparation is simple and results in a heterogeneous mixture of liposomes that range in size from 500 Å to several nm in diameter. These relatively large liposomes are trapped more effectively by macrophages and, as a result, are generally considered to be more immunogenic than other liposome preparations.

3. Physical characteristics

Although the immunopotentiating activity of liposomes is widely recognized, considerable controversy exists concerning the liposomal characteristics responsible for such activity. Numerous groups have reported conflicting results as to the roles of liposome composition, charge, fluidity, preparation method, bilayer number, surface-associated verses entrapped antigen,and lipid to protein ratio in promoting adjuvanticity (154,170-177). The physical characteristics of liposomal bilayers are determined by their phospholipid composition. It is unlikely that any single property accounts for the adjuvant activity at the exclusion of other factors. However, liposomes which have been shown to enhance immune responses do share certain properties such as membrane fluidity (not too solid or too fluid), stable

retention of associated antigen in vivo, and a high phospholipid to antigen ratio (177). In addition, the physical properties of antigens associated with liposome can influence the magnitude and nature of the immune response. Certain proteins and peptides are incorporated into liposomes to a lesser or greater extent, or in different configurations due to isoelectric points, particular sequences of charged amino acids, hydrophobicity, or other unidentified factors. Thus, there is no single ideal lipid formulation for enhancing immunity to associated antigen, and the characteristics of liposomes for inducing optimal responses vary considerably depending on the nature of the antigen used, and may depend on other variables such as the route of immunization, age, and animal species.

4. Adjuvanticity of liposome-associated peptides

A synthetic vaccine based on small synthetic peptides offers great potential for the formulation of safe efficacious vaccines. Unfortunately, the development of peptide subunit vaccines has been hampered by their inadequate immunogenicity and the lack of adjuvants suitable for human use. Classically, the generation of an efficient immune response, which requires the cognate interaction between antigen specific B cells and T cells, has been accomplished by cross linking peptides to macromolecular molecules such as KLH, bovine serum albumin, or tetanus toxiod and immunizing together with CFA. In addition to the phenomenon of carrier-induced epitope suppression previously mentioned, these carrier proteins are also unsuitable for human use because antigenic competition can result and because homology with the pathogen is lacking. Consequently, they will not prime the organism for an

essential T cell memory response which can be recalled by a determinant on the pathogen. Thus, in order to elicit protective immune responses, peptide vaccines will not only have to be formulated in more acceptable immunogenic carrier systems, but will also have to contain relevant B and T cell epitopes derived from the same microorganism. Recently, several innovative techniques have been developed for enhancing the immune response to peptides. These include linking peptides to hepatitis B core antigen (178), proteosomes composed of meningococcal outer membranes (179), phospholipids (180,181), fatty acids (182), anti-immunoglobulin antibodies (183), and antibodies against MHC class II molecules (184). In addition, Tam et al. have designed a chemically defined approach known as the multiple antigen peptide system which links multiple epitopes to produce an immunogenic macromolecular structure (185).

In comparison with the many studies dealing with liposome associated protein antigens, few studies have been done on peptides (186). While the immunogenicity of proteins can be enhanced by presenting them in association with liposomes, the same technology usually does not result in enhanced responses to small antigens such as peptides which were either encapsulated within liposomes or adsorbed to the surface, even when additional adjuvants were included in the immunization protocol (187). The primary reason for this lack of immunogenicity appears to be the inefficient encapsulation of many peptides and the instability of liposome association with peptide that is either passively adsorbed or encapsulated. Several of the techniques mentioned above for enhancing the response to peptides can also be used to facilitate the association of peptide with the lipid bilayer (180-182). Coupling vaccine peptides to fatty acids or phospholipids to obtain lipophilic peptides has been shown to be potentially powerful techniques for enhancing the immunogenicity of liposome associated peptides. The controlled nature of

coupling these moieties to peptides eliminates the potential problem of masking immunogenic residues, one of the major shortcomings of conventional carrier proteins. Furthermore, the ease of preparation of these lipophilic peptides and the capacity to incorporate relevant epitopes that encompass T and B cell recognition sites of multiple proteins of a pathogen, together with synthetic adjuvants, into the same liposome preparation makes them ideal candidates for designing completely synthetic vaccines.

In this study the feasibility of using liposomes containing the HSV gD 1-23 peptide coupled to palmitic acid and immunoadjuvants to elicit immune responses to HSV-1 has been explored. By varying several parameters of the liposome such as composition, presence or absence of immunomodulators, peptide form and the mode of peptide association with the liposome, a chemically defined preparation was found that yielded an effective and long lasting immune response to HSV-1 and the 1-23 peptide. The results obtained show that peptide association is more stable and that superior levels of humoral and cellular immunity against HSV resulted from exposure to palmitilized peptide in liposomes. However, maximum immunogenicity was only achieved by the additionally incorporating synthetic immunomodulators.

Chapter 2

Materials and Methods

A. Virus production and titration

HSV type 1 (HSV-1) strains BK and KOS were used throughout these studies. For producing and titering HSV, stocks were grown in monolayers of Hep-2 cells supplemented with McCoy 5A medium with 10% heat inactivated donor calf serum (DCS) (GIBCO Laboratories, Grand Island, N.Y.), penicillin (100U/ml), and streptomycin (100 μg/ml). After three freeze thaw cycles, the cellular debris was removed (1,000 x g for 10 min) and the virus was divided and stored at -70°C. Samples were titrated in quadruplicate with Vero cell monolayers grown in microdilution plates. After 4 days, the plates were washed in phosphate-buffered saline (PBS), stained with crystal violet, and examined for cytopathic effect. The results were expressed as tissue culture infective dose (50% endpoint) ($TCID_{50}$). Ultraviolet light (UV)-inactivated virus was prepared by exposing 1 ml of virus stock to a germicidal lamp at a distance of 3 cm for five minutes. This procedure reduced infectivity titers from 10^8 to less than 10^1 $TCID_{50}$ / ml

B. Peptide synthesis

The 1-23 peptide, corresponding to the amino-terminal end of HSV-1 gD, was synthesized by solid-phase methods with alpha amino groups protected with *tert* -butyloxycarbonyl groups on a SAM TWO Biosearch synthesizer using

the standard Merrifield resin. Half of the complete peptide was cleaved from the resin with HF (HF/anisole/peptide resine: 10ml/1ml/1g) while the other half was derivatized with hydrophobic palmitic acid at the amino terminal lysine by the anhydride method precisely as the method for amino acid coupling. An 8 M excess of palmitic anhydride was added to peptide-containing resin in the presence of 10 ml of dichloromethane. The peptide has an amino-terminal lysine with two free amino groups, but the internal amino groups were blocked. After being stirred for 2 hours, the resin was washed twice in dichloromethane and dried under high vaccum. Palmitic acid coupling was confirmed by negative ninhydrin analysis. The resulting diacylpeptide was cleaved from the resin by the standard *tert* - butyloxycarbonyl method (188). This dipalmitic derivative was used to facilitate a stable incorporation of the 1-23 peptide into the liposomal bilayer. The crude peptides were purified by gel filtration on silica gel C-18 columns. HPLC and amino acid analysis (Waters Picotag system) verified the purity (>90%) and composition of the peptide.

C. Liposome preparation

Phospholipids were purchased from Avanti Polar Lipid Corporation (Birmingham, AL) and cholesterol was obtained from Sigma Chemical Corp. (St. Louis, MA). Stock solutions of egg phosphatidylcholine, cholesterol, and phosphatidyl-serine were dissolved in methanol-chloroform (1:4) and mixed at a molar ratio of 7:3:1. This mixture was dried to a thin film by rotating the vial in warm water under a stream of nitrogen and was then further dried in a vacuum desiccator. The lipophilic MTP-PE, MPL, and palmitic acid-peptide (acylpeptide) were incorporated into the liposome structure by inclusion in

the original lipid preparation at molar ratios of 0.02:1 (MTP-PE:phosphatidlycholine), 0.05:1 (MPL:phosphatidlycholine), and 0.3:1 (peptide:phosphatidly-choline). The dry mixture was rehydrated with sterile pyrogen-free PBS and vortexed at high speed for 6 min to produce large multilamellar vesicles (MLVs). Unincorporated material was removed by washing three times in PBS (30,000 x g for 10 min) and suspending in PBS at a final concentration of 10 μmol of lipid per ml. The efficiency of incorporation of palmityl-peptide and immunomodulators into the membranes of MLVs was determined by using tracer radiolabeled derivatives of each. The final capture efficiencies of liposome-associated [^{3}H]MTP-PE, [^{125}I]acylpeptide, and [^{125}I]free peptide were calculated from the radioactivity present in the original volume of liposomes and were approximately 90% for MTP-PE, 55% for acylpeptide, and 5% for free peptide. The initial concentrations of acylpeptide and free peptide were manipulated to obtain a final lipid to peptide molar ratio of 30:1 in both preparations. Better immune responses were seen with MLVs containing cholesterol, which stabilized the liposomes in vivo, as opposed to those seen with liposomes composed of only phosphatidylserine and phosphatidylcholine (data not shown). MTP-PE was manufactured by the CIBA GEIGY Corporation and was kindly supplied to us by Biosine Corporation, (Emeryville, CA); MPL was purchased from the RIBI Corporation, (Hamilton, MO).

D. Mice and Immunizations

C3H/HeN mice were obtained from Harlan Sprague Dawley, Inc., (Indianapolis, IN). Six-week-old mice were immunized intraperitoneally with solutions containing 50 μg of peptide in its various forms: MLVs with

acylpeptide with or without immunomodulators; MLVs with free peptide with or without immunomodulators; keyhole limpet hemocyanin (KLH)-peptide in complete Freund adjuvant (CFA); acylpeptide in CFA; free peptide in CFA; or free peptide. Controls included live virus (5 x 10^6 $TCID_{50}$ of HSV-1 strain KOS given IP), MLVs containing immunomodulators only, and saline. The immunomodlator doses given with each injection were 50 µg of MPL and 20 µg of MTP-PE. Before each injection and before sacrifice mice were bled from the retroorbital sinus and the sera tested by ELISA.

E. Antibody measurements

Blood specimens were collected from anesthetized mice via the retro-orbital plexus, allowed to clot and serum was separated by centrifugation. HSV-1 specific antibody in the serum at different days post-immunization was assayed in a solid phase indirect enzyme-linked immunosorbent assay (ELISA) . Polyvinyl chloride microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with 50 µl per well of purified HSV-1 antigen (102) in carbonate buffer(Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g per liter H_2O, pH 9.6) overnight at 4°C. Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) between this and each successive step. PBS containing 3% gelatin was added to each well (50 µl) and incubated one hour to block non-specific binding sites. Next, 50 µl of serum diluted in PBS-T was added to the first well, serially diluted, and incubated at 4°C overnight on a rocker platform. This was followed by 50 µl of horseradish peroxidase-labeled goat anti-mouse immunoglobulins were added (Cappell Laboratories) and incubated for 1 hr at 37°C. After washing with PBS-T 5 times, development with o-phenylene diamine (1mg/ml) (Sigma Chemical Co. St. Louis, MO) was allowed at room temperature for 20 min, then

stopped with 2.5 M sulfuric acid and read on a Fisher ELISA reader. The titer was defined as the highest dilution of serum with an absorbance (488nm) of at least 0.1 and 2 times greater than the negative controls

F. Virus Neutralization assay

Serum was inactivated (56°C for 20 min) and diluted in twofold increments in growth medium, 50 µl were distributed into six replicate wells of a 96-well flat-bottom microtiter plate (Costar, Cambridge, MA). To each well an equal volume of media containing 100 $TCID_{50}$ HSV-1 (KOS) was added and the sealed microtiter plate incubated at 4°C overnight. Subsequently, 50 µl of media containing Low Tox rabbit complement diluted 1/4 were added to one-half of the replicates and the plate was resealed and incubated for 1 hr at 37°C. Finally, 5 x 10^3 viable Vero cells were added to each well (50 µl), and the plates were resealed and incubated at 37°C. The plates were incubated for 4 to 5 days incubation at 37°C in a humidified atmosphere of 5% CO_2, and were examined for cytopathic effect. The serum titers were determined as the dilution that neutralized 50% of the virus.

G. Lymphoproliferation

Spleen cells were obtained 14 days after the last immunization with peptide or virus and single cell suspensions were prepared as described by Lawman et al. (19). Mice from each treatment group were killed by cervical dislocation and their spleen was removed aseptically. Single cell suspensions were prepared by teasing cell gently through 80-mesh stainless-steel screens into Hank's balanced salt solution (HBSS). After one wash, erythrocytes were

lysed by brief exposure to 0.83% NH_4Cl at 37°C. Enriched T cell populations were obtained by passing the spleen cells over nylon wool columns. Approximately 10 ml of the spleen cell suspension was adjusted to 4 x 10^7 cells per ml and loaded onto a nylon wool column which was composed of a 35 ml syringe packed to 24 ml with 2.4 g of washed nylon wool. The column was incubated for 1 hr at 37° C in a humidified 5% CO_2 incubator. Nonadherent cells were collected by passing two column volumes of warm media through the column. The cells were then washed in HBSS twice, the cell viabilities were determined by trypan blue exclusion, and the total number of cells was determined using a standard hemocytometer. The cells were adjusted to 6 x 10^5 viable cells per ml in RPMI 1640-based proliferation medium (RPMI-P) (GIBCO), containing 5% heat inactivated fetal bovine serum and supplemented with HEPES buffer (Research Organics Inc., Cleveland, OH), sodium bicarbonate, oxaloacetic acid, insulin, sodium pyruvate (Sigma), L-glutamine, antibiotic mixture (penicillin (100U/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml)), essential and nonessential amino acids, and MEM vitamin solution (Gibco), and 5 x 10^{-5} M 2-mercaptoethanol, and 100 µl added to each well of a round-bottomed 96-well microtiter plate (Corning). Virus stimulated cultures were incubated with X-irradiated syngeneic spleen cells (5 x 10^5 per well in 100 µl of RPMI-P) infected with UV-irradiated virus (multiplicity of infection of 5 before inactivation). Peptide stimulated cultures were incubated with X-irradiated syngeneic spleen cells (5 x 10^5 per well) previously incubated with 10 µg per ml of 1-23 peptide. A dilution of mitogen (Con A) found to give optimal lymphocyte stimulation in preliminary standardization was included in each test as a positive control. The incorporation of tritiated thymidine into cellular DNA was used as a measure of

lymphocyte proliferation. The plates were incubated for 5 days at 37°C, pulsed with 0.5 μCi of tritiated thymidine ([^{3}H]-TdR) (ICN Biomedicals, Irvine CA) in 50 μl of RPMI-P in each well for the final 6 h of incubation. The cells from each well were harvested onto glass fiber filters and washed using a PHD cell harvester (Cambridge Technology Inc., Watertown MA). The individual filter disks were placed in vials and immersed in 0.5 ml of Ecolume scintillation cocktail (ICN), and harvested counts per minute (cpm) were determined in a Beckman model LS 7000 liquid scintillation spectrometer (Beckman Instruments Inc., Palo Alto CA). Results are expressed as the mean counts per minute (cpm) obtained from eight replicate wells. The stimulation index (SI) was calculated by dividing the values obtained from virus, peptide, and mitogen stimulated wells by the average cpm of eight wells containing cells and RPMI-P (media controls). The lymphocyte proliferation of normal mice or mice injected with adjuvant alone in response to peptide or virus stimulation was similar to the proliferation of these cells in medium alone (the peptide and HSV preparations had no inherent mitogenic activity).

H. Viral clearance assay

Mice were inoculated with 20 μl of virus containing 10^4 or 10^6 $TCID_{50}$ of HSV-1 (KOS) on the dorsal side of the ear pinnae. After 3 days, infected pinnae were removed and stored at -70°C until required. Ear flaps were homogenized in 1 ml of RPMI 1640 containing 10% heat-inactivated fetal calf serum (GIBCO), 7 mM L-glutamine, penicillin (200U/ml), streptomycin (200 μg/ml), and 5 x 10^{-5} M 2-mercaptoethanol. The infectious virus titers were determined by a microtitration assay on Vero cells.

I. Lethality experiments

Immune mice were lethally challenged 2 weeks after the last immunization with an intraperitoneal inoculum of 1 x 10^8 $TCID_{50}$ of HSV-1, BK strain. Mice were monitored daily for clinical signs over a period of 40 days. Results are expressed as the percentage of mice surviving. No more animals died after this time.

J. Interleukin 2 (IL-2) determination

On day 3 of the lymphoproliferation assay, supernatant fluids from the wells were collected and assayed for IL-2 by measuring tritiated thymidine uptake by an IL-2-dependent cell line CTLL-20. The cells were incubated for 24 h in two-fold serial dilutions of the supernatant fluids or known concentrations of recombinant IL-2 as standards. The lower limit of IL-2 detection in this assay was 0.5 U/ml.

K. Peptide retention in vivo

Free peptide and acylpeptide were iodinated by the Iodo-gen method (189) and were injected into mice. Both forms of the peptide were given free and incorporated into liposomes in the hind footpad. Retention of the peptide was determined by measuring radioactivity in the draining lymph nodes at various times after injection. Results are expressed as percentage of input radioactivity at day zero.

L. Statistical Treatment of Data

All experiments were performed at least three times under identical conditions, and the data were statistically analyzed on a Macintosh SE-30 computer using the commercial statistical package Statveiw (Abacus Concepts Inc.).

Chapter 3

Results

A. Palmitilization and Immunomodulators in Liposomes Enhance Immunogenicity

C3H/HeN mice were immunized on four occasions with 50 μg of gD 1-23 peptide (per injection) in various formats, and ELISA antibody titers against HSV were measured at weekly intervals. Although free peptide was barely immunogenic by itself, in liposomes, or when given with CFA, the immunogenicity was enhanced up to fourfold when the carrier ,KLH, was attached to the peptide and given with CFA (Table 1). To facilitate incorporation into liposomes, gD 1-23 was palmitilized by adding two palmitic acid residues onto the terminal lysine. This procedure led to a considerable improvement in the efficiency of incorporation into liposomes (Figure 1) with about 5% of the free peptide and 50 to 55% of the acylpeptide incorporated. Acylpeptide incorporated into liposomes was also more stable than free peptide-associated liposomes (Table 2). Incorporation data were used to ensure that the mice received equal amounts of free and derivatized peptide at the same lipid to protein ratio (30:1). By increasing the amount of free peptide or decreasing the amount of acylpeptide or both in the incorporation procedure, equal ratios of lipid to peptide were obtained.

Although the acylpeptide without added vehicle or adjuvant was barely more immunogenic than was the free peptide, its incorporation into liposomes was more immunogenic, particularly as measured late in the course of

Table 1: MEAN ANTI-HSV TITERS AFTER PEPTIDE IMMUNIZATION[a]

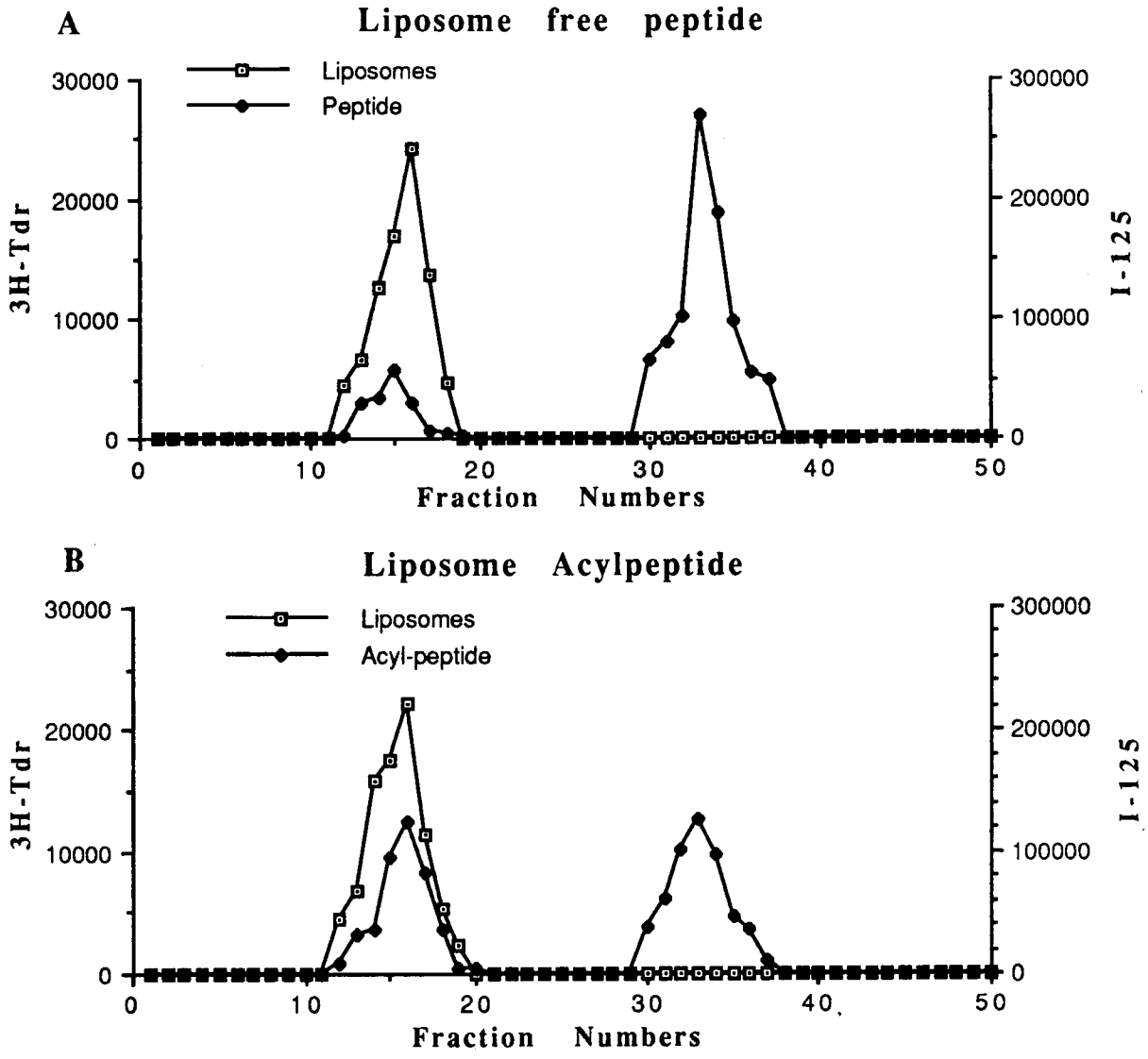
Immunogen[b]	Day 7	Day 14	Day 21	Day 35	Day 42	Day 56
Free peptide	< 25	< 25	< 25	25	25	< 25
Peptide CFA/IFA	< 25	25	50	100	100	67
Peptide Liposome	NT[c]	33	50	133	167	100
KLH-peptide CFA/IFA	< 25	25	100	267	400	333
Free Acylpeptide	< 25	25	50	50	67	NT
Acylpeptide CFA/IFA	25	50	167	333	667	533
Acylpeptide Liposome	25	50	100	200	533	533
Acylpeptide Liposome + MDP	NT	50	333	1066	1866	1600
Acylpeptide Liposome + MTP-PE	25	67	533	2133	3200	2667
Acylpeptide Liposome + MPL	NT	50	200	1600	2667	2667
ALMM	25	100	667	4267	5333	4267
HSV-1	133	1333	4267	8533	8533	8533
Mock infected	< 25	< 25	< 25	< 25	< 25	< 25

[a] Six week-old female C3H/HeN mice were immunized on days zero, 5, 10, and 15, and blood samples were taken on the indicated days. ELISA titers represent the mean of three experiments with five mice per group.

[b] Peptide in CFA for the first immunization; peptide in incomplete Freund adjuvant (IFA) for subsequent injections.

[c] NT, Not tested

Figure 1: INCORPORATION OF FREE AND ACYLPEPTIDE INTO LIPOSOMES



To determine incorporation percentage free and acyl peptides were mixed with a lipid preparation and vortexed. The resulting liposome-peptide preparations were separated from unincorporated peptide by Bio-Gel A 0.5M column chromatography. Fractions were collected and counted for radioactivity (^{125}I: peptide marker; ^{3}H cholestanyl ether: liposome marker), and incorporation calculated as the percent of original peptide in the preparation.

Table 2: STABILITY OF PEPTIDE AND ACYLPEPTIDE ASSOCIATION WITH LIPOSOMES

Peptide form	% Leakage after[a]		
	6 h	12 h	24 h
Free peptide-liposome	36	59	86
Acylpeptide-liposome	2	3.5	4.9

[a] Liposomes were prepared with ^{125}I-labeled peptide and acylpeptide and were incubated in PBS at 37°C for 6,12, and 24 h. Free peptide was determined by separating the liposomes from the leaked material by centrifugation (30,000 X g for 10 min). The radioactivity of the supernatent fluid was compared with the initial radioactivity, and the percentage of leakage was calculated.

immunization (Table 1). It can also be seen that the addition of 50 μg of MPL, 100 μg of MDP, or 20 μg of MTP-PE to the acylpeptide liposome preparations stimulated significantly higher antibody titers compared with the other immunogenic formulations. The incorporation of these immunomodulators into acylpeptide-liposomes was even superior to acylpeptide in CFA. Of the two synthetic muramyl peptide adjuvants, the lipid-soluble form, MTP-PE, was superior, presumably in part because of its more stable incorporation into liposomes. The adjuvant effects of MTP-PE and MPL appeared to be additive, since the highest antibody titers were obtained when both immunomodulators were incorporated into the same acylpeptide-liposome. This combination produced 8-fold-higher titers by week 6 than those achieved by the same dose of acylpeptide in CFA and 256-fold-higher titers than those induced by free acylpeptide. In fact, the combination of acylpeptide-liposome-MTP-PE-MPL (ALMM) induced titers approaching those induced by live virus.

Anti-HSV neutralizing antibody titers were examined (Table 3), and a pattern of results similar to those observed for ELISA titers was obtained. The ALMM construct stimulated significantly higher neuralizing antibody responses in mice than did the other immunogenic 1-23 peptide constructs tested, though this response was threefold less than that induced by live virus.

Immunoglobulin G (IgG) isotypes were examined by an Ig isotype-specific ELISA (Table 4) to determine the distribution of immunoglobulin that was induced by the 1-23 peptide, and the effects of immunomodulators on this distribution were assessed. The form of the antigen appeared to influence the pattern of isotype responses. Accordingly, vaccination with either live HSV or acylpeptide-liposome containing MPL elicited predominantly antibodies of the IgG1 isotype, while vaccination with acylpeptide-liposome constructs containing MTP-PE preferentially elicited IgG2a.

Table 3: SERUM NEUTRALIZING ANTIBODY TITERS FOLLOWING IMMUNIZATION OF C3H/HEN MICE[a]

Immunogen	Titer after experiment 1	2	3	Mean Titer
HSV-1	256	128	128	171
Peptide CFA/IFA	4	4	4	4
Peptide Liposome	4	8	8	6.7
Peptide Liposome: MTP-PE: MPL	8	8	16	10.7
Acylpeptide CFA/IFA	16	16	16	16
Acylpeptide Liposome	16	26	8	13.3
Acylpeptide Liposome: MTP-PE: MPL	64	64	32	53.3
Mock infected	< 4	< 4	< 4	< 4

[a] Six week-old female C3H/HeN mice were immunized on days zero, 5, 10, and 15, and serum samples were collected on day 35 and tested for neutralization activity against HSV-1 (KOS) in a microtiter assay. Data from three indepenent experiments are shown.

Table 4: IMMUNOGLOBULIN SUBCLASS PROFILE IN MICE IMMUNIZED WITH DIFFERENT PREPARATIONS

Immunogen	%Total IgG response with[a]:			
	IgG1	IgG2a	IgG2b	IgG3
HSV-1	61	25	11	3
Acylpeptide-liposome-MTP-PE	25	59[c]	12	3
Acylpeptide-liposome-MPL	50	28	16	5
ALMM	32	50[c]	16	4
Acylpeptide CFA/IFA[b]	25	50[c]	19	3

[a] Serum samples collected from mice on day 42 following four immunizations on days zero, 5, 10, and 15. ELISA titers represent the mean of three experiments with five mice per group.

[b] Acylpeptide in CFA for the first injection (immunization); acylpeptide in incomplete Freund adjuvant (IFA) for subsequent injections.

[c] Statisically significant difference ($P < 0.01$) from the IgG2a titers of the HSV-1 immunized mice

B. Effect on Cell Mediated Immunity

Both antigen-induced cell proliferation and IL-2 production were used as measures of cell-mediated immunity (CMI). Immunization with HSV-1 gave rise to significant responses as measured both by stimulation with UV-inactivated HSV and by exposure to the soluble 1-23 peptide (on syngenic stimulator cells). Mice immunized with acylpeptide incorporated in adjuvant or liposomes with immunomodulators developed significant responses (Table 5). In regard to antigen-induced IL-2 induction, only mice immunized with HSV and the ALMM formulation developed significant responses (data not shown).

C. Induction of Immunity to Local and Systemic Challenge with HSV

To test the effect on antiviral immunity, mice were challenged in the ear pinna with either 10^4 or 10^6 $TCID_{50}$ of the virus. Their ability to clear such virus was measured 3 days later by measuring the quantity of virus in homogenized pinnae. This route of challenge, in contrast to relying on the systemic intraperitoneal route of virus challenge to assess protection, more accurately reflects the normal human cutaneous route of infection. Like the clinical disease in man, this model of HSV clearance from the skin is usually non-lethal and localized to a small region of the epithelium (190). Only mice immunized with infectious HSV-1 were capable of clearing 10^6 $TCID_{50}$ (Table 6). However, mice given the ALMM immunogen and acylpeptide in CFA could clear 10^4 $TCID_{50}$, whereas significant levels of clearance were not observed

Table 5: LYMPHOPROLIFERATION OF IMMUNE T CELLS BY VIRAL AND PEPTIDE ANTIGEN

Priming antigen[a]	Antigenic stimulation	cpm ([^{3}H]) TdR uptake[b]	S.I.[c]
HSV-1	UV HSV-1	26,539 ±2,826	4.8[d]
	1-23	14,893 ±1,531	2.7[d]
	None	5,484 ±1,646	1.0
Free acylpeptide	UV HSV-1	5,439 ±873	0.9
	1-23	6,341 ±1,760	1.1
	None	5,816 ±1,051	1.0
Acylpeptide CFA/IFA	UV-HSV-1	10,231 ±1,698	2.1
	1-23	17,346 ±1,873	3.5[d]
	None	4,940 ±745	1.0
Acylpeptide-liposome	UV-HSV	9,017 ±1,281	1.3
	1-23	12,103 ±2,237	1.7
	None	6,893 ±937	1.0
ALMM	UV-HSV	14,118 ±1,854	2.4[d]
	1-23	18,952 ±2,698	3.4[d]
	None	5,769 ±1,357	1.0

[a] Splenocytes from mice immunized with different forms of the free 1-23 peptide did not exhibit any significant proliferation to viral or peptide antigens

[b] Peptide and virus-immune splenic T cells were prepared as described in Materials and Methods. T cells were stimulated for 5 days with an equal number of X-irradiated syngenic normal splenocytes (APC) which were preincubated with the 1-23 peptide or UV-inactivated HSV-1. The final 12 h of incubation was in the presence of 1.0 μCi per well of [3H]TdR. cpm, counts per minute; TdR, thymidine.

[c] S. I. (stimulation index) = mean cpm antigen stimulated cells/ mean cpm APC only.

[d] Statistically significant difference ($P < 0.01$) from the mean of cells stimulated with antigen presenting cells only.

Table 6: CLEARANCE OF LOCAL INFECTION BY IMMUNIZED C3H/HeN MICE

Immunogen	Clearance[a] after a challenge *dose of:*	
	10^4 PFU	10^6 PFU
HSV-1	<1.5[b]	<1.5[b]
Peptide CFA/IFA[c]	3.8	5.9
Peptide-liposome	3.8	5.8
Peptide-liposome: MTP-PE: MPL	2.3	4.2
Acylpeptide CFA/IFA[d]	1.5[b]	4.3
Acylpeptide-liposome	3.3	5.4
ALMM	<1.5[b]	3.2
Mock infected	4.1	5.9

[a] Quantitative analysis of ear clearance ability. On day 28, groups of five immune mice were challenged in the left pinnae with the dose of virus shown. Three days after challenge, the pinnae were removed and the infectious titers determined (log 50% tissue culture infective dose) on Vero cell monolayers. Results are shown as the average titer with a lower limit of detection of 1.5 log. A similar pattern of results were obtaimed in two repeat experiments.

[b] Significantly below control values

[c] See Table 1, footnote b

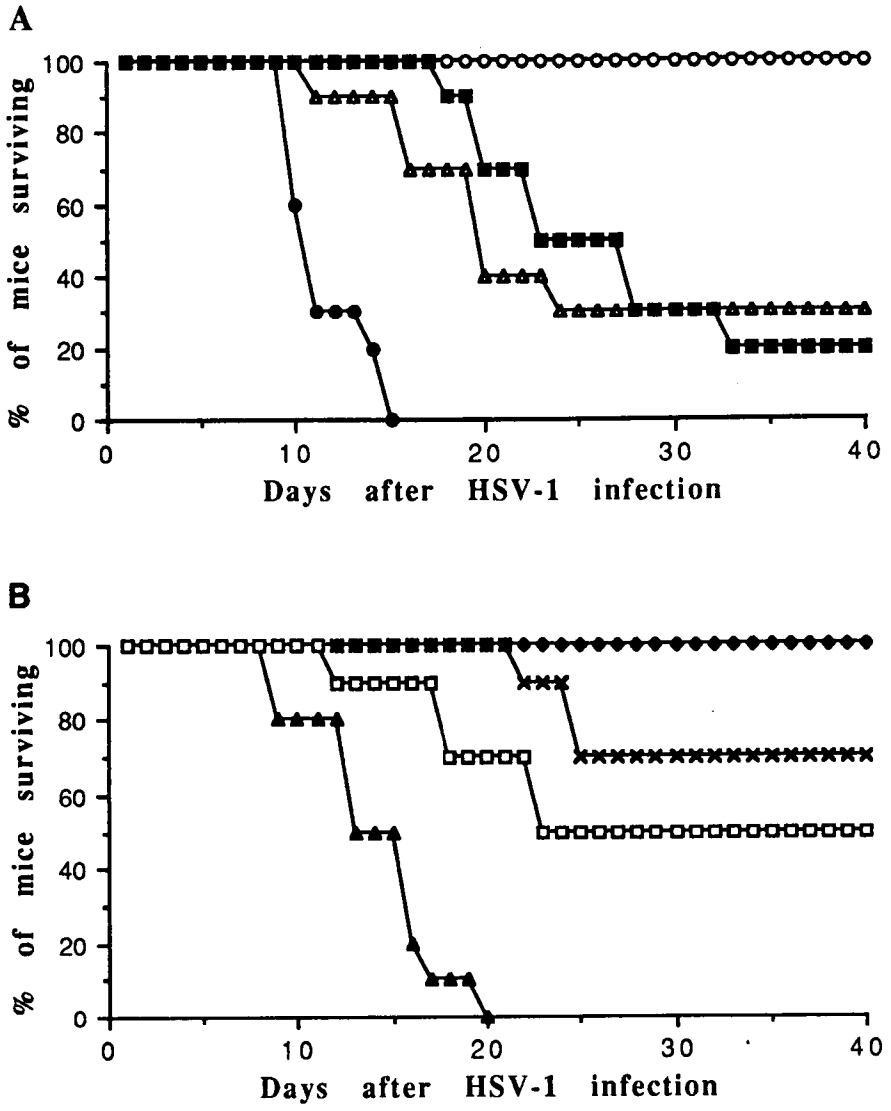
[d] See Table 4, footnote b

with mice immunized with the other peptide formulations. With regard to protection from systemic challenge (Figure 2), complete protection was evident in two groups: those given HSV and those immunized with the ALMM immunogen. Partial levels of protection occurred in other groups which received various acylpeptide formulations, but significant protection did not occur in mice immunized with free peptide.

D. In Vivo Distribution of Acylated and Free Peptide

To determine the distribution and retention characteristics of various immunogenic preparations, ^{125}I-labeled peptides, free and within liposomes, were injected into mice, and at periods thereafter, draining lymph nodes were collected to record levels of radioactivity. The percentage of input material that was retained was highest in mice given acylpeptide in liposomes (Table 7). In these mice, more than 10% of the input radioactivity was still present 21 days after initial injection. This value was greater than that in mice given free peptide in liposomes when measured 4 days after injection.

Figure 2: PROTECTION OF MICE AGAINST LETHAL CHALLENGE BY HSV-1[a]



[a] Female C3H/HeN mice were immunized four times with gD 1-23 peptide in various forms, and then challenged with a lethal dose of HSV-1 (strain BK). Immunizing forms are: HSV-1 (○), acylpeptide- liposome (■) acylpeptide-liposome with MPL (Δ), and PBS (●) (A); ALMM (◆), acylpeptide-liposome with MTP-PE (X), acylpeptide in CFA/IFA (□), and peptide in CFA/IFA (▲) (B).

Table 7: RETENTION OF LABELED PEPTIDES FOLLOWING IMMUNIZATION

Test day	% Peptide retained in draining lymph nodes with[a]: Liposome containing acylpeptide	Acylpeptide	Liposome containing free peptide	Free peptide
4	37	24	9	2
7	33	13	3	<1
10	27	8	2	<1
17	16	6	<1	<1
21	12	2	<1	<1

[a] Mice were given 50 µl of inoculum into the footpads, and at the times given, draining lymph nodes were collected for measurement of radioactivity. Values given represent means of four samples for each time.

Chapter 4

Discussion

This investigation has evaluated liposomes as vehicles for improving the immunogenicity of a synthetic peptide of HSV glycoprotein D (gD 1-23). This peptide is known to induce antibody and T-cell responses that react with the virus itself (5,10,88,89). These results demonstrate that acylated forms of gD 1-23 in liposomes induce superior antibody responses, although in the absence of adjuvant, neither elicited CMI. Excellent humoral responses occurred when synthetic immunomodulators were included along with the acylated peptide, and these responses exceeded those that were induced by peptide emulsified in CFA. Although liposomes containing acylpeptides failed to induce CMI (unless emulsified in CFA), the additional incorporation of immunomodulators did induce such responses. These liposomes also provided a significant level of protective immunity and caused no observable side effects.

Although reasons for the greater immunogenicity of acylated peptide were not established, it probably occurs because of greater stability and longer retention in cells that serve to present and process antigen. In support of this notion, in vitro experiments revealed that antigen leakage from liposomes which contained acylated peptide was far less than occurred with free peptide. Presumably, after in vivo administration, liposomes with free peptide lose much material before being phagocytized by macrophages involved in antigen presentation. However there could also be a variable outcome following macrophage uptake. Thus, in comparisons of distribution and retention time of liposomes with free and acylated peptides, a greater percentage of acylated material was trapped in the local lymph node but

underwent a slower rate of clearance than occured with the soluble antigen-containing liposomes. Slower clearance rates of lipid soluble components as compared to hydrophilic counterparts in liposomes have been reported in other systems (138-140).

Enhancement of peptide immunogenicity was first reported by Hopp (182) with a hepatitis B virus peptide, and the approach was also investigated by Heber-Katz and colleagues (88) with the same HSV peptide used in this study. In the study by Heber-Katz et al., liposomes were also used, although the value of these vehicles was obscurred because the liposomes containing acylpeptide were additionally emulsified in CFA. Accordingly, the adjuvant effect of acylated peptide in liposome could not be independently assessed. Moreover, in marked contrast to the findings in this report, Warari et al. (10) failed to observe a humoral antibody response but did achieve potent cellular responses. In fact, since the vehicles used by Watari et al. could engender protective immunity, they used these observations to argue that protective immunity against HSV would seem to have a cellular explanation. The studies in this thesis could also be taken to indicate that cellular immunity may be more important than antibody responses, at least as measured by clearance of virus from local sites. Thus, in a model in which the ability of mice to clear a local HSV infection from the ear was tested, only animals immunized with preparations which induced significant T cell, as well as antibody, responses could clear virus. Moreover, in a protection from lethality model, although many peptide immunogens were capable of inducing antibody and confered partial protection, the best protection was noted with liposomes which contained acylpeptide and immunomodulators and which also induced a significant CMI response. Indeed, these experiments showed that liposomes which contained acylpeptide along with lipid-soluble immunomodulators were superior to CFA as vehicles to induce immunity; these immunoliposomes also

favored the induction of IgG2a-isotype antibody, which is a subclass of IgG that appears to be instrumental for protection against certain infectious agents (190,101). Although the mechanism responsible for this induction was not investigated, is has been demonstrated that isotype switching in vivo is dependent upon non-specific stimulation properties of the adjuvant to activate different types of T helper cells and cause the production of different lymphokines (192,193). These experiments implicate a role for antibody in viral clearence which, when produced in sufficient titer and of the appropriate isotype, can halt the spread of a low challenge dose of virus to uninfected cells. Antibody as a mechanism of viral clearance and protective immunity has been suggested by others and, in sufficient quantity, may even prevent the establishment of a latent infection (194).

Although liposome-containing acylpeptide and lipid-soluble immunomodulators appear to have great promise, these results indicate that gD 1-23 may not represent an ideal peptide to induce anti-HSV immunity when given alone it is a good candidate for inclusion in a multiple peptide containing HSV vaccine. Immunization with ALMM elicited a protective immune response, but the extent of this immunity was less than that elicited after immunization with HSV-1. The results obtained with the use of a model that more closely mimics the natural pathogenesis of HSV clearly demonstrates that the protection is inadequate. Accordingly, whereas the best synthetic immunogen preparation could induce protection against a low-dose local challenge, immunity to high-dose virus challenge only occurred in mice immunized with live virus. It has been demonstrated by others that immunity to high-dose virus challenge required that numerous aspects of acquired immunity must be induced, including the cytotoxic T lymphocyte system (6,7). Moreover, studies with thymectomized mice depleted in vivo of either Lyt-1^+ or Lyt-2^+ cells (194) and adoptive tranfer of these cells (195) demonstrated that

protection against high-dose challenge requires the participation of both subsets of T cells. Unfortunately, gD appears to have no epitopes that are recognized by murine cytotoxic T lymphocytes, at least in those strains studied so far. Consequently, it will be of interest to perform similar studies on the effect of acylation and liposome incorporation of peptides which are considered part of the repertoire of cytotoxic T lymphocytes. The identification of peptides that can stimulate the production of CTL is central to HSV vaccine design. Complete protection must be confered against persistent infections such as HSV because, once infection has occured and latency has been established, the results of this transpires throughout the life of the infected individual. Such peptides containing CTL epitopes, in conjuction with 1-23 and other HSV peptides which stimulate the production of neutralizing antibody and T helper cell responses, could represent an efficacious vaccine against HSV. Moreover, as shown by others, as well as noted in these studies, the liposome formulation lacks toxicity and provides a vehicle ideal for the delivery of HSV peptides to humans for vaccination purposes.

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Appendix

LIST OF SYNTHETIC PEPTIDES STUDIED[a]

GLYCOPROTEIN D PEPTIDES SYNTHESIZED

1-23: Group VII mAbs bind to this peptide. Induces HSV nAb, T cell proliferation, protective immunity

10-20: Group VII mAb binding, nAb, T cell proliferation, protective immunity

59-78: Predicted amphipathic region

72-91: Predicted amphipathic region

163-178: Predicted amphipathic region

245-260: Immunodominant T helper epitope

261-272: Antibody negative epitope

245-272: Direct linkage for T helper studies

267-281: Sequential epitope group II mAb binding

284-301: Group XI mAb binding

T HELPER PEPTIDES SYNTHESIZED

OVA 323-331: Ovalbumin immunodominant T helper peptide restricted to I-A^d

HA 111-120: Influenza hemagglutinin T helper peptide restricted to I-E^d

a All peptides were synthesized in free form and linked to two palmitic acid moieties via at three residue amino terminal spacer, glycine-glycine-lysine.

LYMPHOPROLIFERATION OF IMMUNE BALB/C T CELLS BY VIRAL, PEPTIDE, AND LIPOSOME ASSOCIATED ANTIGENS

Priming antigen	Antigenic stimulation	cpm ([^{3}H]) TdR uptake[a]	S.I.[b]
HSV-1	UV HSV-1	48,480 ± 2,162	11.4[c]
	10-20	25,744 ± 1,819	6.0
	A10-20 Lip	31,492 ± 1,623	7.4
	None	4,253 ± 1,275	-
Acyl 10-20 Liposome-MTP-PE	UV HSV-1	16,141 ± 1,283	4.4
	10-20	20,541 ± 1,658	5.6
	A10-20 Lip	27,876 ± 1,402	7.6
	None	3,668 ± 826	-
Acyl 10-20 CFA/IFA	UV HSV-1	9,491 ± 1,418	1.6
	10-20	13,204 ± 1,749	2.2
	A10-20 Lip	17,988 ± 1,390	3.0
	None	5,994 ± 1,833	-
10-20 CFA/IFA	UV HSV-1	5,545 ± 1,196	0.9
	10-20	5,924 ± 895	0.9
	A10-20 Lip	6,949 ± 1,227	1.1
	None	6117 ± 1,083	-

[a] Peptide and virus-immune splenic T cells from Balb/c mice were prepared as described in Materials and Methods. T cells were stimulated for 5 days with a equal number of X-irradiated syngenic normal splenocytes (APC) which were preincubated with the 10-20 peptide,UV-inactivated HSV-1, or acyl 10-20 in liposomes. The final 12 h of incubation was in the presence of 1.0 μCi per well of [3H]TdR. cpm, counts per minute; TdR, thymidine.

[b] S. I. (stimulation index) = mean cpm antigen stimulated cells/mean cpm APC only.

[c] Underlined values exhibit statistically significant difference ($P < 0.01$) from the mean of cells stimulated with APC only.

CLEARANCE OF LOCAL INFECTION BY LONG TERM IMMUNE BALB/C MICE

Immunogen[a]	Clearance[b] after a challenge dose of :	
	10^4 PFU	10^6 PFU
HSV-1	<u><1.5</u>[c]	<u><1.5</u>
Acyl 1-23 CFA/IFA[d]	2.4	4.9
Acyl 1-23 Liposome	2.7	5.2
Acyl 1-23 Liposome-MTP-PE	<u><1.5</u>	3.8
Acyl 10-20 CFA/IFA	2.6	5.5
Acyl 10-20 Liposome	3.2	5.9
Acyl 10-20 Liposome-MTP-PE	<u><1.5</u>	4.3
Acyl 245-260 CFA/IFA	3.1	5.9
Acyl 245-260 Liposome-MTP-PE	2.5	6.0
Acyl 267-281 CFA/IFA	3.5	5.8
Acyl 267-281 Liposome-MTP-PE	2.8	6.2
Mock infected	4.2	6.0

[a] Mice immunized with different forms of the free peptides did not exhibit any significant clearance of local HSV-1 infection.

[b] Quantitative analysis of ear clearance ability. On day 180, groups of five immune mice were challenged in the left pinnae with the dose of virus shown. Three days after challenge, the pinnae were removed and the infectious titers determined (log 50% tissue culture infective dose) on Vero cell monolayers. Results are shown as the average titer with a lower limit of detection of 1.5 log. A similar pattern of results were obtained in two repeat experiments.

[c] Underlined values are significantly below control values.

[d] Acylpeptide in CFA for the first immunization; acylpeptide in incomplete Freund abjuvat (IFA) for subsequent injections.

ANTIBODY TITERS IN BALB/C MICE TO PREDICTED AMPHIPATHIC PEPTIDE gD 163-178[a]

Immunogen	n[b]	Anti-HSV titers		Anti-peptide titers	
		Median	Range	Median	Range
163-178 CFA/IFA[c]	3	50	(<50-100)	100	(50-200)
163-178 Liposome	4	<50	(<50-100)	50	(<50-100)
Acyl 163-178 CFA/IFA	3	267	(200-400)	1,333	(800-1600)
Acyl 163-178 Liposome	5	440	(200-800)	1,040	(400-1600)
Acyl 163-178 Liposome MTP-PE	6	1,067	(800-1600)	4,267	(3200-6400)
HSV-1	4	11,200	(6400-12800)	900	(400-1600)

[a] Six week old female Balb/c mice were immunized on days 0, 7, 21, and 35 and blood samples were taken on day 56.

[b] n = number of mice per group.

[c] Peptide in CFA for the first immunization; peptide in incomplete Freund adjuvant (IFA) for subsequent injections.

CLEARANCE OF LOCAL INFECTION BY BALB/C MICE IMMUNIZED WITH AMPHIPATHIC PEPTIDES

Immunogen[a]	Clearance[b] after a challenge dose of :	
	10^4 PFU	10^6 PFU
HSV-1	<u><1.5</u>[c]	<u><1.5</u>
Acyl 163-178 CFA/IFA[d]	2.6	5.7
Acyl 163-178 Liposome-MTP-PE	<u><1.5</u>	4.5
Acyl 59-78 CFA/IFA	3.8	5.8
Acyl 59-78 Liposome-MTP-PE	3.1	5.3
Acyl 72-91 CFA/IFA	3.6	5.7
Acyl 72-91 Liposome-MTP-PE	3.3	5.4
Mock infected	4.0	5.9

[a] Mice immunized with different forms of the free peptides did exhibit any significant clearance of local HSV-1 infection.

[b] Quantitative analysis of ear clearance ability. On day 56 , groups of five immune mice were challenged in the left pinnae with the dose of virus shown. Three days after challenge, the pinnae were removed and the infectious titers determined (log 50% tissue culture infective dose) on Vero cell monolayers. Results are shown as the average titer with a lower limit of detection of 1.5 log. A similar pattern of results were obtained in two repeat experiments.

[c] Underlined values are significantly below control values.

[d] Acylpeptide in CFA for the first immunization; acylpeptide in incomplete Freund abjuvat (IFA) for subsequent injections.

LYMPHOPROLIFERATION OF IMMUNE BALB/C T CELLS BY VIRAL AND AMPHIPATHIC PEPTIDE ANTIGENS

Priming antigen[a]	Antigenic stimulation	cpm ([^{3}H]) TdR uptake[b]	S.I.[c]
HSV-1	UV HSV-1	56,856 ± 1,930	12.1[d]
	163-178	30,242 ± 2,398	6.4
	59-78	9,556 ± 1,125	2.0
	72-91	6,109 ± 1,179	1.3
	None	4,716 ± 932	-
Acyl 163-178 CFA/IFA	UV HSV-1	9,392 ± 1,385	1.6
	163-178	12,438 ± 1,493	2.1
	None	5,805 ± 1,451	-
Acyl 163-178 liposome	UV HSV-1	23,153 ± 1,386	4.2
MTP-PE	163-178	28,734 ± 1,772	5.3
	None	5,431 ± 1,139	-
Acyl 59-78 liposome	UV HSV-1	9,970 ± 1,173	2.5
MTP-PE	59-78	14,933 ± 847	3.8
	None	3,908 ± 975	-
Acyl 72-91 liposome	UV HSV-1	5,625 ± 1,341	1.9
MTP-PE	72-91	6,893 ± 1,371	2.3
	None	2,984 ± 887	-

[a] T lymphocytes from mice immunized with different forms of the free peptides did not exhibit any significant proliferation to viral or peptide antigens.

[b] T cells were stimulated for 5 days with an equal number of X-irradiated syngenic normal splenocytes (APC) which were preincubated with the peptides or UV-inactivated HSV-1. The final 12 h of incubation was in the presence of 1.0 μCi per well of [3H]TdR. cpm, counts per minute; TdR, thymidine.

[c] S. I. (stimulation index) = mean cpm antigen stimulated cells/ mean cpm APC only.

[d] Underlined values exhibit statistically significant difference ($P < 0.01$) from the mean of cells stimulated with APC only.

LYMPHOPROLIFERATION OF IMMUNE C3H/HEN T CELLS BY VIRAL AND AMPHIPATHIC PEPTIDE ANTIGENS

Priming antigen [a]	Antigenic stimulation	cpm ([^{3}H]) TdR uptake[b]	S.I.[c]
HSV-1	UV HSV-1	49,374 ± 2,204	8.7[d]
	163-178	6,806 ± 1,352	1.2
	59-78	12,195 ± 1,514	2.2
	72-91	15,568 ± 1,735	2.8
	None	5,638 ± 1,284	-
Acyl 163-178 Liposome-MTP-PE	UV HSV-1	6,835 ± 1,258	1.4
	163-178	7,582 ± 1,473	1.5
	None	4,931 ± 1,284	-
Acyl 59-78 Liposome-MTP-PE	UV HSV-1	15,304 ± 2,204	2.8
	59-78	18,172 ± 1,738	3.4
	None	5,393 ± 1,126	-
Acyl 72-91 Liposome-MTP-PE	UV HSV-1	21,746 ± 1,848	3.3
	72-91	22,832 ± 1,518	3.4
	None	6,689 ± 1,407	-

[a] T lymphocytes from mice immunized with different forms of the free peptides did not exhibit any significant proliferation to viral or peptide antigens.

[b] T cells were stimulated for 5 days with an equal number of X-irradiated syngenic normal splenocytes (APC) which were preincubated with the peptides or UV-inactivated HSV-1. The final 12 h of incubation was in the presence of 1.0 μCi per well of [3H]TdR. cpm, counts per minute; TdR, thymidine.

[c] S. I. (stimulation index) = mean cpm antigen stimulated cells/ mean cpm APC only.

[d] Underlined values exhibit statistically significant difference ($P < 0.01$) from the mean of cells stimulated with APC only.

LYMPHOPROLIFERATION OF IMMUNE C57/BL6 T CELLS BY VIRAL AND AMPHIPATHIC PEPTIDE ANTIGEN

Priming antigen[a]	Antigenic stimulation	cpm ([^{3}H]) TdR uptake[b]	S.I.[c]
HSV-1	UV HSV-1	61,051 ± 2,246	8.9[d]
	163-178	8,458 ± 1,347	1.2
	59-78	7,226 ± 1,125	1.1
	72-91	28,780 ± 1,804	4.2
	None	6,824 ± 932	-
Acyl 163-178 Liposome-MTP-PE	UV HSV-1	7,062 ± 1,332	1.3
	163-178	8,755 ± 1,384	1.6
	None	5,432 ± 735	-
Acyl 59-78 Liposome-MTP-PE	UV HSV-1	12,352 ± 1,507	2.0
	59-78	13,885 ± 1,404	2.3
	None	6,041 ± 986	-
Acyl 72-91 Liposome-MTP-PE	UV HSV-1	29,905 ± 1,479	4.0
	72-91	35,246 ± 1,893	4.8
	None	7,394 ± 1,251	-

[a] T lymphocytes from mice immunized with different forms of the free peptides did not exhibit any significant proliferation to viral or peptide antigens.

[b] T cells were stimulated for 5 days with an equal number of X-irradiated syngenic normal splenocytes (APC) which were preincubated with the peptides or UV-inactivated HSV-1. The final 12 h of incubation was in the presence of 1.0 μCi per well of [3H]TdR. cpm, counts per minute; TdR, thymidine.

[c] S. I. (stimulation index) = mean cpm antigen stimulated cells/ mean cpm APC only.

[d] Underlined values exhibit statistically significant difference ($P < 0.01$) from the mean of cells stimulated with APC only.

ANTIBODY TITERS IN BALB/C MICE IMMUNIZED WITH T HELPER PEPTIDES[a]

Immunogen	n[b]	Anti-HSV titers Median	Range	Anti-peptide titers Median	Range
Acyl 10-20 Liposome-MTP-PE	6	2,133	(1600-3200)	23,467	(12800-25600)
A10-20 + A163-178 Liposome-MTP-PE	4	2,000	(1600-3200)	25,600	(25600)
A10-20 + AOVA[c] Liposome-MTP-PE	4	9,600	(6400-12800)	76,800	(51200-102400)
A10-20 + AHA[d] Liposome-MTP-PE	6	3,467	(1600-6400)	27,733	(12800-51200)
A10-20 Lip-MTP-PE + AOVA Lip-MTP-PE[e]	4	1,800	(800-3200)	17,600	(6400-25600)
Acyl 10-20 CFA/IFA	4	500	(400-800)	4,800	(3200-6400)
A10-20 + AOVA CFA/IFA	4	900	(400-1600)	8,800	(3200-12800)
A 261-272 Lip-MTP-PE	5	<50	(< 50)	<50	(< 50-100)
A 245-272 Lip-MTP-PE	5	<50	(< 50)	130	(50-200)

[a] Six week old female Balb/c mice were immunized on days 0, 7, 21, and 35 and blood samples were taken on day 56.

[b] n = number of mice per group.

[c] **OVA** = ovalbumin I-A^d restricted T helper peptide (323-339)

[d] **HA** = influenza hemagglutanin I-E^d restricted T helper peptide (111-120)

[e] Acyl 10-20 in liposomes containing MTP-PE and acyl ovalbumin peptide given by a different routes.

SERUM NEUTRALIZING ANTIBODY TITERS FOLLOWING IMMUNIZATION OF BALB/C MICE WITH gD PEPTIDES[a]

Immunogen	Titer after experiment			Mean Titer
	1	2	3	
HSV-1	256	256	128	213
Free 10-20 CFA/IFA	<4	4	4	2.6
Acyl 10-20 CFA/IFA	8	8	16	10.6
Acyl 10-20 Liposome: MTP-PE	32	16	32	26.6
Acyl 10-20 Liposome	8	4	8	6.7
Acyl 267-281 Liposome: MTP-PE	4	4	4	4
Acyl 245-260 Liposome: MTP-PE	<4	<4	<4	<4
Acyl 245-272 Liposome: MTP-PE	<4	<4	<4	<4
Acyl 163-178 Liposome: MTP-PE	<4	<4	<4	<4
Acyl 59-78 Liposome: MTP-PE	<4	<4	<4	<4
Acyl 72-91 Liposome: MTP-PE	<4	<4	<4	<4
Mock infected	<4	<4	<4	<4

[a] Six week old female Balb/c mice were immunized on days 0, 7, 21, and 35, and serum samples were collected on day 56 and tested for neutralization activity against HSV-1 (KOS) in a microtiter assay. Data from three independent experiments are shown.

VITA

Ketil Brynestad was born in Trondheim, Norway on June 14, 1958. He attended primary and secondary schools in Oak Ridge, Tennessee and was graduated from Oak Ridge High School In June, 1976. He recieved a Associate of Science degree in Medical Laboratory Technology from Roane State Community College, Harriman Tennessee, in 1983. Mr Brynestad was awarded a Bachelor of Arts degree in Microbiology from The University of Tennessee, Knoxville Tennessee. in 1986. He entered graduate school at The University of Tennessee, Knoxville, in 1986 and received the Master of Science degree in microbiology in May, 1991.