To the Graduate Council:

I am submitting herewith a dissertation written by Joseph C. Mester entitled "Herpes Simplex Virus: Protective Mechanisms of Antibody and the Ability of Peptides to Induce Viral Reactivity." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Barry T. Rouse, Major Professor

We have read this dissertation
and recommend its acceptance:

Arthur Brown

Robert N. Moore

Leon N. D. Potgieter

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

HERPES SIMPLEX VIRUS IMMUNITY:
PROTECTIVE MECHANISMS OF ANTIBODY
AND THE ABILITY OF PEPTIDES TO INDUCE VIRAL REACTIVITY

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Joseph C. Mester

May 1990

Copyright © Joseph C. Mester, 1990
All rights reserved

ACKNOWLEDGEMENTS

The direction which guided this research came from several sources. My research committee, composed of Arthur Brown, Robert Moore, Leon Potgieter, and Barry Rouse, spent many hours engineering a reputable research project and assuring its accurate documentation. As a result, this effort is mainly due to their input through the years. Joseph Glorioso generously supplied the monoclonal antibody panel, and offered encouragement during unproductive spells in the lab. Steve Highlander's dedication to the analysis of the antigenic structure of glycoprotein B provided the information necessary to construct the peptides corresponding to the site recognized by MAb B6. Alex Osmand and Charles Murphy, as well as assisting in peptide synthesis and analysis, were ever stimulating and supportive in their discussions, and will be sorely missed. Finally, I would like to thank my many lab mates over the years, especially Steve Martin and Steve Norley, for the day to day friendship that made this research enjoyable.

DEDICATION

To Joe Timko, for his inspiration.

To my parents, for their support.

To Laura, for everything.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PART I :

HSV : ANIMAL MODELS AND HUMAN DISEASE

CHAPTER 1

ANIMAL MODELS AND HUMAN DISEASE : A REVIEW OF THE LITERATURE

A. INTRODUCTION

Investigation of the immune response to herpes simplex virus (HSV) has progressed rapidly, mainly through the use of various animal models of human disease. Initial studies demonstrating that immunization with live HSV-1 could protect mice against fatal encephalitis and the establishment of latent infection indicated that effective anti-viral immunity could be generated with a HSV vaccine (Price *et al.*, 82); however, reports of the oncogenic potential of HSV (Duff and Rapp, 22, 23), the possibility of latent and recurrent infection by a live vaccine, and the immunosuppression associated with HSV infection (Cappel, 9) argued against the use of a live or attenuated form of the virus for immunization. Subsequent investigations revealed the protective potential of nucleic acid free and envelope antigen vaccine preparations (Cappel, 9, Chan, 13, Erturk *et al.*, 27, Kites *et al.*, 41, Klein *et al.*, 44) and their ability to stimulate both humoral and cellular immunity against HSV. Current research has focused on defining the role of each of the viral envelope glycoproteins in stimulating a protective B and T cell response in an attempt to identify the minimum effective immunization unit.

The ongoing dissection of the glycoprotein-specific immune response has revealed that the viral subunit and peptide approaches are both powerful and full of potential pitfalls. Most importantly, their evaluation in rodents raises the question of their relevancy to human infection. The following sections review the results of mouse, guinea pig, and

human studies evaluating the response to, and protective potential of, the envelope glycoproteins of HSV.

B. MURINE STUDIES

PASSIVE PROTECTION AGAINST HSV CHALLENGE

INTRODUCTION

Initial passive transfer experiments indicated that immune sera provided a scant degree of protection against lethal HSV challenge. Hyperimmune rabbit sera was shown to prolong the life and delay the sequence of hind-limb paralysis, encephalitis and death in a significant number of mice following footpad challenge, but the absolute prevention of lethality occurred in only a few of the treated mice (Evans *et al.*, 28). Human gamma globulin was shown by others to have only a slight protective effect against mice challenged by the intracranial or intraperitoneal route (Cheever and Daikos, 15). Related studies revealed that HSV could spread directly from infected to uninfected cell *in vitro* in the presence of virus-neutralizing immune serum (Black and Melnick, 6, Hooks *et al.*, 36). From the start, then, humoral immunity, and especially neutalizing antibody, were perceived as having a limited role in combating HSV infection *in vivo*.

MECHANISMS OF ANTIBODY-MEDIATED PROTECTION

Elucidation of the ability of HSV to invade the peripheral nervous system during epithelial infection, establish latent infection in neurons of the local sensory ganglia, and reactivate and initiate a secondary epithelial infection in an immune host has prompted investigation of the role of antiviral antibody in modulating each aspect of infection in greater detail. Other than the extracellular neutralization of infectious virions, antibody may also mediate complement and cellular lysis of infected cells, two mechanisms which, if active early in the infectious cycle, could theoretically reduce or remove the infectious load in the skin and peripheral nervous system, and clear recurrent virus from secondarily infected epidermis. Early investigation of the protective mechanisms of antibody :

(1.) Demonstrated the ability of neutralizing antibody to restrict the amount of virus penetrating the peripheral nervous system, but questioned its ability to prevent ganglionic colonization or protect from lethal challenge,

(2.) indicated that antibody-dependent complement-mediated cytolysis (ADCMC) was not a means of limiting infection, and

(3.) suggested that antibody-dependent cellular cytotoxicity (ADCC) may be involved in the ability of antibody to clear replicating virus from the skin and limit the neurological spread of HSV.

When examining the neurological progression of HSV infection in the mouse, Wildy (112) first noted that the passive transfer of neutralizing HSV-immune sera had no effect on the formation of local vesicles or the onset of paralysis when given eighteen hours following intradermal challenge, and did not influence the progression of the virus from the peripheral to the central nervous system. Cook and Stevens (16) found similar results when they passively transferred high titer HSV-neutralizing antibody to mice five and ten

hours after intradermal infection. In mice immunized with live HSV before challenge, Price *et al.* (82) demonstrated that no correlation existed between the established titer of neutralizing antibody and protection from viral colonization of the local sensory ganglia following HSV challenge.

Several reports, however, found that administration of neutralizing antibody could reduce the amount of virus in the peripheral nervous system following viral challenge. Walz *et al.* (108), when quantitating the number of acutely infected neurons in immune and nonimmune mice following HSV challenge, demonstrated that immune mice had a lower percentage (approximately 0.1% versus approximately 1%) of neurons positive for HSV during the first ten days after challenge. Walz *et al.* also documented a similar restriction in the number of acutely infected neurons for nude mice given high titer anti-HSV neutralizing antibody two days after HSV challenge. McKendall *et al.* (60) found that the passive transfer of immune serum could reduce the number of mice with latent infection of the sensory ganglia ipsilateral to the inoculation site (and prevent death in a significant portion of the mice) as well as completely prevent viral access to the contralateral ganglia after viral challenge. The prevention of contralateral spread was seen even when antibody was given 48 hours after infection, by which time the ipsilateral ganglia had been colonized in all of the mice. While the results of McKendall *et al.* suggested that antibody could block virus spread within the nervous system, the authors did not speculate as to the mechanism responsible for this activity.

In partial agreement with McKendall *et al.* and Walz *et al.*, Klein (43) found that immune serum did not prevent acute or latent infection of the ipsilateral ganglia, but reduced the amount of acutely replicating virus and the amount that could be reactivated in latently infected ganglia. Interestingly, Openshaw *et al.* (77) found that the passive transfer of neutraling serum could prevent acute ganglionic infection in normal mice but

not in nude mice, and Oakes and Rosemond-Hornbeak (74) demonstrated the involvement of a radiosensitive component in the protection from the neurological spread of HSV by neutralizing antisera transferred up to 48 hours post-challenge. These two results suggested that protection by neutralizing antibody involves other immune system components and antibody effector mechanisms, although a direct role for T cells and innate immune mechanisms could only be inferred.

The involvement of innate immunity in protection mediated by the passive transfer of antibody was suggested by Rager-Zisman and Allison (83), who showed that the passive transfer of immune sera did not protect against a lethal intraperitoneal challenge in cyclophosphamide-treated mice unless normal adherent splenocytes were also transferred. Rager-Zisman and Bloom (84) previously demonstrated that ADCC was effective at higher immune serum dilutions than ADCMC *in vitro*. The ability of antibody and complement to influence HSV infection *in vitro* was investigated by Lodmell *et al.* (52), who showed that antibody and complement were not able to influence the cell-to-cell spread of HSV. However, the addition of activated rabbit leukocytes (mainly macrophages and monocytes) along with antibody (alone or with complement) drastically inhibited plaque formation. Importantly, Lodmell *et al.* demonstrated that the appearance of viral antigens on the infected cell surface and the transfer of virus from infected to uninfected cells occurred from six to eight hours post-infection, whereas ADCMC was not detectable until ten to twelve hours post-infection. Shore *et al.* (96, 97) initially described human anti-HSV ADCC *in vitro* , and further demonstrated that the human effectors of ADCC could recognize viral cell surface antigens as early as two hours after infection and produce target cell lysis by three hours post-infection. While the interaction of complement and antibody leading to ADCMC of HSV-infected cells required a high antibody concentration and occurred too late to stop the cell-to-cell spread of virus,

ADCC of infected cells occurred early, before the release of extracellular virions or the cell-to-cell spread of virus, and was effective at low concentrations of antibody.

Recent *in vivo* demonstrations of the importance of ADCC and the F_C region of immunoglobulin in antibody-mediated protection have compared the protective effect of whole immunoglobulin (Ig) with pepsin-generated $F(ab')_2$ fragments and have demonstrated the ability of subneutralizing doses of antibody to confer passive protection when coadministered with the effector cells of ADCC. Oakes and Lausch (75), utilizing the intraocular challenge route, initially reported paradoxical results. They demonstrated that both IgG and $F(ab')_2$ fragments (which retained neutralizing ability but not ADCMC activity) were protective when administered before or at the time of virus challenge, but, when administered at eight hours post-infection, only whole IgG was protective. Hayashida *et al.* (33) presented clearer evidence utilizing zosteriform spread analysis in nude mice, and demonstrated that the transfer of high concentrations of antibody (from a variety of animal species) inhibited the development of zosteriform spread and significantly prolonged the survival of lethally infected mice. However, the $F(ab')_2$ fragment of human gamma globulin, when administered frequently to maintain serum neutralizing titer, had no protective effect. In a similar comparative study, McKendall (59) documented that passive administration of whole IgG led to quicker clearance of virus from the epidermis (than in untreated controls), substantial reduction of the amount of latent virus detectable in ganglia ipsilateral to the inoculation site, and prevention of the contralateral spread of virus after the mice were challenged in the footpad. $F(ab')_2$ fragments were not as effective as whole IgG, and yielded a slower clearance of virus from the footpad, a less significant reduction in the amount of virus detected in the ipsilateral ganglia, and no prevention of the contralateral spread of the virus, even though they were administered repeatedly to maintain serum neutralizing levels. McKendall

suggested that the superior protection mediated by whole IgG was due not only to ADCC but also to noncytolytic interactions of the F_C region of Ig and F_C receptor bearing effector cells, leading to lymphokine release and the production of tissue active substances which would induce inflammation and accelerate the B and T cell specific anti-viral response. Oakes and Lausch, Hayashida *et al.*, and McKendall also examined the contribution of ADCMC to the protection mediated by whole IgG in their models through the use of several complement deficient mouse strains, and all agreed that complement activation played no detectable role in the enhanced protective capacity of whole Ig. Kohl and Loo (45) also gave evidence for the protective action of ADCC *in vivo* by demonstrating that the combination of a subneutralizing dose of human gamma globulin and human leukocytes protects neonatal mice from lethal intraperitoneal challenge, whereas neither antibody nor leukocytes alone were protective.

Convincing recent arguments for the role of antibody in immunity to HSV were derived from studies of B cell suppressed mice. Kapoor *et al.* (39) and Simmons and Nash (100) documented a more severe infection of the peripheral nervous system in normal and HSV-immune animals depleted by anti-μ antiserum administration before exposure to HSV. While *in vivo* anti-μ depletion had no effect on the titer of virus replicating in the skin of naive or immune mice, the B cell suppressed naive mice had higher acute virus titers in the ipsilateral ganglia after challenge than did the normal controls, and were the only mice susceptible to the contralateral spread of HSV. Additional reports by Kapoor *et al.* (40) and Simmons and Nash (99) suggested that the ability of antibody to limit the spread of virus to and within the nervous system was due to its neutralizing ability.

In addition to its effect on acute primary infection, anti-viral antibody may play a role in latency and reactivation of HSV. Stevens and Cook (103) first suggested that anti-viral

antibody was involved in the *in vivo* maintenance of latent neurological infection by HSV, when they transplanted latently infected spinal ganglia into the spleen or peritoneal cavity of normal mice and prevented reactivation by the administration of anti-viral IgG. Oakes and Lausch (76) later demonstrated that antiviral antibody could markedly suppress virus growth in cultured explants of acutely infected trigeminal ganglia or in neuroblastoma cells infected *in vitro*, in the absence of complement or immunologically reactive cells. Recently, Schneweis *et al.* (90) suggested that passively transferred antibody induces a latent infection *in vivo* by interacting directly with the ganglion and nonspecific immune effector cells. Additional reports, however, have refuted the role of antibody in the maintenance of latency. Sekizawa *et al.* (94) demonstrated the *in vivo* presence of latent HSV-1 in trigeminal ganglia in the absense of neutralizing antibody. Additionally, Openshaw *et al.* (77) demonstrated that cytotoxic agents (cyclophosphamide and X-irradiation) can reactivate HSV both *in vitro* and *in vivo* in the presence of neutralizing antibody. Nevertheless, in the zosteriform spread model which mimics HSV recurrence, Simmons and Nash (99) documented the ability of neutralizing monoclonal antibodies to block the spread of virus from acutely infected ganglia to uninfected epidermis.

PROTECTION MEDIATED BY HSV GLYCOPROTEIN-SPECIFIC ANTIBODY

The production of a vast number of monoclonal antibodies (MAbs) to HSV glycoproteins B, C, D, and E (gB, gC, gD, and gE) has allowed a more exquisite analysis of antibody effector mechanisms active in protection against HSV, and has led to the identification of specific glycoproteins as well as the epitopes of the glycoproteins that

are important targets for antibody-mediated immunity. The importance of the envelope glycoproteins in the humoral response to infection has been documented by Glorioso, *et al.* (31), who used HSV mutants defective in the synthesis of gB and gC to demonstrate that a major portion of cytolytic antibody in HSV immune serum was directed against gB and gC, and Para *et al.* (80), who demonstrated that gD represents the prime target for HSV neutralizing MAbs. The significance of epitope recognition in antibody-mediated immunity has been demonstrated by Ashe and Notkins (1), Centifanto *et al.* (12), and La Thangue *et al.* (50). Ashe and Notkins first demonstrated that polyclonal immune serum could sensitize HSV by attaching to the virion and blocking the attachment of neutralizing antibody. Thereby, nonneutralizing fractions of immune serum reacted with the virus in a manner that allowed infectivity in the presence of an excess amount of neutralizing antibody. Centifanto *et al.* discovered this phenomena *in vivo* in the rabbit ocular challenge model, and suggested that sensitization of HSV by IgG may explain why recurrent infections occur in competent immune animals. Via immune electron microscopy, LaThangue *et al.* suggested that neutralizing epitopes are exposed on the surface of the envelope glycoprotein fringe, and that nonneutralizing epitopes are found within the glycoprotein fringe (closer to the viral envelope).

Several investigators have documented the ability of MAbs specific for gB, gC, gD, and gE to protect mice against lethal challenge. Dix *et al.* (19) demonstrated that passive transfer of a gC-1 or gD-1 specific MAb up to 24 hours post-infection protected mice from a footpad challenge. A correlation between *in vitro* neutralization and *in vivo* protection was also shown, although the authors suggested additional mechanisms were involved in the protection mediated by the day one post-infection passive transfer. Balachandran *et al.* (3) demonstrated protection from lethal footpad challenge by the passive transfer of MAbs specific for gB-2, gC-2, gD-2, and gE-2 and a correlation

between *in vivo* protection and *in vitro* ADCC. Interestingly, Rector *et al.* (85) found that while MAbs specific for gB-1, gC-1, gD-1, and gE-1 were protective *in vivo* against a lethal ocular challange, no correlation existed with the *in vitro* activities of neutralization, ADCC, or ADCMC. Metcalf *et al.* (65) verified these results in the ocular challenge model with an independent panel of MAbs specific for gB-1, gC-1, gD-1, and gE-1, and suggested that several mechanisms may be involved in *in vivo* protection. Kapoor *et al.* (40), however, found that a neutralizing gD-1 specific MAb was protective in nude mice challenged in the ear pinna, whereas a nonneutralizing gD-1 specific MAb was not. Similarly, Simmons and Nash (99) found a correlation between neutralization and protection from zosteriform spread mediated by gB and gD specific MAbs. With MAbs specific for thirteen unique epitopes of gB, gC, and gD, Kumel *et al.* (47) found no correlation between protection against intracranial challenge and MAb isotype or *in vitro* neutralization titer. Generally, the MAbs reactive with gC were effective *in vivo* at the lowest doses, followed by the gB and then the gD specific MAbs. Still, a marked variation in protective capacity among the gB and gC specific MAbs was evident when equivalent neutralization doses were administered *in vivo*, which suggested the involvement of alternative protective mechanisms and the importance of epitope recognition.

We have performed an in depth analysis of passive protection mediated by MAbs in the zosteriform spread model by examining such parameters as MAb dose and affinity for the challenge virus, the ability of each MAb to neutralize HSV and mediate ADCC and ADCMC of HSV-infected cells, and the role of epitope recognition in protection (63). Our results indicated that neutralizing gB and gD specific MAbs protect against both ganglionic colonization by HSV and its zosteriform spread. The gC specific MAbs mediated a high degree of ADCC, and protected against zosteriform spread but not

against ganglionic colonization. Finally, while all of the gC and gD reactive MAbs were protective, only one of the four gB reactive MAbs prevented zosteriform spread. Thereby, the targets of protective antibody on gB appeared more limited than those on gC and gD.

SUMMARY

In summary, investigation of the role of HSV-immune sera in protection from primary infection, ganglionic colonization, and death indicated that antibody does play a role in restricting the flow of virus from the initial epithelial infection to the local sensory ganglia and may further affect viral replication in the skin and spread through the nervous system. Neutralization, ADCC, and F_C-dependent functions are mechanisms involved in antibody-mediated protection, although in several reports no firm correlation of protection to any one *in vitro* activity was documented. Investigations utilizing monoclonal antibodies specific for the major envelope glycoproteins of HSV (gB, gC, gD, and gE) and a variety of challenge routes (footpad, intracranial, and ocular) have demonstrated that all of the glycoproteins examined could serve as targets for protective antibody. Additional reports suggested that not all of the glycoproteins, nor the epitopes of each glycoprotein, were equivalent as targets of protective antibody.

More information concerning the mechanism(s) of antibody-mediated protection and the role of epitope recognition in the ability of the monoclonal antibodies to confer protection *in vivo* is needed. Recent reports of the ability of antibody to inhibit viral replication both at the site of epithelial inoculation and in the acutely infected ganglion,

and to induce a switch from a replicative infection to a latent one in the ganglion warrant more extensive exploration of the many facets of antiviral humoral immunity.

ADOPTIVE PROTECTION AGAINST HSV CHALLENGE

INTRODUCTION

Evidence for the dominant role of T cell mediated immunity (CMI) in protection from HSV infection was initially suggested by the *in vitro* cell-to-cell spread of the virus in the presence of neutralizing antibody (Lodmell *et al.*, 52) and the ability of adoptively transferred HSV-immune splenocytes to protect mice against a lethal intracranial challenge (Ennis, 26). Further accounts revealed that mice immunosuppressed by cyclophosphamide or anti-thymocyte serum were made susceptible to lethal infection by HSV and remained so even after receiving the passive transfer of neutralizing antibody, but were completely protected by the adoptive transfer of HSV-immune T cells generated in immunocompetent mice (Oakes, 73, Rager-Zisman and Allison, 83). An additional report demonstrated that the acute susceptibility of congenitally athymic nude mice to lethal infection by HSV could be precluded by the adoptive transfer of immune T cells, whereas the passive transfer of neutralizing antibody merely prolonged the survival time of the mice (Nagafuchi *et al.*, 67). Adoptively transferred T cells mediated the clearance of infectious virus from the epidermis of athymic mice (Kapoor *et al.*, 40) and B cell suppressed mice cleared infectious virus from the skin as efficiently as immunocompetent

mice (Kapoor *et al.*, 39, Simmons and Nash, 100). Cell mediated immunity has also been implicated in the control of recurrent infections by HSV (Openshaw *et al.*, 77).

MECHANISMS OF T-CELL MEDIATED PROTECTION

The potential protective mechanisms of CMI against HSV include lymphokine release by T_{Helper} cells, delayed-type hypersensitivity (DTH) elicited by T_{DTH}, and cytolysis of infected cells by T_{CTL}. Lymphokines secreted by T_{Helper} cells :

(1.) are responsible for the generation of antiviral IgG by providing helper factors for HSV-specific B cells (IL-4, IL-5, IL-6),

(2.) augment T_{CTL} activity (IL-2, IL-4, gamma-IFN),

(3.) augment the inflammatory response and macrophage (MØ) and natural killer cell (NK) activity (IL-2, gamma-IFN), and

(4.) directly inhibit viral replication (gamma-IFN).

Investigation of the role of the separate T cell lineages, including $T_{Suppressor}$ cells, in HSV immunity has elucidated the complexity and interrelatedness of the various T cell effector mechanisms. Howes *et al.* (38) suggested that either major histocompatibility (MHC) class I or class II restricted T cells (in the classical murine system : T_{CTL} and T_{Helper} / T_{DTH}, respectively) were able to confer adoptive protection from lethal intraperitoneal challenge, while Nash *et al.* (71) initially suggested that the interaction between MHC class I and II restricted T cells was necessary for the clearance of infectious virus from the skin. Later reports from Nash and Gell (69) demonstrated the simultaneous transfer of DTH and protection from ear pinna challenge by HSV-immune Lyt-1+ T cells (MHC class II restricted T_{Helper} / T_{DTH}). The protective capacity of Lyt-

1+ T cells was also demonstrated by Yasumoto *et al.* (116), Nagafuchi *et al.* (66), and Simmons and Nash (98) in the zosteriform spread model using normal or nude mice. Interestingly, Nash *et al.* (70) demonstrated that mice tolerized for HSV-specific DTH (by intravenous inoculation with HSV) were also primed for enhanced clearance of virus from the skin as compared to untreated mice.

Together, these results indicate that MHC class II restricted, Lyt-1+ T_{Helper} cells are able to protect against lethal challenge and enhance viral clearance in a nonlethal challenge model. This protective effect is enhanced by the simultaneous transfer of Lyt-2+ T_{CTL}. Communications reported by Wildy and Gell (113), based on *in vivo* Lyt-1+ and Lyt-2+ depletions and demonstrating that Lyt 2+ T_{CTL} were necessary for protection against a high dose of virus, whereas Lyt-1+ T_{Helper} and T_{DTH} cells were able to clear a low virus dose in the ear flap, support this notion and indicate that lymphokine release and DTH may be sufficient mechanisms for protection against limited amounts of virus but T_{CTL} are necessary for protection against large infectious challenges. An additional report of *in vivo* T cell depletion prior to low dose challenge has suggested that the T_{Helper} / T_{DTH} subset is responsible for clearance from the epidermis, while the T_{CTL} subset is responsible for eradicating the acute phase of ganglial infection (Nash *et al.*, 68).

The ability of class I restricted T_{CTL} to be protective against lethal intraperitoneal challenge when adoptively transferred was demonstrated by Sethi *et al.* (95) and Larsen *et al.* (48). However, the *in vivo* relevance of *in vitro*-generated T_{CTL} is unclear (immune cells must be cultivated *in vitro* for three to five days before HSV-specific CTL activity is detectable). Sethi *et al.* noted that high serum gamma-IFN levels accompanied the adoptive transfer of their T_{CTL}s, and speculated that protection may be a result of IFN-

enhanced NK and MØ activity or a direct IFN effect on viral replication and spread as suggested by the earlier *in vitro* studies of Lodmell and Notkins (53).

PROTECTION MEDIATED BY HSV GLYCOPROTEIN-SPECIFIC CMI

The main targets of cellular immunity appear to be the viral glycoproteins. Lawman *et al.* (51) and Carter *et al.* (11) documented that a majority of T_{CTL} generated in C3H and C57/Bl mice are glycoprotein specific. Glorioso *et al.* (30) further demonstrated that most HSV-specific T_{CTL} clones generated in C57/Bl mice recognize gC, and Rosenthal *et al.* (88) found that gC, and not gB, gD, or gE, were recognized by HSV-specific T_{CTL} generated in CBA mice. Blacklaws *et al.* (7) showed weak T_{CTL} recognition of gB and no T_{CTL} recognition of gD in CBA mice. Blacklaws *et al.*, Martin *et al.* (55), and Schrier *et al.* (91) agreed that gB, gC, and gD, as immunopurified proteins, or expressed on the surface of transfected cells or in vaccinia virus vectors, could elicit HSV-specific DTH.

Interestingly, on the epitopic level, Wyckoff *et al.* (114) and Yamashita and Herber-Katz (115) demonstrated that HSV-immune T cells from Balb/c and C3H mice proliferate to a large number of unique synthetic peptides spanning the full range of gD. This finding suggests that potentially many T cell sites exist on the viral glycoproteins. Furthermore, some of these sites were found to stimulate $T_{Suppressor}$ cells (Wyckoff *et al.*). Finally, using synthetic peptides representing computer predicted antigenic sites of gB-1, we have identified two epitopes recognized by HSV-specific T_{CTL} generated in Balb/c mice (Horohov *et al.*, 37).

Reports of protection mediated by the adoptive transfer of glycoprotein or peptide-specific T cells are lacking. However, Chan *et al.* (14) demonstrated that L3T4+ gB-specific T cells could confer protection against lethal intraperitoneal challenge, presumably because of their "helper abilities" and not because of DTH. Alternatively, Watari *et al.* (109) found a transferable protective Lyt-2+ T cell response elicited by a gD peptide corresponding to the first twenty-three amino acids of the N-terminus of the glycoprotein. Our experience with acute immune lymph node cells elicited by vaccinia-gB and gD constructs in the zosteriform spread model indicated that only a limited degree of protection (approximately 50%) was conferrable in C3H mice (Appendix A). Whether this incomplete protection was due to the inability of the HSV glycoprotein to act as a suitable immunogen or to deleterious effects of its expression in the vaccinia virus vector is uncertain. Clearly more information is needed regarding the ability of the viral glycoproteins to stimulate a protective T cell response.

SUMMARY

The role of CMI in combating infection by HSV, and the importance of glycoprotein recognition in CMI are well established. However, the details of specific glycoprotein recognition and the intricacies involved in the stimulation of one or more of the functional subclasses of T cells are largely unresolved. While HSV-immune T cell populations apparently recognize and proliferate in response to many epitopes on gB, gC, and gD *in vitro*, the types of T cell stimulated and the epitopes involved are mostly unknown. This mystery is further complicated by analyses in inbred mouse strains, where recognition of strain-specific T cell epitopes is likely to occur. Ideally, regions of each glycoprotein will

be identified that specifically stimulate T_{Helper}, T_{CTL}, T_{DTH}, and $T_{Suppressor}$ cells. Whether or not these regions will be similarly active in several inbred mouse strains will provide essential information on the reliability of the mouse model for making predictions concerning the role of the viral glycoproteins in human herpetic disease.

ACTIVE PROTECTION AGAINST HSV CHALLENGE

INTRODUCTION

Glycoproteins B, C, and D of HSV have been demonstrated to be the targets of protective humoral and cellular immunity. Through the use of glycoprotein specific MAbs and immunoaffinity columns, and recombinant DNA techniques which allow protein synthesis from cloned genes and the construction of HSV gene vectors by the insertion of glycoprotein genes into nonessential regions of vaccinia and adenovirus, it has been possible to assess the ability of gB, gC, and gD to act as effective immunogens against HSV. Both the immunopurified and virus vector-expressed forms of the glycoproteins have been shown to stimulate HSV-specific and protective immunity.

PROTECTION GENERATED BY IMMUNIZATION WITH HSV gB

Reports of gB-induced HSV immunity are isolated and diverse. Chan *et al.* (14) demonstrated that immunization of CBA mice with immunopurified gB-1 in saline is capable of generating a strong anti-HSV antibody (nonneutralizing) and DTH response, a strong HSV-specific T cell proliferative response, and a L3T4+ T cell-dependent (and transferable) protective response. Balb/c mice immunized with gB-1 in Freunds Complete Adjuvant (FCA) or alum gel were completely protected against lethal challenge and ganglionic colonization by both HSV-1 and 2, and generated HSV-specific DTH and neutralizing antibody (Kino *et al.*, 42). Blacklaws *et al.* (7) utilized L cells transfected with and expressing the gB-1 gene as immunogens, and showed a protective response and the inhibition of the establishment of latency in the local sensory ganglia, accompanied by the generation of HSV-specific DTH, nonneutralizing antibody, and Lyt-2+ T_{CTL}s in CBA mice. Pachl *et al.* (78) demonstrated that a truncated form of gB-1, lacking 194 carboxyl-terminal amino acids, induced high antibody levels and a limited degree of protection, after administration in FCA, in outbred Swiss Webster mice. A vaccinia virus vector expressing gB-1 and an adenovirus vector expressing gB-2 both generated neutralizing antibody and protection from lethal challenge in Balb/c mice (Cantin *et al.*, 8, and McDermott *et al.*, 58, respectively). Martin *et al.* (55) documented that vaccinia-gB-1 elicited HSV-specific lymphoproliferation, DTH, and antibody, and primed C3H mice for clearance of infectious challenge from the ear pinna. Finally, with a peptide corresponding to an antigenic site of gB-1 (gB20.2mer, corresponding to amino acids 91-110 of the primary translation product), which appears to be highly homologous among strains of both HSV types, we (Mester *et al.*, 64) generated neutralizing antibody

in Balb/c mice which was protective when passively transferred into naive mice before lethal challenge.

PROTECTION GENERATED BY IMMUNIZATION WITH HSV gC

Immunopurified gC-1 and gC-1 expressd in a vaccinia virus vector have also been shown to generate protective immunity in murine challenge models. Schrier *et al.* (91) demonstrated that immunopurified gC-1 administered in borate buffer stimulated HSV-specific DTH, and protection in A/J mice in the absence of detectable anti-HSV antibody. Roberts *et al.* (86) also demonstrated protection by immunization of Balb/c mice with purified gC-1 in Incomplete Freunds Adjuvant along with the generation of neutralizing antibody. Expressed in vaccinia virus, gC-1 generated neutralizing antibody, HSV-specific T cell proliferation, and protection against lethal challenge in C3H mice (Weir, *et al.*, 111).

PROTECTION GENERATED BY IMMUNIZATION WITH HSV gD

The ability of gD to act as a protective immunogen was analyzed similarly to gB and gC, and with similar results. Long *et al.* (54) and Eisenberg *et al.* (25) documented that immunopurified gD-1 in FCA elicited neutralizing antibody against HSV-1 and HSV-2 and protected Balb/c mice against lethal challenge with either HSV type. A truncated form of gD-1 (containing residues 1 through 275 of the mature protein) also protected mice against a lethal HSV-1 or HSV-2 challenge (Laskey *et al.*, 49). Blacklaws *et al.*

generated neutralizing antibody and HSV-specific DTH by immunizing CBA mice with gD-1 expressing L cells, and protected the mice against ear challenge and reduced the incidence of latent infection in the corresponding dorsal root ganglia. With vaccinia-gD-1 constructs, Cremer *et al.* (17) and Rooney *et al.* (87) protected Balb/c mice against lethal HSV-1 and HSV-2 infection and markedly reduced the number of surviving mice harboring latent HSV. Paoletti *et al.* (79) also demonstrated protection of outbred mice by prior immunization with a unique vaccinia gD-1 construct. Interestingly, Martin *et al.* (57) demonstrated that vaccinia-gD-1 generated HSV-specific DTH and antibody, and conferred protection against a low dose HSV-1 ear challenge in C3H mice. This protection was mediated by L3T4+ T cells and not by a DTH or CTL mechanism. Martin *et al.* (56) also documented that suppressor factors, which acted on T_{CTL} generation *in vitro*, were generated by gD-1 expressed in vaccinia, and speculated that suppression accounted for their inability to detect gD-1-specific T_{CTL}.

By immunization with a peptide corresponding to the first 23 amino acids of mature gD-1 or gD-2 emulsified in FCA, Eisenberg *et al.* and Watari *et al.* (109) were able to protect Balb/c mice from lethal HSV-2 infections. Eisenberg *et al.* correlated protection with the generation of neutralizing antibody, whereas Watari *et al.*, who used a palmitilated peptide / liposome preparation, found no neutralizing antibody but documented a strong Lyt-2+ T cell response. Furthermore, the gD1-23mer-elicited Lyt-2+ T cells were able to transfer protection to naive mice.

SUMMARY

The ability of individual HSV glycoproteins to act as immunogens when introduced in recombinant viruses, as purified proteins, or in tranfected L cells, has been thoroughly investigated for whole glycoproteins B, C, and D. Protection from HSV challenge was achieved with gB, gC, and gD as purified proteins administered in FCA, alum gel, or aqueous buffer, as well as in transfected L cells and adenovirus and vaccinia virus vectors. The ability of truncated, cell-secreted versions of gB and gD to protect from lethal challenge was also verified. Essentially no difference was found between the ability of the purified protein administered in adjuvant, and the protein expressed via recombinant virus vectors or on the surface of transfected cells, to stimulate humoral or cellular immunity against HSV. The postulated mechanisms of protection, when proposed, have varied from correlations to neutralizing antibody or DTH, CTL, or "helper" activity, or to both humoral and cellular mechanisms. Unfortunately, a significant number of the reports relied on systemic intraperitoneal challenge to assess protection, a route which does not accurately reflect normal human mucocutaneous infection by HSV. On the epitopic level, only two peptides have been shown to generate a protective response: the gD1-23mer and the gB20.2mer.

C. GUINEA PIG MODEL

INTRODUCTION

A major deficiency of murine models of HSV infection are their inability to consistently reproduce the phenomenon of HSV recrudescence. This phenomenon, however, is the hallmark of the guinea pig model of HSV-2 infection. Initially described by Scriba (92), guinea pigs infected with HSV-2 demonstrate frequent spontaneous recurrent infections of a duration ranging from a few to more than twenty days. However, this model is not an accurate mimic of the human situation, as it was subsequently revealed that latent HSV-2 infection occurred in the epidermis of the animals as well as in the nervous system (Scriba and Tatzber, 93). Additionally, the incidence of HSV recurrence in humans is thought to be controlled immunologically, and as a result the majority of latently infected humans do not suffer from frequent spontaneous recurrent herpetic lesions.

PROTECTIVE CAPACITY OF HSV GLYCOPROTEINS

Both truncated, cell-secreted forms of gB-1 and gD-1 when administered in FCA or alum gel, were demonstrated to be both protective against primary vaginal infection by HSV-2, and effective in reducing the frequency and severity of recurrent lesions once primary infection had been established (Berman *et al.*, 4, Stanberry *et al.*, 101, 102). The mechanism behind this protection, however, was not detailed. Additionally, a gD-1-expressing vaccinia virus vector was demonstrated to be effective in protecting guinea

pigs from primary and recurrent infections (Wachsman *et al.*, 110). The authors suggested that protection resulted from the stimulation of T cell immunity, leading to lymphokine release and an enhancement of natural killer cell function. Finally, in HSV-2 infected guinea pigs, an increased anti-gD ELISA titer was shown to be associated with a reduction in the number and severity of recurrent episodes (Bernstein *et al.*, 5). Reports of the immune response to other HSV glycoproteins and the epitopes of all of the glycoproteins in this model are absent from the literature.

D. HUMAN SYSTEM

INTRODUCTION

A majority of the human population is seropositive with respect to HSV (Straus, 104). In the significant percentage who suffer from recurrences, acyclovir therapy can decrease the frequency and duration of the recurrent episodes (Peacock *et al.*, 81). However, acyclovir treatment has no effect on latency and subsequent reactivation (Peacock *et al.*), and its widespread use may foster the development of resistant strains (Straus). Since recurrent HSV infections occur in an immune host, there is little hope for a subunit vaccine which would be effective for those already infected with HSV. In nonimmune humans, however, it may be possible to reduce the severity of primary infection and its sequelae (latency and recrudescence) by generating HSV-specific immunity via immunization.

HUMORAL IMMUNE RESPONSE

The inability of neutralizing antibody to prevent recurrent infections (as demonstrated by Douglas and Couch, 21) suggested that humoral immunity may not play a role in controlling human infection by HSV. Nevertheless, the possibility exists that the production of "sensitizing" antibody (Ashe and Notkins, 1) during primary infection blocks the ability of neutralizing antibody to limit viral spread. Additionally, as suggested by Rager-Zisman and Bloom (84), ADCC may be the more relevant mechanism of antibody-mediated protection *in vivo*.

Antiviral reactivity of seropositive human sera is strongest against gB and somewhat less and more variably reactive to gC and gD (Eberle and Mou, 24, Vestergaard, 107). Interestingly, Kuhn *et al.* (46) and Zweerink and Corey (119) demonstrated that, while the antibody response during primary infection is generated against immediate early, early, and late viral proteins, those patients with antibody reactivity more restricted to gB and gD experienced less frequent recurrences. Glycoprotein-specific antibodies are generated too late in the course of primary infection to prevent viral access to the local sensory ganglia, but if they were present before primary infection, they would significantly alter the course of infection and possibly prevent the establishment of latency in the neurons.

CELLULAR IMMUNE RESPONSE

The importance of CMI in controlling infection by HSV is demonstrated by the propensity of patients with acquired T cell immunodeficiences or those undergoing

immunosuppressive therapy to suffer severe recurrent infections (Merigan and Stevens, 62, Douglas *et al.*, 20). The human cellular immune response to HSV appears to be MHC class II-restricted for both T_{Helper} and T_{CTL} anti-HSV activities. (Schmid, 89, Zarling *et al.*, 117, 188). Recognition of gB-1 and gD-1 by human T_{Helper} and T_{CTL} was documented by Zarling *et al.* (118), and the gD1-23mer peptide was demonstrated by Defrietas *et al.* (18) to activate human T cells *in vitro* . These results further demonstrate the important role of the viral glycoproteins in human immunity to HSV, and suggest potential candidates for a HSV subunit vaccine.

E. CONCLUSION

Animal and human studies have indicated that the viral glycoproteins are the major immunogens of HSV, and have allowed the precise definition of recognition and effector function in anti-HSV immunity. While there is no consensus on the mechanism of antibody and T cell-mediated protection, the convergence of potential protective mechanisms points to several areas where anti-viral intervention is active and targetable.

HSV may exploit the phenomenon of T cell mediated suppression in order to gain an advantage on the immune system and facilitate its ability to cause recurrent lesions in immune hosts. Thereby, analysis of the T cell response to glycoproteins considered to be prime candidates for effective subunit vaccines should be particularly aware of potential suppressive effects .

The ultimate application of the animal models would be in the delineation of epitopes recognizable by the protective effector mechanisms of human immunity. The initial studies with the gD1-23mer have shown a degeneracy (*i.e.*, association of a single peptide with more than one unique MHC molecule) in epitope recognition between the human and murine systems (DeFrietas *et al.*, 18, Herber-Katz and Dietzschold, 34, Heber-Katz *et al.*, 35) which was not found in related studies of the influenza system (McMichael *et al.*, 61, Townsend *et al.*, 106). Recent investigations in the influenza and respiratory syncytial virus murine models, however, have indicated an unexpected degree of degeneracy in T cell epitope recognition (Gao *et al.*, 29, Nicholas *et al.*, 72). Undoubtedly, nondegenerate epitopes will also be found in the murine HSV system, but they will still provide a basis from which more suitable investigations can be attempted.

Both humoral and cellular immunity against HSV have been generated in man using DNA free and glycoprotein mixture subunit vaccines (Ashley *et al.*, 2, Cappel *et al.*, 10). The recently identified glycoproteins G and H (Gompels and Minson, 32, Sullivan and Smith, 105) may also be prime targets of protective immunity in mouse and man. The challenge now lies in identifying regions of the glycoproteins which are able to stimulate the desired immunity in the human system.

F. REFERENCES

(1) Ashe, W. K. and A. L. Notkins. 1967. Kinetics of sensitization of herpes simplex virus and its relationship to the reduction in the neutralization rate constant. Virology. **33:** 613-617.

(2) Ashley, R., G. Mertz, H. Clark, M. Schick, D. Salter, and L. Corey. 1985. Humoral immune response to herpes simplex virus type 2 glycoproteins in patients receiving a glycoprotein subunit vaccine. J. Virol. **56:** 475-481.

(3) Balachandran, N., S. Bacchetti, and W. E. Rawls. 1982. Protection against lethal challenge of Balb/c mice by passive transfer of monoclonal antibodies to five glycoproteins of herpes simplex virus type 2. Infect. Immun. **37:** 1132-1137.

(4) Berman, P. W., T. Gregory, D. Crase, and L. A. Lasky. 1985. Protection from genital herpes simplex virus type 2 infection by vaccination with cloned type glycoprotein D. Science. **227:** 1490-1492.

(5) Bernstein, D. I., L. R. Stanberry, C. J. Harrison, R. Shukla, J. C. Kappes, and M. G. Meyers. 1987. Antibody response to herpes simplex virus glycoprotein D: effects of acyclovir and relation to recurrence. J. Inf. Diseases. **156:** 423-429.

(6) Black, F. L. and J. L. Melnick. 1955. Micro-epidemiology of poliomyelitis and herpes-B infections. Spread of the virus within tissue cultures. J. Immunol. **74:** 236-242.

(7) Blacklaws, B. A., A. A. Nash, and G. Darby. 1987. Specificity of the immune response of mice to herpes simplex virus glycoproteins B and D constituitively expressed on L cell lines. J. Gen. Virol. **68:** 1103-1114.

(8) Cantin, E. M. , R. Eberle, J. L. Baldick, B. Moss, D. E. Willey, A. L. Notkins, and H. Openshaw. 1987. Expression of herpes simplex virus 1 glycoprotein B by a recombinant vaccinia virus and protection of mice against lethal herpes simplex virus 1 infection. PNAS (USA). **84:** 5908-5912.

(9) Cappel, R. 1976. Comparison of the humoral and cellular immune responses after immunization with live, UV inactivated herpes simplex virus and a subunit vaccine and efficacy of these immunizations. Arch. Virol. **52:** 29-35.

(10) Cappel, R., S. Sprecher, F. Rickaert, and F. deCuyper. 1982. Immune response to a DNA free herpes simplex virus vaccine in man. Arch. Virol. **73:** 61-67.

(11) Carter, V. C., P. C. Schaffer, and S. S. Tevethia. 1981. The involvement of herpes simplex virus type 1 glycoproteins in cell-mediated immunity. J. Immunol. **126:** 1655-1660.

(12) Centifano, Y. M., J. M. Little, and H. E. Kaufman. 1970. The relationship between virus chemotherapy, secretory antibody formation and recurrent herpetic disease. Ann. N. Y. Acad. Sci. **173:** 649-655.

(13) Chan, W. L. 1983. Protective immunization of mice with specific HSV-1 glycoproteins. Immunology. **49:** 343-352.

(14) Chan, W. L., M. L. Lukig, and F. Y. Liew. 1985. Helper T cells induced by an immunopurified herpes simplex virus type 1 (HSV 1) 115 kilodalton glycoprotein (gB) protect mice against HSV-1 infection. J. Exp. Med. **162:** 1304-18.

(15) Cheever, F. S. and G. Daikos. 1950. Studies on the protective effect of gamma globulin against herpes simplex virus infection in mice. J. Immunol. **65:** 135-141.

(16) Cook, M. L. and J. G. Stevens. 1973. Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection. Infect. Immun. **7:** 272-288.

(17) Cremer, K. J., M. MacKett, C. Wohlenberg, A. L. Notkins, and B. Moss. 1985. Vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D prevents latent herpes in mice. Science. **228:** 737-740.

(18) DeFreitas, E. C., B. Dietzschold, and H. Koprowski. 1985. Human T-lymphocyte response in vitro to synthetic peptides of herpes simplex virus glycoprotein D. PNAS (USA). **82:** 3425-3429.

(19) Dix, R. D., L. Pereira, and J. R. Baringer. 1981. Use of monoclonal antibody directed against herpes simplex virus glycoproteins to protect mice against acute virus-induced neurological disease. Infect. Immun. **34:** 192-199.

(20) Douglas, R. G., M. S. Anderson, J. G. Weg, T. Williams, D. E. Jenkins, V. Knight, A. C. Beall. 1969. Herpes simplex virus pneumonia. Occurrence in an allotransplanted lung. JAMA. **210:** 902-904.

(21) Douglas, R. G. and R. B. Couch. 1970. A prospective study of chronic herpes simplex virus infection and recurrent herpes labialis in humans. J. Immunol. **104:** 289-295.

(22) Duff, R. and F. Rapp. 1971. Oncogenic transformation of hamster cells after exposure to herpes simplex virus type 2. Nature. **233:** 48-50.

(23) Duff, R. and F. Rapp. 1973. Oncogenic transformation of hamster embryo cells after exposure to inactivated herpes simplex virus type 1. J. Virol. **12:** 209-217.

(24) Eberle, R. and S. W. Mou. 1983. Relative titers of antibodies to individual polypeptide antigens of herpes simplex virus type 1 in human sera. J. Inf. Diseases. **148:** 436-444.

(25) Eisenberg, R. J, C. P. Cerini, C. J. Heilman, A. D. Joseph, B. Dietzschold, E. Golu, D. Long, M. P. Leon, and G. H. Cohen. 1985. Synthetic glycoprotein D - related peptides protect mice against herpes simplex virus challenge. J. Virol. **56:** 1014-1017.

(26) Ennis, F. A. 1973. Host defense mechanisms against herpes simplex virus. II. Protection conferred by sensitized spleen cells. J. Inf. Diseases. **127:** 632-638.

(27) Erturk, M., R. Jennings, D. Hockley, and C. W. Potter. 1989. Antibody responses and protection in mice immunized with herpes simplex virus type 1 antigen immune-stimulating complex preparations. J. Gen. Virol. **70:** 2149-2155.

(28) Evans, C. A., H. B. Slavin, and G. P. Berry. 1946. Studies on herpetic infections in mice. IV. The effect of specific antibodies on the progression of virus within the nervous system of young mice. J. Exp. Med. **84:** 429-447.

(29) Gao, X.-M., F. Y. Liew, and J. P. Tite. 1989. Identification and characterization of T helper epitopes in the nucleoprotein of influenza A virus. J. Immunol. **143:** 3007-3014.

(30) Glorioso, J. C., U. Kees, G. Kumel, H. Kirchner, and P. H. Krammer. 1985. Indentification of herpes simplex virus type 1 (HSV-1) glycoprotein gC as the immunodominant antigen for HSV-1-specific memory cytotoxic T lymphocytes. J. Immunol. **135:** 575-582.

(31) Glorioso, J., C. H. Schroder, G. Kumel, M. Szczesiul, and M. Levine. 1984. Immunogenicity of herpes simplex virus glycoproteins gC and gB and their role in protective immunity. J. Virol. **50:** 805-812.

(32) Gompels, U. and A. Minson. 1986. The properties and sequence of glycoprotein H of herpes simplex virus type 1. Virology. **153:** 230-247.

(33) Hayashida, I.,S. Nagafuchi, Y. Hayashi, Y. Kino, R. Mori, H. Oda, N. Ohtomo, and A. Tashiro. 1982. Mechanism of antibody-mediated protection against herpes simplex virus infection in athymic nude mice: requirement of Fc portion of antibody. Microbiol. Immunol. **26:** 497-509.

(34) Heber-Katz, E. and B. Dietzschold. 1986. Immune response to synthetic herpes simplex virus peptides: the feasibility of a synthetic vaccine. Curr. Topics Microbiol. Immunol. **130:** 51-64.

(35) Heber-Katz, E., S. Valentine, B. Dietzschold, and C. Burns-Purzycki. 1988. Overlapping T cell antigenic sites on a synthetic peptide fragment from herpes simplex virus glycoprotein D, the degenerate MHC restriction elicited, and functional evidence for antigen-Ia interaction. J. Exp. Med. **167:** 275-287.

(36) Hooks, J. J., W. Burns, K. Hayashi, S. Geis, and A. L. Notkins. 1976. Viral spread in the presence of neutralizing antibody: mechanisms of persistence of foamy virus infection. Infect. Immun. **14:** 1172-1178.

(37) Horohov, D. W., J. Mester, S. Pourciau, A. Baghian, B. T. Rouse, and K. G. Kousoulas. 1989. Identification of cytotoxic T lymphocyte epitopes on herpes simplex virus glycoprotein B. 14th. International Herpesvirus Workshop. Abstract, p. 149.

(38) Howes, E. L., W. Taylor, N. A. Mitchison, and E. Simpson. 1979. MHC matching shows that at least two T-cell subsets determine resistance to HSV. Nature. **277:** 67-68.

(39) Kapoor, A. K., A. A. Nash, P. Wildy. 1982. Pathogenesis of herpes simplex virus in B cell suppressed mice: the relative roles of cell-mediated and humoral immunity. J. Gen. Virol. **61:** 127-131.

(40) Kapoor, A. K., A. A. Nash, P. Wildy, J. Phelan, C. S. McLean, and H. J. Field. 1982. Pathogenesis of herpes simplex virus in congenitally athymic mice: the relative roles of cell-mediated and humoral immunity. J. Gen. Virol. **60:** 225-233.

(41) Kites, E. N., P. S. Morahan, J. G. Tew, and B. K. Murray. 1977. Protection from oral herpes simplex virus infection by a nucleic acid-free virus vaccine. Infect. Immun. **16:** 955-960.

(42) Kino, Y., T. Eto, K. Nishiyama, N. Ohtomo, and R. Mori. 1986. Immunogenicity of purified glycoprotein gB of herpes simplex virus. Arch. Virol. **89:** 69-80.

(43) Klein, R. J. 1980. Effect of immune serum on the establishment of herpes simplex virus infection in trigeminal ganglia of hairless mice. J. Gen. Virol. **49:** 401-405.

(44) Klein R. J., E. Buimovici-Klein, H. Moser, R. Moucha, and J. Hilenhaus. 1981. Efficacy of a virion envelope herpes simplex virus vaccine against experimental skin infections in hairless mice. Arch. Virol. **68:** 73-80.

(45) Kohl, S. and L. S. Loo. 1982. Protection of neonatal mice against herpes simplex virus infection: probable in vivo antibody-dependent cellular cytotoxicity. J. Immunol. **129:** 370-376.

(46) Kuhn, J. E., G. Dunkler, K. Munk, and R. W. Braun. 1987. Analysis of the IgM and IgG antibody response against herpes simplex virus type 1 (HSV-1) structural and nonstructural proteins. J. Med. Virol. **23:** 135-50.

(47) Kumel, G., H. C. Kaerner, M. Levine, C. H. Schroder, and J. C. Glorioso. 1985. Passive immune protection by herpes simplex virus-specific monoclonal antibodies and monoclonal antibody-resistant mutants altered in pathogenicity. J. Virol. **56:** 930-937.

(48) Larsen, H. S., R. G. Russel, and B. T. Rouse. 1983. Recovery from lethal herpes simplex virus type 1 infection is mediated by cytotoxic T lymphocytes. Infect. Immun. **41:** 197-204.

(49) Laskey, L. A., d. Dowbenko, C. C. Simonsen, and P. W. Berman. 1984. Protection of mice from lethal herpes simplex virus infection by vaccination with a secreted form of cloned glycoprotein D. Biotech. **2:** 527-529.

(50) LaThangue, N. B., W. L. Chan, and J. D. Almeida. 1984. Monoclonal antibodies to herpes simplex virus type 1 glycoproteins show that epitope location influences virus neutralization. J. Med. Virol. **13:** 227-242.

(51) Lawman, M. J. P., R. J. Courtney, R. Eberle, P. A. Schaffer, M. K. O'Hara, and B. T. Rouse. 1980. Cell-mediated immunity to herpes simplex virus: specificity of cytotoxic T cells. Inf. Immun. **30:** 451-461.

(52) Lodmell, D. L., A. Niwa, K. Hayashi, and A. L. Notkins. 1973. Prevention of cell-to-cell spread of herpes simplex virus by leukocytes. J. Exp. Med. **137:** 706-720.

(53) Lodmell, D. L. and A. L. Notkins. 1974. Cellular immunity to herpes simplex virus mediated by interferon. J. Exp. Med. **140:** 764-778.

(54) Long, D., T. J. Madara, M. Ponce de Leon, G. H. Cohen, P. C. Montgomery, and R. J. Eisenberg. 1984. Glycoprotein D protects mice against lethal challenge with herpes simplex virus types 1 and 2. Inf. Immun. **43:** 761-764.

(55) Martin, S., E. Cantin, and B. T. Rouse. 1988. Cytotoxic T lymphocytes. Their relevancy in herpesvirus infections. Annals NY Acad. Sci. **532:** 257-272.

(56) Martin, S., B. Moss, P. W. Berman, L. A. Lasky, and B. T. Rouse. 1987. Mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: cytotoxic T cells. J. Virol. **61:** 726-734.

(57) Martin, S. and B. T. Rouse. 1987. The mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: clearance of local infection. J. Immunol. **138:** 3431-3437.

(58) McDermott, M. R., F. L. Graham, T. Hanke, and D. C. Johnson. 1989. Protection of mice against lethal challenge with herpes simplex virus by vaccination with an adenovirus vector expressing HSV glycoprotein B. Virology. **169:** 244-247.

(59) McKendall, R. R. 1985. IgG-mediated viral clearance in experimental infection with herpes simplex virus type 1: role for neutralization and F_C-dependent functions but not C' cytolysis and C5 chemotaxis. J. Inf. Diseases. **151:** 464-470.

(60) McKendall, R. R., T. Klassen, and J. R. Baringer. 1979. Host defenses in herpes simplex infections of the nervous system: effect of antibody on disease and viral spread. Infect. Immun. **23:** 305-311.

(61) McMichael, A. J., F. M. Gotch, and J. Rothbard. 1986. HLA B37 determines an influenza A virus nucleoprotein epitope recognized by cytotoxic T lymphocytes. J. Exp. Med. **164:** 1397.

(62) Merigan, T. C. and D. A. Stevens. 1971. Viral infections in man associated with acquired immunological deficiency states. Fed. Proc. **30:** 1858-1864.

(63) Mester, J. C., J. C. Glorioso, and B. T. Rouse. 1989. Neutralization, antibody-dependent cellular cytotoxicity, and epitope recognition in monoclonal antibody-mediated protection against the zosteriform spread of herpes simplex virus 1. Submitted.

(64) Mester, J. C., S. L. Highlander, A. Osmand, J. C. Glorioso, and B. T. Rouse. 1989. HSV-1-specific immunity induced by peptides corresponding to an antigenic site of glycoprotein B. Submitted.

(65) Metcalf, J. F., S. Chatterjee, J. Koga, and R. J. Whitley. 1988. Protection against herpetic ocular disease by immunotherapy with monoclonal antibodies to herpes simplex virus glycoproteins. Intervirol. **29:** 39-49.

(66) Nagafuchi, S., I. Hayashida, K. Higa, T. Wada, and R. Mori. 1982. Role of Lyt-1 positive immune T cells in recovery from herpes simplex virus infection in mice. Microbiol. Immunol. **26:** 359-362.

(67) Nagafuchi, S., H. Oda, R. Mori, and T. Taniguchi. 1979. Mechanism of aquired resistance to herpes simplex virus infection as studied in nude mice. J. Gen. Virol. **44:** 715-723.

(68) Nash, A. A., A. Jayasuriya, J. Phelan, S. P. Cobbold, H. Waldmann, and T. Prospero. 1987. Different roles for L3T4+ and Lyt 2+ T cell subsets in the control of an acute herpes simplex virus infection of the skin and nervous system. J. Gen Virol. **68:** 825-833.

(69) Nash, A. A. and P. G. H. Gell. 1983. Membrane phenotype of murine effector and suppressor T cells involved in delayed hypersensitivity and protective immunity to herpes simplex virus. Cellular Immunol. **75**: 348-355.

(70) Nash, A. A., P. G. H. Gell, and P. Wildy. 1981. Tolerance and immunity in mice infected with herpes simplex virus: simultaneous induction of protective immunity and tolerance to delayed-type hypersensitivity. Immunology. **43:** 153-159.

(71) Nash, A. A., J. Phelan, and P. Wildy. 1981. Cell-mediated immunity in herpes simplex virus-infected mice: H-2 mapping of the delayed-type hypersensitivity response and the antiviral T cell response. J. Immunol. **126:** 1260-1262.

(72) Nicholas, J. A., M. E. Levely, M. A. Mitchell, and C. W. Smith. 1989. A 16-amino acid peptide of respiratory syncytial virus 1A protein contains two overlapping T cell-stimulating sites distinguishable by class II MHC restriction elements. J. Immunol. **143:** 2790-2796.

(73) Oakes, J. E. 1975. Role for cell-mediated immunity in the resistance of mice to subcutaneous herpes simplex virus infection. Infect. Immun. **12:** 166-172.

(74) Oakes, J. E. and H. Rosemond-Hornbeak. 1978. Antibody-mediated recovery from subcutaneous herpes simplex virus type 2 infection. Infect. Immun. **21:** 489-495.

(75) Oakes, J. E. and R. N. Lausch. 1981. Role of F_C fragments in antibody-mediated recovery from ocular and subcutaneous herpes simplex virus infections. Infect. Immun. **33:** 109-114.

(76) Oakes, J. E., and R. N. Lausch. 1984. Monoclonal antibodies suppress replication of herpes simplex virus type I in trigeminal ganglia. J. Virol. **51:** 656-661.

(77) Openshaw, H., L.V. S. Asher, C. Wohlenberg, T. Sekizawa, and A. L. Notkins. 1979. Acute and latent infection of sensory ganglia with herpes simplex virus: immune control and virus reactivation. J. Gen. Virol. **44:** 205-215.

(78) Pachl, C., R. L. Burke, L. L. Stuve, L. Sanchez-Pescador, G. V. Nest, F. Masiarz, and D. Dina. 1987. Expression of cell-associated and secreted forms of herpes simplex virus type 1 glycoprotein gB in mammalian cells. J. Virol. **61:** 315-325.

(79) Paoletti, D. E., B. R. Lipinskas, C. Samsonoff, S. Mercer, and D. Panicali. 1984. Construction of live vaccines using genetically engineered poxviruses: biological activity of vaccinia virus recombinants expressing hepatitis B virus surface antigen and the herpes simplex virus glycoprotein. PNAS (USA). **81:** 193-197.

(80) Para, M. F., M. L. Parish, A. G. Noble, and P. G. Spear. 1985. Potent neutralizing activity associated with anti-glycoprotein D specificity among monoclonal antibodies selected for binding to herpes simplex virions. J. Virol. **55:** 483-488.

(81) Peacock, J. E., L. G. Kaplowitz, P. F. Sparling, D. T. Durack, J. W. Gnann, R. J. Whitely, M. Lovett, Y. J. Bryson, R. J. Klein, A. E. Friedman-Kien, G. M. Knowlton, and L. G. Davis. 1988. Intravenous acyclovir therapy of first episodes of genital herpes: a multicenter double-blind, placebo-controlled trial. Am. J. Med. **85:** 301-306.

(82) Price, R. W., M. A. Walz, C. Wohlenberg, and A. L. Notkins. 1975. Latent infection of sensory ganglia with herpes simplex virus: efficacy of immunization. Science. **188:** 345-348.

(83) Rager-Zisman, B. and A. C. Allison. 1976. Mechanism of immunologic resistance to herpes simplex virus (HSV-1) infection. J. Immunol. **116:** 38-40.

(84) Rager-Zisman, B. and B. R. Bloom. 1974. Immunological destruction of herpes simplex virus 1 infected cells. Nature. **251:** 542-543.

(85) Rector, J. T., R. N. Lausch, J. E. Oakes. 1982. Use of monoclonal antibodies for analysis of antibody-dependent immunity to ocular herpes simplex virus type 1 infection. Infect. Immun. **38:** 168-174.

(86) Roberts, P. L., B. E. Duncan, T. J. G. Raybould, and D. H. Watson. 1985. Purification of herpes simplex virus glycoproteins B and C using monoclonal antibodies and their ability to protect mice against lethal challenge. J. Gen. Virol. **66:** 1073-1085.

(87) Rooney, J. F., C. Wohlenberg, K. J. Cremer, B. Moss, and S. L. Notkins. 1988. Immunization with a vaccinia virus recombinant expressing herpes simplex virus type 1

glycoprotein D: long-term protection and effect of revaccination. J. Virol. **62:** 1530-1534.

(88) Rosenthal, K. L., J. R. Smiley, S. South, and D. C. Johnson. 1987. Cells expressing herpes simplex virus glycoprotein C but not gB, gD, or gE are recognized by murine virus specific cytotoxic T lymphocytes. J. Virol. **61:** 2438-2447.

(89) Schmid, D. Scott. 1988. The human MHC-restricted cellular response to herpes simplex virus type 1 is mediated by $CD4^+$, $CD8^-$ T cells and is restricted to the DR region of the MHC complex. J. Immunol. **140:** 3610-3616.

(90) Schneweis, K. E., M. Brado, B. Ebers, A. Friedrich, M. Olbrich, and W. Schuler. 1988. Immunological mechanisms giving rise to latency of herpes simplex virus in the spinal ganglia of the mouse. Med. Microbiol. Immunol. **177:** 1-8.

(91) Schrier, R. D., L. I. Pizer, and J. W. Moorhead. 1983. Type-specific delayed hypersensitivity and protective immunity induced by isolated herpes simplex virus glycoprotein. J. Immunol. **130:** 1413-1418.

(92) Scriba, M. 1975. Herpes simplex virus infection in guinea pigs: an animal model for studying latent and recurrent herpes simplex virus infection. Infect. Immun. **12:** 162-165.

(93) Scriba, M. and F. Tatzber. 1981. Pathogenesis of herpes simplex virus infections in guinea pigs. Infect. Immun. **34:** 655-661.

(94) Sekizawa, T., H. Openshaw, C. Wohlenberg, and A. L. Notkins. 1980. Latency of herpes simplex virus in absence of neutralizing antibody: model for reactivation. Science. **210**: 1026-1028.

(95) Sethi, K. K., Y. Omata, and K. E. Schneweis. 1983. Protection of mice from fatal herpes simplex virus type 1 infection by adoptive transfer of cloned virus-specific and H-2-restricted cytotoxic T lymphocytes. J. Gen. Virol. **64:** 443-447.

(96) Shore, S. L., T. L. Cromeans, and T. J. Romano. 1976. Immune destruction of virus-infected cells early in the infectious cycle. Nature. **262:** 695-696.

(97) Shore, S. L., A. J. Nahmias, S. E. Star, P. A. Wood, and D. E. McFarlin. 1974. Detection of cell-dependent cytotoxic antibody to cells infected with herpes simplex virus. Nature. **251:** 350-352.

(98) Simmons, A. and A. A. Nash. 1984. Zosteriform spread of herpes simplex virus as a model with which to study the immunology of recrudescent disease, pp. 55-69. In F. Rapp (ed.). Herpesvirus. A. R. Liss. New York.

(99) Simmons, A. and A. A. Nash, 1985. Role of antibody in primary and recurrent herpes simplex virus infection. J. Virol. **53:** 944-48.

(100) Simmons, A. and A. A. Nash. 1987. Effect of B cell suppression on primary infection and reinfection of mice with herpes simplex virus. J. Inf. Diseases. **166:** 649-654.

(101) Stanberry, L. R., D. I. Bernstein, R. L. Burke, C. Pachl, and M. G. Myers. 1987. Vaccination with recombinant herpes simplex virus glycoproteins: protection against initial and recurrent genital herpes. J. Inf. Diseases. **155:** 914-919.

(102) Stanberry, L. R., R. L. Burke, and M. G. Myers. 1988. Herpes simplex virus glycoprotein treatment of recurrent genital herpes. J. Inf. Diseases. **157:** 156-163.

(103) Stevens, J. G. and M. L. Cook. 1974. Maintenance of latent herpetic infection: an apparent role for anti-viral IgG. J. Immunol. **113:** 1685-1693.

(104) Straus, S. E. 1985. Herpes simplex virus infection: biology, treatment, and prevention. Ann. Int. Med. **103:** 404-419.

(105) Sullivan, V. and G. L. Smith. 1987. Expression and characterization of herpes simplex virus type 1 (HSV-1) glycoprotein G (gG) by recombinant vaccinia virus: neutralization of HSV-1 infectivity with anti-gG antibody. J. Gen. Virol. **68:** 2587-2598.

(106) Townsend, A. R. M., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael. 1986. The epitopes of influenza nucleoprotein recognised by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell. **44:** 959.

(107) Vestergaard, B. F. 1980. Herpes simplex virus antigens and antibodies: a survey of studies based on quantitative immunoelectrophoresis. Rev. Inf. Diseases. **2:** 899-913.

(108) Walz, M. A., H. Yamamoto, and A. L. Notkins. 1976. Immunological response restricts number of cells in sensory ganglia infected with herpes simplex virus. Nature. **264:** 554-556.

(109) Watari, E., B. Dietzschold, G. Szokan, and E. Heber-Katz. 1987. A synthetic peptide induces long-term protection from lethal infection with herpes simplex virus 2. J. Exp. Med. **165:** 459-470.

(110) Wachsman, M., L. Aurelian, C. C. Smith, B. R. Lipinskas, M. E. Perkus, and E. Paoletti. 1987. Protection of guinea pigs from primary and recurrent herpes simplex virus (HSV) type 2 cutaneous disease with vaccinia virus recombinants expressing HSV glycoprotein D. J. Inf. Diseases. **155:** 1188-1197.

(111) Weir, J. P., M. Bennett, E. M. Allen, K. L. Elkins, S. Martin, and B. T. Rouse. 1989. Recombinant vaccinia virus expressing the herpes simplex virus type 1 glycoprotein C protects mice against herpes simplex virus challenge. J. Gen. Virol. **70:** 2587-2594.

(112) Wildy, P. 1967. The progression of herpes simplex virus to the central nervous system of the mouse. J. Hyg., Camb. **65:** 173-192.

(113) Wildy, P. and P. G. H. Gell. 1985. The host response to herpes simplex virus. Brit. Med. Bul. **41:** 86-91.

(114) Wycoff, J. H., A. P. Osmand, R. J. Eisenberg, G. H. Cohen, and B. T. Rouse. 1988. Functional T cell recognition of synthetic peptides corresponding to continuous antibody epitopes of herpes simplex virus type 1 glycoprotein D. Immunobiol. **177:** 134-148.

(115) Yamashita, K. and E. Herber-Katz. 1989. Lack of immunodominance in the T cell response to herpes simplex virus glycoprotein D after administration of infectious virus. J. Exp. Med. **170:** 997-1002.

(116) Yasumoto, S., N. Okabe, and R. Mori. 1986. Role of epidermal langerhans cells in resistance to herpes simplex virus. Arch. Virol. **90:** 261-271.

(117) Zarling, J. M., P. A. Moran, L. A. Lasky, and B. Moss. 1986. Herpes simplex virus (HSV)-specific human T-cell clones recognize HSV glycoprotein D expressed by a recombinant vaccinia virus. J. Virol. **59:** 506-509.

(118) Zarling, J. M., P. A. Moran, R. L. Burke, C. Pachl, P. W. Berman, and L. A. Lasky. 1986. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells IV. Recognition and activation by cloned glycoproteins gB and gD. J. Immunol. **136:** 4669-4672.

(119) Zweerink, H. J. and L. Corey. 1982. Virus-specific antibodies in sera from patients with genital herpes simplex virus infection. Infect. Immun. **37:** 413-421.

CHAPTER 2

RATIONALE / OBJECTIVES

A. OVERVIEW

In man, the immune response to HSV-1 takes place too late during the course of a primary mucocutaneous infection to prevent colonization of the local sensory ganglia. As a consequence, an individual harboring latent virus is subject to recurrent infection, even though he or she has demonstratable "immunity" to the virus. There exists then, a dichotomy between immunoreactivity and protection in the case of recurrent infections. This may be due to several factors, including the possibility of the virus being "invisible" with respect to the immune system until causing a clinical lesion, immunosuppression at the onset of recurrence hindering the initial elimination of the virus, or the inability of the host to generate sufficient immunity following primary exposure.

Efforts to generate a HSV vaccine have encompassed approaches at the level of the whole virus, whole viral protein, and viral protein subunit. With respect to the whole virus, efforts here are based on the belief that the overall anti-viral response is beneficial, while those focusing on whole proteins seek to enhance a more specific response in hopes that it will provide a greater stimulus of the protective response. Both of these approaches, however, may generate a situation similar to that seen in human recurrent infections -- in other words, nonprotective immunity. Furthermore, an attenuated virus vaccine could express immunosuppressive proteins, which would generate a population of memory cells destined to suppress responsiveness upon reexposure to the virus. This may well be the case in the natural history of HSV-1 infections : "immunosuppressive"

epitopes encountered during primary infection leave an imprint on the immune system and allow the virus an advantage when reinfection occurs. Alternatively, the wide range of proteins made by the virus may serve as a screen to detract immune reactivity to essential proteins or epitopes, which, if seen exclusively, would be capable of generating a protective response. Nonsense viral peptides may outcompete the relevant "protective" peptides by binding cellular antigen presenting molecules with greater affinity. In this way, the full spectrum of viral proteins may act as non-specific "noise", eliciting an immune response to many irrelevant antigens and hindering immune reactivity with "essential" epitopes.

A similar argument can be invoked against vaccine attempts utilizing whole virus proteins. Once again, extraneous or suppressive epitopes may interefere with recognition and reactivity to essential (protective) epitopes. For instance, antibody generated to several regions of viral envelope glycoprotein X may impede the ability of neutralizing antibody to interact with the neutralizing epitope of glycoprotein X either by steric hindrance or through the induction of a conformational change in the glycoprotein. Additionally, the existence of "hindratopes", protein segments which hinder the recognition of other segments, has been documented for myoglobin and foot-and-mouth disease virus VP1 recognition (1,3). Herein lies the advantage of subunit vaccines over whole proteins or the virus itself : the immune response generated is focused upon the "essential" immunogen.

The initial analysis here seeks to identify protective epitopes of glycoproteins B, C, and D of HSV-1 by examining the ability of monoclonal antibodies, reactive with discrete regions of these glycoproteins, to protect against a murine model of human infection. Once identified, one of these regions will be synthesized as short overlapping peptides and assessed for its ability to induce HSV-specific -- and protective -- immunity.

B. PART II

As described in Chapter I, antibody directed against glycoproteins B, C, D, and E is protective in murine HSV challenge models; however, there is no consensus on the mechanism involved, and no indication of the regions of the respective glycoproteins important in recognition by protective antibody. Most of the studies did not address such parameters as the role of antibody affinity and dose in protection, and as a result, did not have a firm basis on which to judge the mechanism of protection in their models.

This study on antibody-mediated protection utilizes a panel of ten MAbs representative of larger panels of glycoprotein B, C, and D specific MAbs whose reactivity was previously classified into overlapping and discrete antigenic sites (6, 8, 10). Each representative MAb recognizes a discrete antigenic site on its respective glycoprotein. Ongoing studies have further defined the epitopes recognized by a majority of these MAbs (7, J. Glorioso, unpublished data), so that once a MAb is determined to be protective or nonprotective, the region and possibly the epitope recognized by the MAb is well-defined.

The murine zosteriform spread model was chosen to evaluate protection because of its well-defined parameters (16) and relatedness to human disease. The initial infection is performed at a cutaneous site, where the virus replicates and gains access to the peripheral nervous system. After replication in the local sensory ganglia, the virus reemerges in the skin at sites distant from the site of the original inoculation, mimicking the formation of recurrent lesions and producing an easily observable zosteriform band. The infected mice usually quickly progress through symptoms of encephalitis and die, and although this does not mimic the usual course of herpetic disease in humans, it provides a lethal challenge model which further emphasizes an antibody's protective capacity.

Additionally, protected mice will be dissected and the relevant dorsal root ganglia examined for the presence of HSV to determine the ability of an antibody to block viral access to the nervous system.

A variety of functional *in vitro* assays will be performed in an attempt to correlate such parameters as antibody dose and affinity, and the ability to neutralize the challenge virus or mediate lysis of virally-infected cells with protection from zosteriform spread and prevention of colonization of the dorsal root ganglia.

C. PART III

With the finding of a single protective gB-reactive MAb and the knowledge of its site of recognition, it is possible to test whether or not the epitope recognized by a protective antibody can function as a protective immunogen. To do this, three overlapping synthetic peptides, corresponding to the region of gB recognized by the protective MAb, were conjugated to poly-(L)-lysine, a minimally or nonimmunogenic carrier (17, 18) by a water soluble carbodiimide (EDC). Before conjugation, the reactive amino groups of the peptides were blocked with citraconic anhydride to block polymerization of the epitope, which could create unique "nonsense" configurations, distorting the integrity of the epitope and compromising its ability to be an effective immunogen. Additionally, initial blocking of the free amino groups of the peptides assures the orientation-specific coupling of the carboxyl-terminus of the peptides to the amino groups of the poly-lysine molecule.

The peptides, in both their free and conjugated forms, will be used to immunize three strains of inbred mice, to test the immunogenic potential of the free versus the conjugated peptide configurations, and to assess the role of major histocompatibility complex

restriction in their recognition. The sera and immune cells resulting from peptide immunization will be screened for cross-reactivity with whole virus *in vitro*, and the ability of the peptides to act as protective immunogens against HSV will be examined.

The successful outcome of this portion of the experiments will indicate a novel subunit immunogen capable of eliciting an anti-viral response. The advantage of synthetic peptides as immunogens is in their specificity, stability, and noninfectivity. Several disadvantages, such as potential nonrecognition and the need for coadministration of adjuvant, may be overcome by advances in adjuvant design and application and novel synthetic techniques. Recent documentation of the adjuvant activity of interleukin 1 (and its nonpyrogenic, noninflammatory fragment) and interleukin 2 (12, 13, 14, 19) as well as proteosome / lipopeptide (9) and lipophilic muramyl tripeptide (15) formulations, has provided evidence that nontoxic compounds are available which are potent immunostimulants. Additionally, interleukin 1 can surmount nonresponsiveness in mice (4), and chemical coupling to a variety of T_{Helper} peptides has potentiated poorly immunogenic peptides (2, 5, 11).

D. REFERENCES

(1) Brett, S. J., K. B. Cease, and J. A. Berzofsky. 1988. Influences of antigen processing on the expression of the T cell repertoire: evidence for MHC-specific hindering structures. J. Exp. Med. **168:** 357-374.

(2) Cox, J. H., J. Ivanyi, D. B. Young, J. R. Lamb, A. D. Syred, and M. J. Francis. 1988. Orientation of epitopes influences the immunogenicity of synthetic peptide dimers. Eur. J. Immunol. **18:** 2015-2019.

(3) Doel, T. R., C. Gale, G. Brooke, and R. DiMarchi. 1988. Immunization against foot-and-mouth disease with synthetic peptides representing the C-terminal region of VP1. J. Gen. Virol. **69:** 2403-2406.

(4) Durum, S. K., C. Higuchi, and Y. Ron. 1984. Accessory cells and T cell activation. The relationship between two components of macrophage accessory function: I-A and IL1. Immunobiol. **168:** 213-231.

(5) Francis, M. J., G. Z. Hastings, A. D. Syred, B. McGinn, F. Brown, and D. J. Rowlands. 1987. Non-responsiveness to a foot-and-mouth disease virus peptide overcome by addition of foreign helper T-cell determinants. Nature. **330:** 168-170.

(6) Highlander, S. L., W. Cai, S. Person, M. Levine, and J. C. Glorioso. 1988. Monoclonal antibodies define a domain on herpes simplex virus glycoprotein B involved in virus penetration. J. Virol. **62:** 1881-1888.

(7) Highlander, S. L., D. J. Dorney, P. J. Gage, T. C. Holland, W. Cai, S. Person, M. Levine, and J. C. Glorioso. 1989. Identification of 'mar' mutations in herpes simplex virus type 1 glycoprotein B which alter antigenic structure and function in virus penetration. J. Virol. **63:** 730-738.

(8) Highlander, S. L., S. L. Sutherland, P. J. Gage, D. C. Johnson, M. Levine, and J. C. Glorioso. 1987. Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J. Virol. **61:** 3356-3364.

(9) Lowell, G. H., W. R. Ballou, L. F. Smith, R. A. Wirtz, W. D. Zollinger, and W. T. Hockmeyer. 1988. Proteosome-lipopeptide vaccines: enhancement of immunogenicity for malaria CS peptides. Science. **240:** 800-802.

(10) Marlin, S. D., T. C. Holland, M. Levine, and J. C. Glorioso. 1985. Epitopes of herpes simplex virus type 1 glycoprotein gC are clustered in two distinct antigenic sites. J. Virol. **53:** 128-136.

(11) Milich, D. R., J. L. Hughes, A. McLachlan, G. B. Thorton, and A. Boriarty. 1988. Hepatitis B synthetic immunogen comprised of nucleocapsid T-cell sites and an envelope B-cell epitope. PNAS (USA). **85:** 1610-1614.

(12) Nencioni, L., L. Villa, A. Tagliabue, G. Antoni, R. Presentini, F. Perin, S. Silvestri, and D. Boraschi. 1987. In vivo immunostimulating activity of the 163-171 peptide of human IL-1ß. J. Immunol. **139:** 800-804.

(13) Nunberg, J. H., M. V. Doyle, S. M. York, and C. J. York. 1989. Interleukin 2 acts as as adjuvant to increase the potency of inactivated rabies virus vaccine. PNAS (USA). **86:** 4240-4243.

(14) Reed, S. G., D. L. Pihl, P. J. Conlon, and K. H. Grabstein. 1989. IL-1 as adjuvant. Role of T cells in the augmentation of specific antibody production by recombinant human IL-1 alpha. J. Immunol. **142:** 3129-3133.

(15) Sanchez-Pescador, L., R. L. Burke, G. Ott, and G. Van Nest. 1988. The effect of adjuvants on the efficacy of a recombinant herpes simplex virus glycoprotein vaccine. J. Immunol. **141:** 1720-1727.

(16) Simmons, A. and A. A. Nash. 1984. Zosteriform spread of herpes simplex virus as a model of recrudescence and its use to investigate the role of immune cells in prevention of recurrent disease. J. Virol. **52:** 816-821.

(17) Stason, W. B., M. Vallotton, and E. Haber. 1967. Synthesis of an antigenic copolymer of angiotensin and succinylated poly-L-lysine. Biochim. Biophys. Acta. **133:** 582-584.

(18) Van Vunakis, H., J. T. Farrow, H. B. Gjika, and L. Levine. 1971. Specificity of the antibody receptor site to D-lysergamide: model of a physiological receptor for lysergic acid diethylamide. PNAS (USA). **68:** 1483-1487.

(19) Weinberg, A. and T. C. Merigan. 1988. Recombinant interleukin 2 as an adjuvant for vaccine-induced protection. Immunization of guinea pigs with herpes simplex virus subunit vaccines. J. Immunol. **140:** 294-299.

PART II :

NEUTRALIZATION, ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY, AND EPITOPE RECOGNITION IN MONOCLONAL ANTIBODY-MEDIATED PROTECTION AGAINST THE ZOSTERIFORM SPREAD OF HSV-1

CHAPTER 1

ABSTRACT

The *in vivo* protective role of herpes simplex virus (HSV-1)-specific antibody was analyzed using a panel of monoclonal antibodies (MAbs) reactive with discrete antigenic sites on glycoproteins B, C, and D (gB, gC, gD) in the murine zosteriform spread model of HSV-1. Of four anti-gB MAbs, only one yielded significant protection, whereas all three anti-gC MAbs and three anti-gD MAbs protected against zosteriform lesion formation. In ganglial explant experiments, the protective capacity of the MAbs was shown to correlate with their ability to prevent the establishment of ganglial latency. *In vitro* antibody affinity determinations demonstrated that the most significantly protective MAbs were of relatively low affinity for the BK strain of HSV-1 used. *In vitro* neutralization assays revealed that the protective anti-gB and two of the protective anti-gD MAbs had relatively high neutralizing activity against HSV-1_{BK}, whereas the protective anti-gC MAbs and one protective anti-gD MAb had relatively low neutralizing activity. Three anti-gB MAbs, which had little or no protective ability, had high HSV-1_{BK} neutralizing activity. A majority of the protective MAbs exhibited very high antibody-dependent cell-mediated cytotoxicity (ADCC) against HSV-1-infected cells, including the weakly neutralizing anti-gD and anti-gC MAbs and one of the strongly neutralizing anti-gD MAbs. Removal of the F_C portion of two of the anti-gC MAbs abrogated their protective capacity. These studies, then, utilizing a murine model which mimics the natural course of human infection, designate specific epitopes recognized by antibodies that can protect mice from a primary infection and the establishment of ganglial latency by HSV-1. Whereas the protection afforded by the gB and one of the gD-reactive MAbs appears to be due to neutralizing ability, the protective capacity of the MAbs reactive with the three gC epitopes and one gD epitope may be a function of their ability to mediate ADCC. One of the three protective gD-reactive MAbs may rely on both neutralization and ADCC to block viral access to the dorsal root ganglia.

CHAPTER 2

INTRODUCTION

Herpes Simplex Virus (HSV)-1 is known to code for at least seven envelope glycoproteins (gB, gC, gD, gE, gG, gH, and gI), which are expressed on the virion envelope as well as on the membrane of HSV-1-infected cells. Glycoproteins B, D, and H are essential for cell entry (5, 14, 15, 33, 52). Glycoprotein H also plays a role in mediating virus release from infected cells (16). Glycoprotein C and the gE-gI complex are able to bind complement component C3b and immunoglobin F_C regions, respectively (2, 10, 11, 24). Glycoprotein G has been immunologically identified with monoclonal antibodies, but has yet to be linked to a particular role in viral pathogenesis (57).

The immune response to a primary mucocutaneous HSV infection is multifarious, relying predominantly on T cell-mediated immunity to clear the initial site of infection and antibody produced by B cells to limit the amount of virus invading the local sensory ganglia (25, 27, 32, 43). Both mice and humans generate an antibody response to a wide range of both structural (envelope and core) and nonstructural (regulatory) HSV proteins during infection, although in humans the structural proteins, especially gB, gC, and gD, appear to be the main antibody targets (8, 30, 39). However, the antibody response arises too late during primary infection to prevent colonization of the local sensory ganglia, and appears to be unable to prevent recurrent infections in a significant portion of individuals harboring latent HSV. In mice, passive transfer experiments have demonstrated the ability of monoclonal antibodies (MAbs) specific for HSV glycoproteins gB, gC, gD, and gE to protect naive mice against a lethal HSV challenge (1, 7, 31, 41, 50). The ability of polyclonal immune sera and gD-specific MAbs to protect in a model of recurrent disease has also been demonstrated (55).

The specific *in vivo* protective mechanism of HSV-specific antibody is uncertain. Other than the extracellular neutralization of virions, the mechanisms by which antibody

may effect anti-viral immunity depend on the binding of viral antigens expressed on the infected cell surface, and include the triggering of the classical complement cascade (and infected cell lysis via antibody-dependent complement-mediated cytotoxicity, ADCMC), the activation of F_C-receptor-bearing killer cells (antibody-dependent cellular cytotoxicity, ADCC), the suppression of viral replication (mediated by the viral protein - antibody interaction at the infected cell surface, 12, 13), and the blocking of direct cell-to-cell spread. Earlier reports have suggested either neutralization or ADCC as a mechanism of protection against lethal HSV challenge (1, 55). An additional report, however, found the protective ability of passively transferred antibody to have no correlation with the *in vitro* activities of neutralization, ADCMC, or ADCC (50). *In vitro* studies have demonstrated that HSV-specific antibody can inhibit cell-to-cell spread of the virus (20, 22) and suppress viral replication, via a putative transmembrane signal following antibody - viral glycoprotein interaction at the cell surface, in infected neuronal cells (45). The potential contribution of these mechanisms to *in vivo* protection is unclear. Finally, previous accounts of antibody-mediated protection against HSV *in vivo* have relied on anti - gB, gC, gD, and gE-reactive MAbs of undefined epitope specificity, and as a result have yielded no indication of the areas of the respective glycoproteins important in the generation of a protective or nonprotective antibody response.

In this report we have examined the ability of a panel of monoclonal antibodies, specific for discrete antigenic sites on gB, gC, and gD, to protect against the zosteriform spread of the BK strain of HSV-1 in C3H mice, and to prevent the colonization of the dorsal root ganglia innervating the inoculation site. We have also examined the affinity of each MAb for the challenge virus, and the ability of each MAb to neutralize HSV-1_{BK} and mediate ADCC against infected cells *in vitro*. Our results point to areas of gB, gC, and gD that are important targets for antibody-mediated immunity, and demonstrate that the protective activity of the MAbs reactive with these sites may be a function of either neutralization or ADCC, or both.

CHAPTER 3

MATERIALS AND METHODS

A. VIRUSES

HSV-1 KOS 321, and a clinical isolate of HSV-1 : strain BK, were propagated in HEp-2 cells, maintained on McCoy's 5a medium with 10% donor calf serum (DCS) (Gibco, Grand Island, NY), as previously described (4). Infectious particles were recovered after 3 freeze/thaw cycles, aliquoted, and stored at -80℃. Titration on Vero cell monolayers (17) revealed titers of 1.8×10^9 $TCID_{50}$'s / ml for KOS 321 and 5.6×10^8 $TCID_{50}$'s / ml for $HSV\text{-}1_{BK}$.

B. MONOCLONAL ANTIBODIES (MAbs)

The production of the HSV-specific MAbs B3, B4, B6, B8, C3, C4, C8, D2, D7, and D8 has been previously described (23). Each was produced as mouse ascites fluid, clarified by ammonium sulfate precipitation, suspended in phosphate-buffered saline and stored at -80℃. The previously reported properties of the MAbs (20, 31, 35) are summarized in Table 1.

C. MICE / INOCULATIONS

Six week old male C3H/TEN (University of Tennessee Memorial Research Center Hospital, Knoxville, TN) and C3H/HeJ (The Jackson Laboratory, Bar Harbor, ME)

Table 1 : Properties of the Monoclonal Antibody Panel.

MAb	Isotype	Antigenic Site	Neutralization Titer HSV-1	Neutralization Titer HSV-2	Plaque Inh. (HSV-1)
B3	IgG3	gB I	2,560	-	-
B4	IgG3	gB III	10,240	640	-
B6	IgG2b	gB IV	10,240	-	+
B8	IgG2a	gB II	102,400	2,560	-
C3	IgG2a	gC IIa	6,400	-	-
C4	IgG2a	gC I	6,400	-	-
C8	IgG2a	gC IIb	20,480	-	-
D2	IgG3	gD I	81,920	25,600	+
D7	IgG2a	gD IX	4,096	2,560	+
D8	IgG2a	gD X	5,120	2,560	+

Previously-defined properties of the MAbs used in this study (20, 22, 35). Antigenic sites refer to the discrete areas of the envelope glycoproteins B, C, and D (gB, gC, gD) of HSV-1 recognized by the MAbs. Antigenic sites for gB and gC are diagrammed in Figure 6. Glycoprotein D's antigenic sites have been described by Cohen, *et al.* (6). Neutralization titers shown are reciprocal 50% endpoints for HSV-1 KOS 321 and HSV-2 186.111. Plaque inhibition refers to the ability of the MAbs to hinder plaque formation *in vitro*.

mice were infected as described by Simmons & Nash (54). One day before infection, their dextral flanks were shaved and depilated with Nair (Carter Products, NY, NY). Two hours prior to infection, 100 µl of a MAb (as concentrated ascities fluid) was administered intraveneously in 200 µl of Hank's Balanced Salt Solution (HBSS). Control animals received 300 µl of HBSS alone. The mice were then coded and mixed so that their treatment was unknown at the time of infection. After anesthetizing the mice with Metofane (Pitman-Moore, Washington Crossing, NJ), each was percutaneously inoculated, with a 28-gauge needle, on the mid-flank region by scratching through a 10 µl drop containing 2.8×10^5 $TCID_{50}$'s of HSV-1_{BK}, leaving an abraded area of approximately $9mm^2$. This inoculation scheme yielded a mid-flank zosteriform band in approximately 90% of untreated control mice on day 5 post-inoculation.

D. DETERMINATION OF PROTECTION

Monoclonal antibody-mediated protection was determined by examining the banding pattern of the infected mice for up to two weeks post-infection. Almost all of the mice which formed a zosteriform band died or were sacrificed following symptoms of encephalitis. Protection was recorded as positive when no zosteriform spread was evident. Significant protection was determined using Chi-Square contingency table comparison (Statview 512+ Macintosh software of Brainpower Inc., Calabasas CA) of each treatment group with the respective number of control mice.

E. AFFINITY DETERMINATION

Individual MAb affinities for HSV-1_{BK} were determined using an enzyme-linked immunosorbent assay (ELISA, 36) variation of the method of Frankel & Gerhard (9).

Briefly, BK virions were purified by 4 hour, 80,000g centrifugation (Beckman L3-50 Ultracentrifuge, Beckman, Palo Alto, CA) over a 20-50% continuous potassium tartrate (Aldrich Chemical Co., Milwaukee, Wisconsin) gradient in 25 X 89 mm centrifuge tubes (Beckman) and used as antigen. Binding curves were determined by coating the ELISA plate wells with a constant amount of purified BK and adding a series of twelve two-fold dilutions of each antibody (in quadruplicate). Quantitation of the total amount of antibody able to bind BK (for the determination of binding constants) was performed using a constant amount of antibody (equivalent to the last relevant dilution of the antibody binding curve) and reacting it with varying amounts of pre-bound BK antigen. Absorbance was read at 490nm on an automated reader (Bio-Tek model EL310). Comparison to a standard curve (ABS_{490} by nanograms of gamma globulin) generated by coating ELISA plates with known amounts of mouse gamma globulin (Accurate Chemical & Scientific Corp., Westbury, NY) allowed the precise conversion of each ABS_{490} reading into nanograms of antibody bound.

F. DETERMINATION OF GANGLIAL LATENCY

Mice surviving infection were kept for 2-3 months, sacrificed, and dissected. Dorsal root ganglia innervating the inoculation site were explanted into 1ml of McCoy's 5A medium with 5% DCS in 2ml tubes (Corning, Corning, NY). After 3-5 days incubation at 37℃, this culture was transferred to a media-evacuated $25cm^2$ flask of Vero cells seeded at 1×10^6 cells per flask the previous day. After 2-4 hours, 5 additional mls of McCoy's 2% DCS were added. Flasks were observed daily for cytopathic effect (cpe) for an additional 7 days. Afterwards, two rounds of cell pelleting, 3 freeze/thaw cycles, and replating of the supernant fluid on a fresh $25cm^2$ flask of Vero cells (observed for an additional 7 days for cpe) were performed to assay for slowly replicating virions.

G. NEUTRALIZATION ASSAY

The ability of each MAb to neutralize HSV-1_{BK} was examined by pre-incubating 80 $TCID_{50}$'s of HSV-1_{BK} with series of two-fold dilutions of each MAb, in the presence of complement (Low-Tox-M rabbit complement, Cedarlane Laboratories LTD, Accurate Chemical & Scientific Corp.) at a final dilution of 1:10, in a total volume of 75 µl in 96 well microtiter plates, for three hours at 37℃. Twenty-five microliters of the mixture was then transferred (in triplicate) to confluent monolayers of Vero cells (maintained in McCoy's 5a medium with 10% DCS). After a one hour adsorption period, three drops of Modified Eagle Medium (Gibco) with 2% DCS and 1.25% low melting point (LMP) agarose (BRL, Gaithersburg, MD) (warmed to 37℃) were added to each well. After three days, the number of plaques was counted. Neutralization units are expressed as the total amount of each MAb initially added to the well where the 50% endpoint of plaque-reduction occurred.

H. ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC) ASSAY

MAb-mediated, anti-HSV ADCC activity was determined following the procedure of Kohl, *et al.* (28). Adherent mouse peritoneal cells were prepared from mice, injected with 1 ml of thioglycollate broth (BBL Microbiology Systems, Cockeysville MD) 5 days earlier, by peritoneal lavage with 4 ml of ice cold RPMI 1640 medium (Gibco) with HEPES buffer (0.025M final), 0.2% sodium bicarbonate, 10% fetal calf serum, PSN antibiotic mixture (Gibco), 50 µg/ml L-glutamine, and 2 mercaptoethanol (5×10^{-5} M final) (complete RPMI). The peritoneal cells were plated in 100 X 20mm tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ) in complete RPMI and incubated at 37℃, 5% CO_2 for 4-6 hours. Nonadherent cells were then removed by vigorous washing with HBSS, and the adherent effector cells collected after

trypsinization of the plates with 1 ml EDTA / 0.125% trypsin (Gibco) for 4 minutes. Vero cells, infected with HSV-1 strain KOS 321 at a multiplicity of infection of 1.5 18 hours before use, and labeled by incubating 2×10^6 cells in 1ml of complete RPMI with 150 μCi of ^{51}Cr (Amersham) for four hour at 37℃, served as targets (after three washes in HBSS to remove excess label).

Determination of the MAbs' ability to mediate ADCC was performed in 96-well round-bottom microtiter plates (Corning) by adding dilutions of each MAb (quadruplicate samples in complete RPMI) to 5×10^3 targets and from $2\text{-}2.5 \times 10^5$ adherent cells (an effector to target cell ratio of 40-50 : 1) for a final volume of 200 μl. After 16 hour incubation at 37℃, 100 μl of the media supernant of each well was collected, an additional 100 μl of 2N NaOH added, and the total remainder collected in a separate glass tube. The ^{51}Cr release in the supernant, and the ^{51}Cr in the cell pellet were counted by a LKB-Wallac 1274 RiaGamma Counter (Wallac Oy, Turku, Finland). Percent ^{51}Cr release was determined with the following formula :

$$\%\ ^{51}\text{Cr release} = {}^{2A}/_{A+B} \times 100,$$

where A equals the average of the media release counts (from the initial 100 μl sample), and B the average of the retained counts in the cell pellet (to which the NaOH was added). The percent specific release (percent ADCC) mediated by the MAbs was determined by subtracting the $\%\ ^{51}Cr$ release value of the negative control wells {containing peritoneal cells and targets (no serum control) or peritoneal cells and targets along with dilutions of normal mouse serum (normal serum control)} from the $\%\ ^{51}Cr$ release value of the MAb wells. In all experiments, the $\%\ ^{51}Cr$ release values for either negative control were equivalent (from 5 - 10% above the spontaneous release by the targets themselves, which ranged from 10-15%). The standard errors of the $\%\ ^{51}Cr$ release sample means were 10% or less. Units were determined by titrating each MAb-

mediated ADCC from zero to maximum, and assigning one ADCC unit value to the amount of each MAb added to the assay causing 50% maximum ^{51}Cr release from the targets.

I. $F(ab')_2$ FRAGMENT GENERATION

$F(ab')_2$ fragments were generated by incubating the MAbs with pepsin (porcine stomach mucosa, 2X cryst.,Calbiochem, La Jolla CA) at low pH. Initial analytical experiments to determine the optimum pH and digestion time were performed as recommended by P. Parham in Handbook of Experimental Immunology (47), and the results monitored by FPLC (Pharmacia) with a 50 X 5mm Mono Q HR 5/5 anion exchange column (20mM Tris buffer pH=7.6, 0 - 0.5M NaCl gradient) and electrophoresis over 7.5% polyacrylamide gels (SDS-PAGE, as in Maniatas, *et al.*, 34). MAbs C3 and C4 were fragmented by incubating them at a concentration of 2 mg/ml with pepsin at 25 µg/ml in 0.1M citrate buffer (pH=3.8) for 6 hours at 37℃. The reaction was terminated by raising the pH above 7 by addition of 3.0 M Tris-HCL, pH=8.6, and the mixture desalted by centrifugation over Centricon-30 microconcentrators (30,000 MW cutoff, Amicon Corp., Danvers, Mass.) for $^1/_2$ hour at 2500 x g and 4℃ in a Sorvall Superspeed RC2-B with a fixed angle SS-34 rotor (Sorvall). Before passive transfer to mice in protection studies, the digestion was confirmed by SDS-PAGE, and the fragments were diluted in HBSS so that each mouse received a 0.5 mg dose in a 300 µl iv. injection.

CHAPTER 4

RESULTS

A. MAb PROTECTION AGAINST THE ZOSTERIFORM SPREAD OF HSV-1

We initially examined the ability of MAbs, administered two hours before infection, to block both zosteriform lesion formation and the colonization of the dorsal root ganglia innervating the site of infection. Our results, as summarized in Tables 2 and 3, and Figure 1, indicate that MAbs specific for HSV-1 gB, gC, and gD were able to protect against zosteriform lesion formation (and death) as well as the colonization of the local sensory ganglia. Specifically, mice given MAbs B6, C3, C4, C8, D2, D7, and D8 had a significantly lower frequency of zosteriform lesion formation than control mice given HBSS. Mice that received MAbs B6, C3, D2, D7, and D8 and did not form a zosteriform band were also protected against viral colonization of the dorsal root ganglia.

Table 2 additively summarizes several separate experiments performed in both C3H/TEN and C3H/HeJ mice. Each experimental trial analyzed a select group of MAbs in treatment groups of from four to six mice each. Since each trial utilized several but not all of the MAbs, and each trial used a varying number of mice (from six to eight) as controls, pooling of the results for each MAb for overall Chi-Square analysis necessitated the pooling of the control mice for each trial that a single MAb was evaluated in. As a result, the number of respective control mice used for the evaluation of statistically significant protection varied for each MAb, and is individually cited in Table 2. For Figure 1, the percentage of nonbanding (or protected) mice in each control group was subtracted from the percentage of nonbanding mice in each MAb treatment group to determine the percent of protection afforded by antibody administration. As

Table 2 : MAb Protection Against Zosteriform Spread of HSV-1

MAb	Zosteriform Spread # (+)	# (-)	Total # Mice	% Positive	p - value
B3	13	4	17	77	NS
control	28	5	33	85	
B4	16	5	21	76	NS
control	35	5	40	88	
B6	2	17	19	11	.0001
control	26	2	28	93	
B8	15	0	15	100	NS
control	22	2	24	92	
C3	5	25	30	17	.0001
control	33	5	38	87	
C4	3	11	14	21	.0001
control	20	3	23	87	
C8	6	8	14	43	.006
control	19	3	22	86	
D2	10	6	16	63	.05
control	18	2	20	90	
D7	1	14	15	7	.0001
control	23	4	27	85	
D8	2	18	20	10	.0001
control	26	4	30	87	

Additive summarization of several separate experiments performed in both C3H/TEN and C3H/HeJ mice. 100 µl of each MAb preparation was administered intravenously two hours prior to HSV-1 challenge. Protection was determined by examining the banding pattern of the infected mice for up to two weeks post-infection, and was recorded as positive when no zosteriform spread was evident. Each experimental trial analyzed a select group of MAbs in treatment groups of from four to six mice each. Since each trial utilized several but not all of the MAbs, and each trial used a varying number of mice (from six to eight) as controls, pooling of the results for each MAb for overall Chi-Square analysis necessitated the pooling of the control mice for each trial that a single MAb was evaluated in. As a result, the number of respective control mice used for the evaluation of statistically significant protection varied for each MAb, and is individually cited.

NS : not significant ($p > 0.05$).

Table 3 : MAb Prevention of Latent Ganglial Infection

MAb - Specificity	# of Mice	# With Latent HSV-1
gB (B6)	10	0
gC	12	5
gD	13	0
(+) Controls	16	9

Two to three months after infection, the dorsal root ganglia innervating the site of inoculation were explanted onto Vero cell monolayers, which were subsequently examined for HSV-induced cytopathic effect (cpe). Mice were recorded as with latent HSV if the initial or twice repeated passage over fresh Vero cells yielded HSV-induced cpe. The gC and gD-reactive MAbs are grouped into their glycoprotein specificity. Positives controls were mice which formed zosteriform lesions and survived.

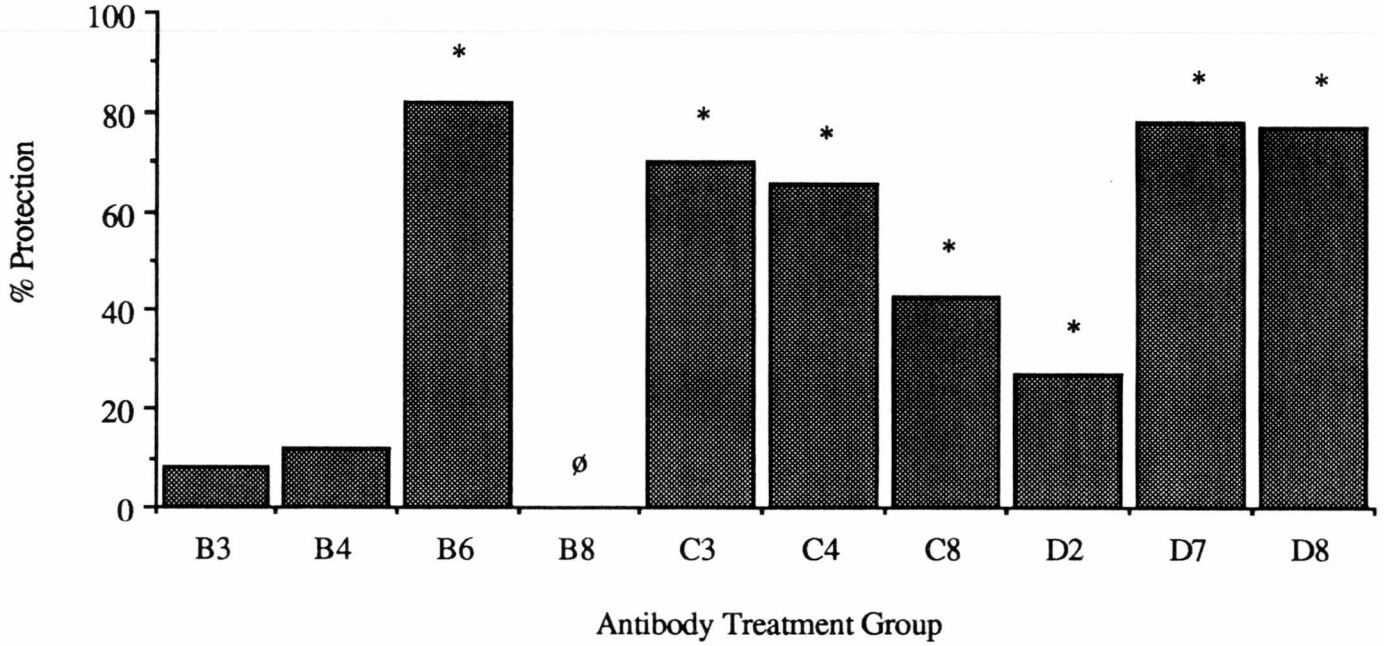


Figure 1 : Protection Against Zosteriform Spread by a Panel of HSV-1 Glycoprotein-Specific Monoclonal Antibodies.

The ability of each MAb to prevent zosteriform lesions is shown by the percentage of nonbanding treated mice minus the percentage of nonbanding control mice (from Table 2) as percent protection. MAbs yielding significant protection, as determined by Chi-Square analysis, are indicated by an asterisk.

shown, MAb B8 conferred no improvement over controls, and MAbs B3 and B4 yielded an approximate ten percent improvement over controls. MAb D2 yielded nearly thirty percent protection, and C8 protected forty percent of the mice. MAbs C3 and C4 yielded approximately sixty-five percent protection, and MAbs B6, D7, and D8, eighty percent protection. Two trends are apparent : (1.) antibodies directed against discrete antigenic areas of gC and gD are all significantly protective, and (2.) only one of four gB-specific MAbs provides a high and significant degree of protection.

The examination of ganglial latency revealed that MAb B6 and the three gD-specific MAbs were able to prevent colonization of the dorsal root ganglia innervating the inoculation site by HSV-1 (Table 3). The gC-specific MAbs, although protective against zosteriform spread, were not as effective as MAbs B6, D2, D7, and D8 in preventing the establishment of ganglial latency. Sixty-seven percent of the mice protected by MAb C3 administration had no detectable latent HSV, but on the whole, forty-two percent of the gC Mab-treated mice were found to harbor latent HSV-1, compared to fifty-six percent of the positive control group, and zero percent of the mice receiving the gD-specific MAbs or MAb B6.

B. THE RELATIONSHIP BETWEEN PROTECTION AND MAb DOSE AND AFFINITY FOR THE CHALLENGE VIRUS

We examined several parameters of the MAbs to evaluate whether they had any relationship to the MAbs' ability to protect against zosteriform lesion formation and the colonization of the local sensory ganglia by HSV-1_{BK}. Initially, 100 µl of concentrated ascites fluid was administered intravenously as a 1:3 dilution in 200 µl of HBSS. Quantitation of the amount of gamma globulin in each MAb preparation, performed via ELISA (using a series of five-fold dilutions of each MAb as antigen, and comparing the final ABS_{490} readings to a standard curve as in **MATERIALS AND METHODS E. AFFINITY DETERMINATION**), revealed that the MAbs' IgG content fell into

three groups (Table 4), averaging : (I.) 5.2 mgs / 100 µl, (II.) 1.2 mgs / 100 µl, and (III.) 0.3 mgs / 100 µl. Inspection of the individual MAbs contained in groups I and II reveals that the high concentration groups contain both protective and nonprotective antibodies. Additional protection experiments were performed using several dilutions of the more concentrated ascites preparations, and no significant variation was found in the ability of MAbs B6, C3, C4, D7, and D8 to protect when 0.2-0.3 mgs of each was administered per mouse (Table 5). Furthermore, MAb D8 was 75% protective when 0.01 mgs was administered per mouse, while MAb B6 was minimally protective (25%) at a dose of 0.01 mg / mouse. On the whole then, although the total IgG content of each MAb initially administered varied, higher doses of immunoglobulin do not correlate with zosteriform protection, and the protective MAbs were equivalently effective when given at a lower concentration of 0.2-0.3 mgs per mouse.

Affinity determinations were performed to assess whether the protective MAbs had a higher affinity for the BK strain of HSV-1 used as a challenge virus in this study. The results of subjecting the points of each of the MAb BK-binding curves (Figure 2) to scatchard analysis, and using the linear portion of the scatchard plots (Figure 3) to determine binding constants (9), indicate that the affinity of the MAbs for HSV-1_{BK} has no relationship to their ability to protect against zosteriform spread or ganglial colonization by HSV-1 (Table 6). MAbs B6, D7, and D8, which gave the greatest protection against zosteriform spread, had the lowest binding affinities for HSV-1_{BK} of all the MAbs analyzed. The three gC-specific MAbs had somewhat greater affinities for the challenge virus than MAbs B6, D7, and D8, but were protective to a lesser extent. Additionally, the nonprotective MAbs B3, B4, and B8 had higher affinities than MAb B6.

Table 4 : Final Concentration of Each MAb Administered.

Group		MAb	Amount IgG in 100 µls (mgs)
I		B3	5.6
	*	C3	4.0
	*	C4	4.8
	*	D7	7.2
	*	D8	4.6
II		B4	1.0
	*	B6	1.2
		B8	1.3
III	*	C8	0.2
	*	D2	0.3

A series of eight five-fold dilutions of each MAb (in quadruplicate) was used as antigen to coat polyvinyl chloride microtiter ELISA plates. Final MAb-related ABS_{490} readings in the range of the ABS_{490} readings of the linear portion of the standard curve (of ABS versus concentration of mouse gamma globulin) were used to determine the amount of IgG in each MAb sample. Astericks (*) denote MAbs yielding significant protection from the zosteriform spread of HSV-1. Groupings of MAb preparations with similar IgG content yielded three concentration subsets as shown.

Table 5 : Protection Against Zosteriform Spread by Reduced Quantities of MAbs.

	% Protection (total # of mice) Amt. Transferred per Mouse (mg)							
MAb	0.7	0.6	0.5	0.4	0.3	0.2	0.05	0.01
B6		100 (6)	100 (4)		100 (4)		25 (4)	25 (4)
C3			100 (4)			60 (5)		
C4			100 (4)			80 (5)		
D7	100 (4)		100 (4)		100 (6)			
D8			100 (6)	100 (4)		100 (4)	67 (3)	75 (4)

Protection against zosteriform spread mediated by dilutions (in HBSS) of the MAb ascites preparations. Each mouse received from 0.01 to 0.7 mgs of antibody intravenously in a total volume of of 300 µls. Percent protection refers to the percentage of mice which did not band after challenge with HSV-1_{BK}. Untreated (HBSS) controls had a "background" percent protection of approximately 8%.

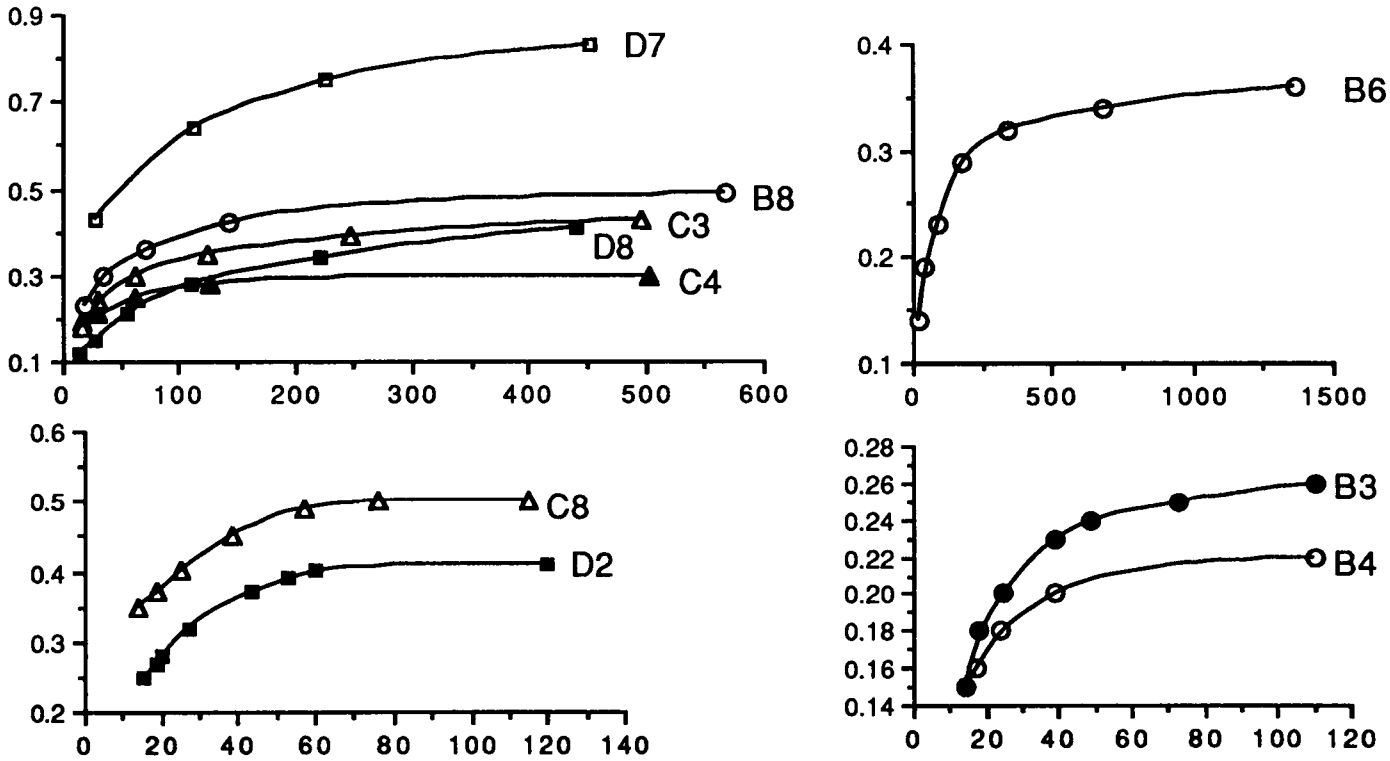


Figure 2 : Monoclonal Antibody Binding Curves to the BK Strain of HSV-1.

Binding studies were performed via ELISA, using a constant amount of purified HSV-1_{BK} as antigen and a series of two-fold dilutions of each MAb. The results are grouped into four graphs of MAbs with similar binding ordinants; ordinant : ngs of MAb added, abscissa : ABS 490.

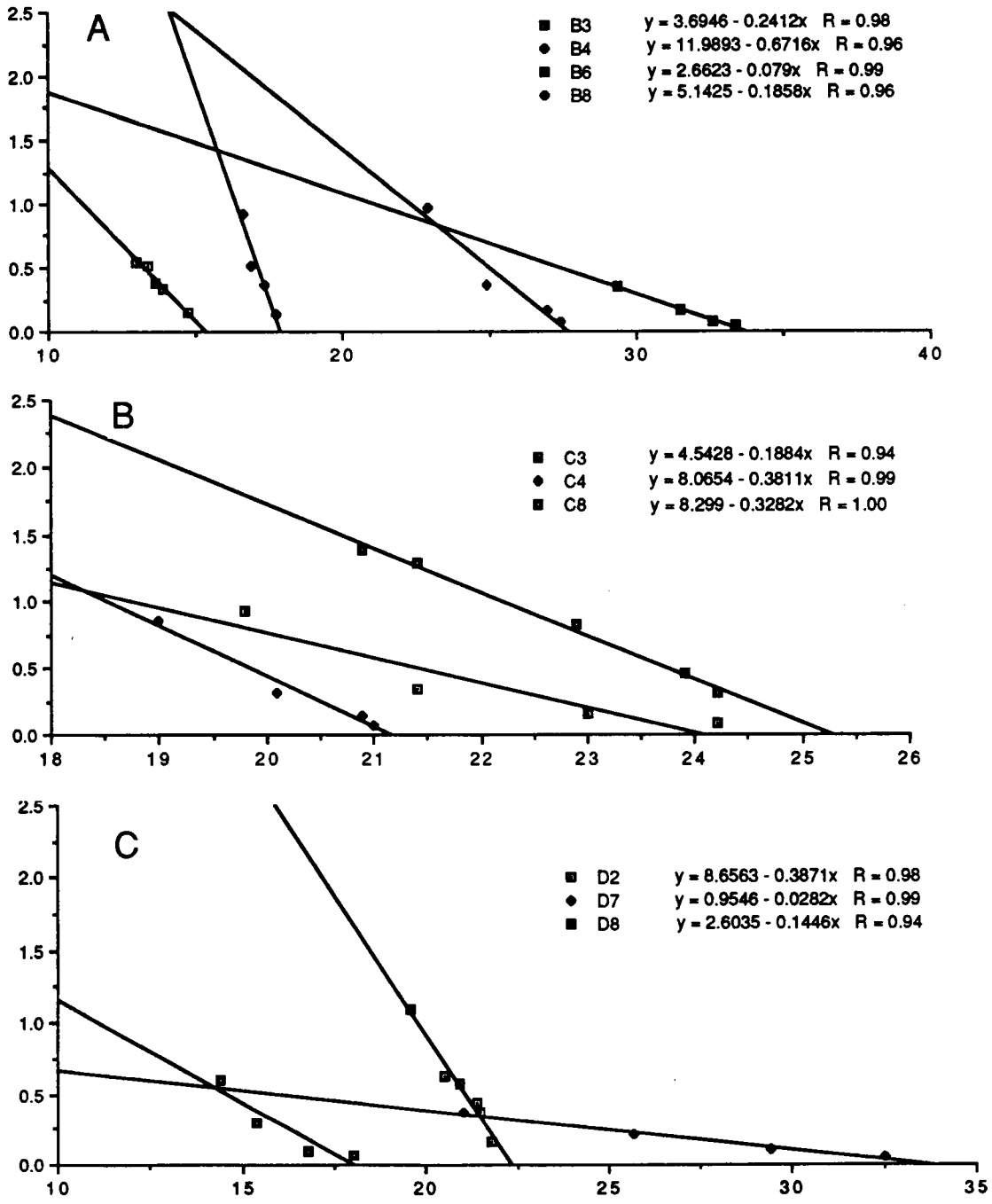


Figure 3 : Affinity Determination Plots.

The linear portion of the scatchard plots based on the MAb binding curves is represented as bound/free versus the concentration of MAb bound X 10^{10} M-1. The equations indicate the y-intercept and slope of the approximated straight line, and the the fit of the data points to the straight line (R : correlation coefficient). MAbs are grouped into their glycoprotein specificities : (A.) MAbs B3, B4, B6, and B8, (B.) MAbs C3, C4, and C8, and (C.) MAbs D2, D7, and D8.

Table 6 : MAbs in Order of Highest to Lowest Affinity for HSV-1_{BK}.

	MAb	K_o (M-1)
	B4	6.7×10^9
*	D2	3.9×10^9
*	C4	3.8×10^9
*	C8	3.3×10^9
	B3	2.4×10^9
*	C3	1.9×10^9
	B8	1.9×10^9
*	D8	1.5×10^9
*	B6	7.9×10^8
*	D7	2.8×10^8

Summary of the affinity determinations based on subjecting the MAb binding curves to scatchard analysis. K_o was determined from the slope of the affinity determination Scatchard plots (of Figure 3) for each MAb. MAbs are ranked in order of highest to lowest affinity, and those yielding significant protection from zosteriform spread are indicated by an asterick (*).

C. THE ROLE OF NEUTRALIZATION AND ADCC IN PROTECTION

To further examine the *in vitro* activities of the MAbs, each was serially diluted to the ng/ml range, and portions of the dilutions added to neutralization and ADCC assays. As shown in Table 7, each MAb could neutralize HSV-1_{BK} and most could act in anti-HSV ADCC. Units were assigned to the amount of MAb necessary to achieve 50% neutralization and 50% of the maximum amount of ADCC mediated by that MAb (Figure 4).

When comparing the neutralization and ADCC units (N-Us and ADCC-Us, respectively) contained in the 0.2 mg / mouse doses of the protective MAbs, a division into two general groups is apparent. The protective MAbs B6, D2, and D8, at 0.2 mg / mouse, have N-Us in the range of 1-5 X 10^4. The other protective MAbs, C3, C4, and D7, have N-Us approximately ten-fold lower (from 1-4 X 10^3). Oddly, the nonprotective MAbs B3, B4, and B8, at their original dose concentrations, have relatively high N-Us (1-4 X 10^4). A greater division is found among the ADCC-Us of the protective MAbs at 0.2 mg / mouse : C3, C4, C8, D7, and D8 have ADCC-Us ranging from 2 X 10^5 to 2 X 10^6, whereas B6 and D2 have ADCC-Us of approximately 4 X 10^3. Here, the nonprotective MAbs B3 and B4 have relatively low ADCC-Us per their original dose (<100 and 8.2 X 10^3, respectively). Nonprotective MAb B8 is an unusual case in that although its 50% maximum ADCC activity occurred at a low MAb amount (1.3 ng), its maximum ADCC never exceeded 10% specific lysis (Figure 4), whereas most of the other MAbs reached a maximum specific lysis of 40%. Therefore, although B8 has a high ADCC-U at its original dose, the total ADCC mediated by B8 is inferior to the ADCC mediated by the other MAbs, and its ADCC-U amount is not relevant to the ADCC-U amounts of the other MAbs.

The difference between 10^4 and 10^3 N-Us, in terms of protection, is apparent when considering the protection mediated by MAbs B6 and D8 at concentrations of 0.05 and

Table 7 : MAb Neutralization and ADCC Units for HSV-1.

MAb	Neutralization Unit	Neutralization Units		ADCC Unit	ADCC Units	
		Original Dose	0.2mg/Mouse		Original Dose	0.2mg/Mouse
B3	340 ng	1.6×10^4		680 ng	8.2×10^3	
B4	31 ng	3.9×10^4		(>10 μg)	(<100)	
B6	18 ng	6.7×10^4	1.1×10^4	55 ng	2.2×10^4	3.7×10^3
B8	78 ng	1.7×10^4		1.3 ng	1.0×10^6	
C3	70 ng	5.7×10^4	2.9×10^3	0.1 ng	4.0×10^7	2.0×10^6
C4	51 ng	9.4×10^4	3.9×10^3	1.0 ng	4.8×10^8	2.0×10^5
C8	130 ng	1.5×10^3	1.5×10^3	0.1 ng	2.0×10^6	2.0×10^6
D2	6.3 ng	4.8×10^4		75 ng	4.0×10^3	
D7	94 ng	7.7×10^4	2.1×10^3	0.1 ng	7.2×10^7	2.0×10^6
D8	17 ng	2.7×10^5	1.2×10^4	0.1 ng	4.6×10^7	2.0×10^6

The ability of each MAb to neutralize HSV-1_{BK} was examined by pre-incubating the virus with a series of two-fold dilutions of each MAb (in the presence of complement) for 3 hours at 37℃, and then adding the mixture to a confluent layer of Vero cells. After a one hour adsorption period, 3 drops of MEM with 1.25% LMP agarose were added. The number of plaques was counted 3 days later. The results are expressed as neutralization units, being the amount of MAb added to the well where the 50% endpoint of plaque reduction occurred. Monoclonal antibody-mediated ADCC was evaluated by adding series of ten -fold dilutions of the MAbs to fixed numbers of adherent mouse peritoneal cells (as effectors) and HSV-1-infected and ^{51}Cr-labeled Vero cells (as targets), and incubating the assay for 16 hours at 37℃. The amount of ^{51}Cr-label released into the culture supernant was used to calculate % ADCC. The amount of each MAb, which when added to the assay led to a 50% maximal ^{51}Cr-label release, was assigned 1 ADCC unit value. Both total Neutralization Units and ADCC Units for the doses of MAbs given *in vivo* to prevent zosteriform spread are listed. Original dose refers to the amount of each MAb listed in Table 4, which was passively tranferred to mice in the initial experiments summarized in Tables 2 and 3. Neutralization and ADCC units at 0.2mg/mouse refers to the passive transfers performed with weight-equilibrated MAb amounts of the protective MAbs.

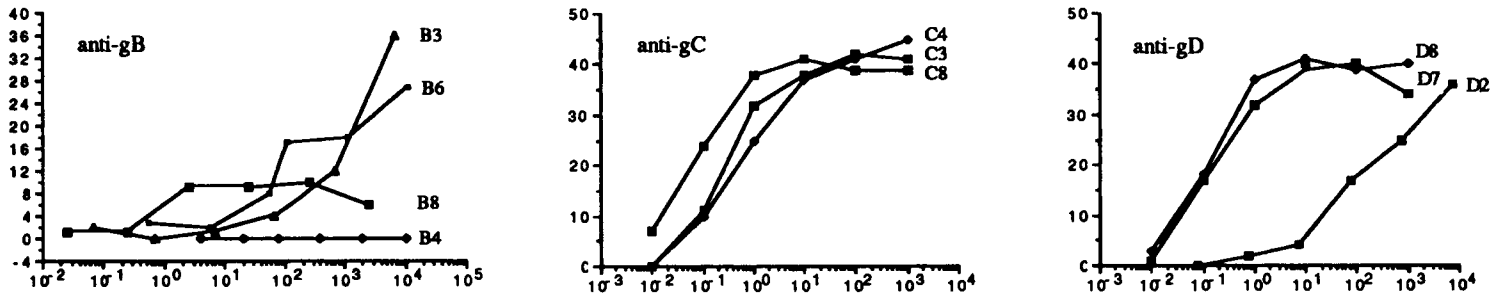


Figure 4 : MAb - Mediated ADCC.

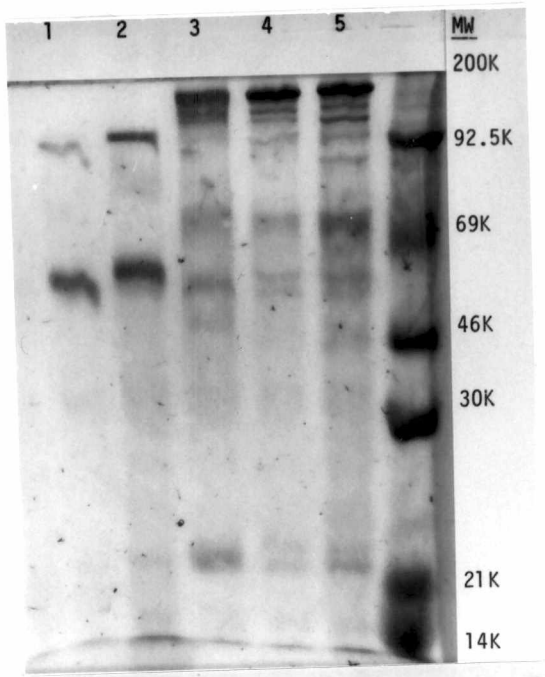
Monoclonal antibody-mediated ADCC is shown as % specific lysis versus nanograms of MAb added per microtiter well. ADCC was evaluated by adding series of ten -fold dilutions of the MAbs to fixed numbers of adherent mouse peritoneal cells (as effectors) and HSV-1-infected and ^{51}Cr-labeled Vero cells (as targets), and incubating the assay for 16 hours at 37℃. The amount of ^{51}Cr-label released into the culture supernant was used to calculate % specific lysis. B3, B4, B6, B8, C3, C4, C8, D2, D7, and D8 refer to the MAbs as described in Table 1.

0.01 mgs per mouse (Table 5). At a concentration of 0.2-0.3 mgs / mouse, both MAbs have total N-Us above 10^4. At 0.05 and 0.01 mgs / mouse, the total N-Us per dose are approximately 3 X 10^3 and 6 X 10^2, respectively, and the protective capacity of both MAbs is compromised. MAb B6 shows the greatest loss of protection (from 100 to 25%), and the ability of D8 to protect (75%) at N-Us of 10^3 or less may be due to its ability to mediate ADCC (at a 500-fold greater level than B6 : the total ADCC-Us at 0.01 mg / mouse are 1 X 10^5 for D8 and 2 X 10^2 for B6). It is therefore possible that protection mediated solely by neutralizing ability requires a minimum of 10^4 N-Us. However, a dose of this magnitude is not necessarily sufficient in itself for protection, as MAbs B3, B4, and B8 were not protective at this level.

The importance of ADCC in protection is suggested by the divergent results of the low dose transfers of B6 and D8, and is further supported by the protection mediated by the gC-specific MAbs at 0.2 mgs / mouse, where the total N-Us per mouse are below 10^4 and total ADCC-Us per mouse above 10^5. All of the mice receiving greater than 10^5 ADCC-Us were protected from zosteriform spread (with the single exception being the unusual ADCC case of B8). Of the mice receiving less than 10^4 ADCC-Us in 0.2 mgs, only those given B6 and D2 were protected (both B6 and D2 have 0.2 mg N-Us above 10^4). Finally, when the F_C portion of MAbs C3 and C4 were removed by pepsin treatment (Figure 5), and 0.5 mg of the $F(ab')_2$ and Fab fragments administered to mice before zosteriform challenge, no protection was seen (Table 8).

In summary, using total unit neutralization and ADCC doses as a gauge for predicting *in vivo* protective mechanisms, it appears that MAbs B6 and D2 are protective mainly because of their neutralizing ability, whereas MAbs C3, C4, C8, and D7 are protective due to ADCC. MAb D8 may mediate protection by both mechanisms, and since the other protective MAbs exhibited both functional capacities (even though one or the other was at a markedly reduced level), the potential contribution of both

A



B

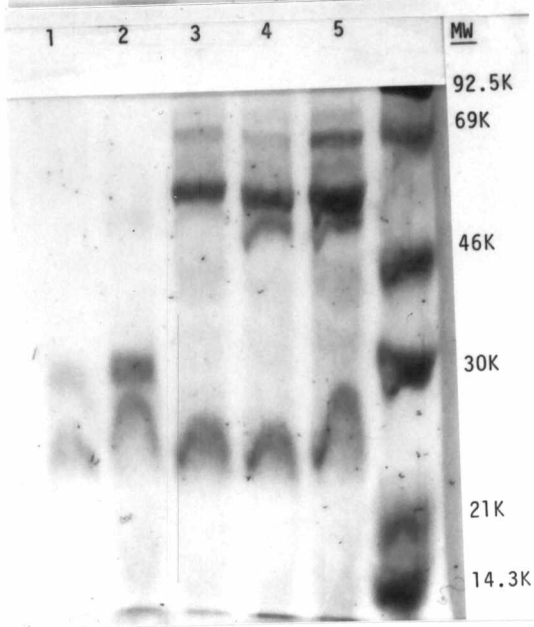


Figure 5 : Pepsin Fragmentation of MAbs C3 and C4.

SDS-PAGE of whole and pepsin digested IgGs : (A.) nonreducing gel, (B.) reducing gel. Lanes 1 and 2: $F(ab')_2$ and Fab fragments from pepsin digestion of C4 and C3. Lanes 3, 4, and 5 : whole MAbs D8, C4, and C3. MAbs C3 and C4 were fragmented by incubating them at a concentration of 2 mg/ml with pepsin at 25 µg/ml in 0.1M citrate buffer (pH=3.8) for 6 hours at 37℃. The reaction was terminated by raising the pH above 7 by addition of 3.0 M Tris-HCL, pH=8.6, and the mixture desalted by centrifugation over Centricon-30 microconcentrators.

Table 8 : Effect of Delayed Transfer or Fragmentation on Protection.

MAb	Transfer Day	MAb Form	% Protection (total # mice)
B6	0	Whole IgG	100 (4)
C3	0	Whole IgG	100 (4)
C4	0	Whole IgG	100 (4)
D7	0	Whole IgG	100 (4)
D8	0	Whole IgG	100 (6)
C3	0	$F(ab')_2$ / Fab	0 (4)
C4	0	$F(ab')_2$ / Fab	0 (4)
B6	2	Whole IgG	100 (3)
C3	2	Whole IgG	100 (3)
C4	2	Whole IgG	0 (3)
C8	2	Whole IgG	67 (3)
D7	2	Whole IgG	60 (5)
D8	2	Whole IgG	100 (4)

Protection against zosteriform spread by the passive transfer of MAbs two hours before (day 0) or two days after (day 2) infection and the $F(ab')_2$ and Fab fragments of MAbs C3 and C4 generated by pepsin cleavage. The mice received a 300 µl suspension containing 0.5 mg of each MAb preparation intravenously. Negative (HBSS) controls had a "background" percent protection of approximately 8%.

neutralization and ADCC in MAb-mediated protection should not be overlooked.

CHAPTER 5

DISCUSSION

The role of HSV-specific antibody in human infections remains an enigma. While humoral immunity is endowed with a range of potential anti-viral activities, the majority of previous investigations, using various animal and *in vitro* models, have both supported and refuted the ability of HSV-specific antibody to : (1.) clear infectious virus from, or limit the extent of viral replication in, the skin (25, 26, 37, 55, 56), (2.) protect the local sensory ganglia from viral infection (25, 26, 27, 38, 46, 48, 55, 56, 58), and (3.) block the formation of recurrent lesions (46, 55, 56). The discrepancy among these results may be due to differences in models, virus strains, and monoclonal antibodies (which may vary in affinity, immunoglobulin subclass, and epitope specificity). The accurate determination of the ability of antibody to act on HSV-infected skin is of primary importance, since the severity of ganglial infection and its corollaries (subsequent latent infection and reactivation) are directly related to the severity of the initial cutaneous infection (27, 58). Additionally, gauging the ability of immune sera to block virus trafficking from the skin to the nervous system, and vice versa, is important for determining the ability of antibody to protect from ganglionic colonization and subsequent reactivation.

During primary HSV infection in humans, ganglionic colonization is achieved before a substantial amount of HSV-specific antibody is generated. Normally, in the course of the primary immune response, the virus is eliminated from the skin and establishes a latent neuronal infection. After recovering from a primary infection, most patients demonstrate strong antibody reactivity to gB, gC, and gD (8, 30), but the role of these antibodies in resolving primary infection and preventing recurrent infection is unclear. While some reports find no difference in the quantitative or qualitative aspects of the HSV-specific antibody response in those with frequent recurrences versus those without (8, 51, 60), others have reported that individuals whose antibody response was

generated to a greater variety of viral proteins were prone to more frequent recurrences (30, 59), while those with an antibody response primarily directed against gB and gD were not (30). Additionally, in the guinea pig model, an increased anti-gD titer was found to be associated with a reduced number of recurrent episodes and days with recurrent lesions (3). At this point, however, one may only speculate that an enhanced gB and / or gD-specific antibody response will decrease the frequency of recurrent lesion formation, or, if generated before infection, be able to protect an individual from ganglionic colonization by HSV.

The zosteriform spread model used in this study mimics the natural course of human HSV infection in that the primary infection is initiated at a cutaneous site and spreads to the local sensory ganglia, where active viral replication occurs (54). Reinfection of the epithelium occurs via retrograde axonal transport of the virus through the sensory nerve projections of the infected ganglion, causing the formation of a confluent (zosteriform) lesion in that dermatome (54). Since this model involves the shuttling of the virus between the epithelium and peripheral nervous system, it is useful for gauging the ability of passively transferred antibody to block viral access to either the sensory ganglia (as a model of protection against a primary infection) or areas of the epithelium remote from the original inoculation site (as a model of recurrent infection).

Our approach initially entailed administration of MAbs, reactive with discrete epitopes of gB, gC, and gD, to naive mice to assess potential antibody-mediated protection from primary infection. Our focus was on the MAbs' ability to block viral access to the nervous system, and by examining the *in vitro* characteristics of the MAbs, we sought correlates to *in vivo* protection. Our results demonstrate that antibody to gB, gC, and gD can protect against viral spread through the nervous system, and that this protection is related to ADCC and neutralizing ability and depends on the epitope specificity of the antibody. Passive transfer of several of the MAbs to infected mice after ganglionic colonization demonstrated that they were also effective in impeding the flow of virus from the infected ganglion to the skin.

The MAbs which best prevented ganglionic colonization by HSV were B6, D2, D7,

and D8, and three of these, B6, D7, and D8, had relatively low affinities for HSV-1_{BK}. Since the assessment of the MAbs' ability to prevent ganglionic colonization was done using "original dose" antibody concentrations (Table 3), it is not clear whether the activities of neutralization or ADCC are most important for B6, D7, and D8's protection (all had neutralization and ADCC unit activities above 10^4). MAb D2, however, appears to have protected mainly by neutralization. Whether or not the gC-specific MAbs were effective in preventing ganglionic colonization is uncertain due to the 56% recovery of reactivatible virus in the surviving positive control mice. However, the data indicate that the gC-reactive antibodies are less effective than the gD-reactive antibodies and MAb B6 in blocking viral access to the dorsal root ganglia.

With the formation of a zosteriform lesion as the gauge of virus spread between the epithelium and the nervous system, our results demonstrate that both neutralization and ADCC may be effective methods of antibody-mediated *in vivo* protection. When administered before the initial epithelial infection or two days afterwards (Table 8), when the virus has reached the sensory ganglia (54), antibody doses with high N-Us (MAbs B6 and D8), as well as antibody doses with high ADCC-Us and low N-Us (MAbs C3, C4, C8, and D7) were protective. Although most of the protective MAbs mediated both neutralization and ADCC, three results serve to differentiate between the two activities : (1.) all of the MAbs which mediated a high degree of ADCC were protective (C3, C4, C8, D7, and D8) whereas strongly neutralizing doses of MAbs B3, B4, and B8 were not, (2.) MAb D8 was protective when given at a dose with low neutralizing activity but high ADCC activity, and (3.) when the F_C portion was removed from MAbs C3 and C4 before transfer, no protection was conferred. Other reports have also demonstrated protection mediated by nonneutralizing MAbs (1, 50) and the requirement of the F_C portion of immunoglobulin for protection (18, 44).

The *in vivo* relevance of ADCC has been demonstrated in HSV and tumor cell challenge studies in mice. Kohl and Loo (29) demonstrated that only when subneutralizing doses of human immune globulin were combined with human (ADCC effector) leukocytes *in vivo* were neonatal mice protected from lethal HSV-1 challenge.

Herlyn and Koprowski (19) and Seto *et al.* (53) showed a correlation between antibodies mediating tumor cell regression *in vivo* and the ability of the antibodies to mediate ADCC *in vitro.* While neutralizing antibody may be important in limiting the spread of free virus, it may only function *in vivo* when present in high titer, and even then may do little to prevent viral pathogenesis (49). Conversely, ADCC is apparently effective at low antibody concentration both *in vitro* and *in vivo.*

Additional mechanisms which may have contributed to the protective capacity of the MAbs include the inhibition of cell to cell spread of HSV-1, which has been shown to be an activity of MAbs B6, D2, D7, and D8 (Table 1), and ADCMC. All of the MAbs used in this study mediated ADCMC against HSV-1-infected Vero cells in the presence of rabbit complement (Appendix B, Table 1). Whether or not they could differentially activate mouse complement *in vivo* is unknown; however, several other investigators have found no role for *in vivo* ADCMC as a potential mechanism of MAb-mediated protection (1, 37, 44, 50).

The antigenic sites recognized by the gB and gC-specific MAbs are diagrammed in Figure 6. Previous analysis of the reactivity of panels of gB, gC, and gD-specific MAbs with mutant herpes simplex viruses (mar mutants : monoclonal antibody resistant) containing point mutations in discrete areas of the genes coding for gB, gC, and gD, has allowed the localization of discrete antigenic sites within each of these glycoproteins. Sequence analysis localizing the specific point mutations of each mar mutant has further defined the epitopes involved in MAb reactivity (21, S. D. Marlin, unpublished data). Both the overall antigenic sites, for which the MAbs analyzed here are representative of, and the specific mar virus mutation points resulting in resistance to neutralization by the specific MAbs analyzed are shown for gB and gC. Our protection results indicate that the epitopes recognized by MAbs B6, C3, C4, C8, D2, D7, and D8 are important targets for antibody-mediated immunity against HSV-1, and that the protective gB and gD-reactive MAbs are most effective in preventing ganglionic

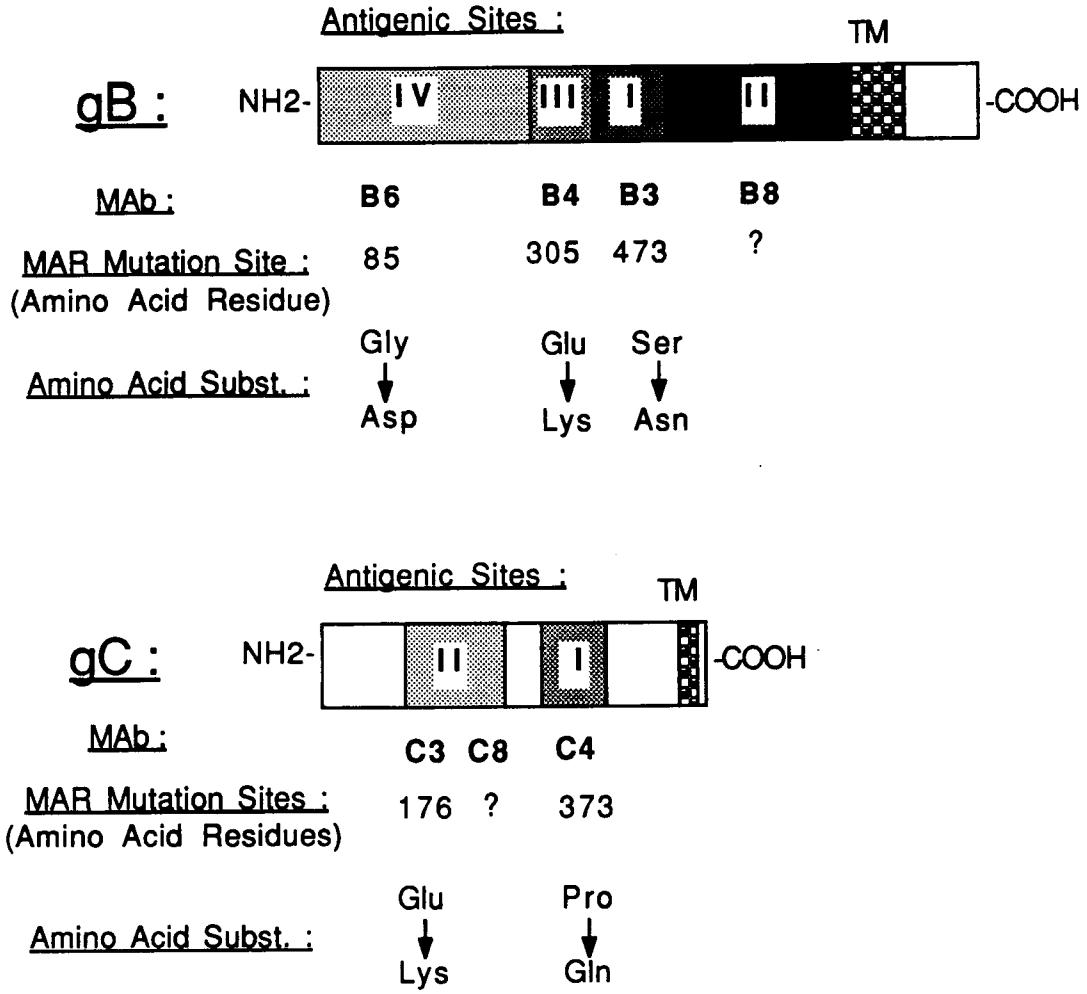


Figure 6 : Antigenic Sites Identified by the gB & gC-Specific MAbs.

The antigenic sites defined by each of the gB- and gC-specific MAbs. The amino acid residues of the mature protein (beginning with the amino-terminal methionine as "1") corresponding to the antigenic sites are as follows: for gB : IV : 44-233, III : 283-380, I : 381-441, II : 441-727, for gC : II :129-247, I : 307-373. The MAR mutation site numbers refer to the amino acid residue site of the single point mutation in each unique MAR mutant which confers resistance to neutralization by one of the MAbs. Amino acid subst. refers to the amino acid substitution at the mutation site.

TM : transmembrane region.

colonization.

The region of gB important in this regard has been localized to the first 233 amino acids of the amino terminus of the glycoprotein (20). MAb B6 reactivity has been mapped to amino acid position 85 within this linear antigenic site (21). Conversely, gB-specific MAbs reactive to antigenic sites I (amino acid residues 381-441), II (residues 441-727), and III (residues 283-380) do not identify targets of protective antibody-mediated immunity in this model, even though these MAbs react with the challenge virus with high avidity and have relatively high neutralizing ability *in vitro*.

Protection against zosteriform spread was achieved with the gC-specific MAbs reactive with antigenic sites I (amino acid residues 307-373) and II (residues 129-147) (35). Protection mediated by the gC site II reactive MAb C3 was slightly better than that mediated by the site I reactive MAb C4 in that MAb C3 yielded a lower percentage of latent HSV in the ganglia of surviving mice (Appendix C, Table 1) and was able to protect mice when administered two days after challenge, whereas MAb C4 was not (Table 8).

The antigenic sites recognized by the protective gD-specific MAbs are incompletely defined (22). MAb D2 recognizes a discontinuous site (site Ib of Muggeridge, *et al.*, 42), and its corresponding mar D2.1 single mutation site is at amino acid residue 157 of the mature gD-1 protein, causing a glutamine to leucine substitution (V. J. Bhagat, personal communication).

In sum, the ability of all the gC and gD-specific MAbs to protect against zosteriform spread indicates that several epitopes exist on these molecules that induce protective immunity, whereas antibody directed to three of the four antigenic sites of gB was ineffective *in vivo*.

Construction of synthetic immunogens based on the epitopes identified by the protective MAbs may be complicated by the discontinuous nature of many of the sites. However, we have made synthetic peptides corresponding to the epitope recognized by MAb B6, and have generated HSV-1-neutralizing, protective antibody using one as an immunogen in mice (40). It would be of interest to further dissect the epitopes

recognized by the gC and gD-reactive MAbs, and evaluate their protective potential as immunogens.

REFERENCES

(1) Balachandran, N., S. Bacchetti, and W. E. Rawls. 1982. Protection against lethal challenge of Balb/c mice by passive transfer of monoclonal antibodies to five glycoproteins of herpes simplex virus type 2. Infect. Immun. **37:** 1132-1137.

(2) Bauke, R. B. and P. G. Spear. 1979. Membrane proteins specified by herpes simplex viruses V. identification on an Fc-binding glycoprotein. J. Virol. **32:** 779-789.

(3) Bernstein, D. I., L. R. Stanberry, C. J. Harrison, R. Shukla, J. C. Kappes, and M. G. Meyers. 1987. Antibody response to herpes simplex virus glycoprotein D: effects of acyclovir and relation to recurrence. J. Inf. Diseases. **156:** 423-429.

(4) Bone, D. R. and R. J. Courtney. 1974. A temperature sensitive mutant of herpes simplex virus type 1 defective in the synthesis of the major capsid polypeptide. J. Gen. Virol. **24:** 17.

(5) Cai, W., B. Gu, and S. Person. 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. **62:** 2596-2604.

(6) Cohen, G. H., W. C. Wilcox, D. L. Sadora, D. Long, J. Z. Levin, and R. J. Eisenberg. 1988. Expression of herpes simplex virus type 1 glycoprotein D deletion mutants in mammalian cells. J. Virol. **62:** 1932-1940.

(7) Dix, R. D., L. Pereira, and J. R. Baringer. 1981. Use of monoclonal antibody directed against herpes simplex virus glycoproteins to protect mice against acute virus-induced neurological disease. Infect. Immun. **34:** 192-199.

(8) Eberle, R. and S. W. Mou. 1983. Relative titers of antibodies to individual polypeptide antigens of herpes simplex virus type 1 in human sera. J. Inf. Diseases. **148:** 436-444.

(9) Frankel, M. E. and W. Gerhard. 1979. The rapid determination of binding constants for antiviral antibodies by a radioimmunoassay. An analysis of the interaction between hybridoma proteins and influenza virus. Mol. Immunol. **16:** 101-106.

(10) Friedman, H. M., G. H. Cohen, R. J. Eisenberg, C. A. Seidel, and D. B. Cines. 1984. Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. Nature. **309:** 633-35.

(11) Friedman, H. M., J. C. Glorioso, G. H. Cohen, J. C. Hastings, S. L. Harris, and R. J. Eisenberg. 1986. Binding of complement component C3b to glycoprotein gC of herpes simplex virus type 1: mapping of gC-binding sites and demonstration of conserved C3b binding in low-passage clinical isolates. J. Virol. **60:** 470-475.

(12) Fujinami, R. S. and M. B. A. Oldstone. 1979. Antiviral antibody reacting on the plasma membrane alters measles virus expression inside the cell. Nature. **279:** 529-530.

(13) Fujinami, R. S. and M. B. A. Oldstone. 1980. Alterations in expression of measles virus polypeptides by antibody: molecular events in antibody-induced antigenic modulation. J. Immunol. **125:** 78-85.

(14) Fuller, A. O., R. E. Santos, and P. G. Spear. 1989. Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. J. Virol. **63:** 3435-3443.

(15) Fuller, A. O. and P. G. Spear. 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. PNAS (USA). **84:** 5454-5458.

(16) Gompels, U. and A. Minson. 1986. The properties and sequence of glycoprotein H of herpes simplex virus type 1. Virology. **153:** 230-247.

(17) Grist, N. R., E. J. Bell, E. A. C. Follett, and G. E. D. Urquhart. 1979. Neutralization tests, pp. 84-85. In N. R. Grist and G. E. D. Urquhart (eds.). Diagnostic methods in clinical virology, 3rd ed. Blackwell Scientific Publications, Ltd., Oxford.

(18) Hayashida, I.,S. Nagafuchi, Y. Hayashi, Y. Kino, R. Mori, H. Oda, N. Ohtomo, and A. Tashiro. 1982. Mechanism of antibody-mediated protection against herpes simplex virus infection in athymic nude mice: requirement of Fc portion of antibody. Microbiol. Immunol. **26:** 497-509.

(19) Herlyn, D. and H. Koprowski. 1982. IgG2a monoclonal antibodies inhibit human tumor growth through interaction with effector cells. PNAS (USA). **79:** 4761-4765.

(20) Highlander, S. L., W. Cai, S. Person, M. Levine, and J. C. Glorioso. 1988. Monoclonal antibodies define a domain on herpes simplex virus glycoprotein B involved in virus penetration. J. Virol. **62:** 1881-1888.

(21) Highlander, S. L., D. J. Dorney, P. J. Gage, T. C. Holland, W. Cai, S. Person, M. Levine, and J. C. Glorioso. 1989. Identification of 'mar' mutations in herpes simplex virus type 1 glycoprotein B which alter antigenic structure and function in virus penetration. J. Virol. **63:** 730-738.

(22) Highlander, S. L., S. L. Sutherland, P. J. Gage, D. C. Johnson, M. Levine, and J. C. Glorioso. 1987. Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. J. Virol. **61:** 3356-3364.

(23) Holland, T. C., S. D. Marlin, M. Levine, and J. C. Glorioso. 1983. Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. J. Virol. **45:** 672-682.

(24) Johnson, D. C., M. C. Frame, M. W. Ligas, A. M. Cross, and N. D. Stowe. 1988. Herpes simplex virus immunoglobulin G F_C receptor activity depends on a complex of two viral glycoproteins, gE and gI. J. Virol. **62:** Y1347-1354.

(25) Kapoor, A. K., A. A. Nash, P. Wildy, J. Phelan, C. S. McLean, and H. J. Field. 1982. Pathogenesis of herpes simplex virus in congenitally athymic mice: the relative roles of cell-mediated and humoral immunity. J. Gen. Virol. **60:** 225-233.

(26) Kino, Y., Y. Hayashi, I. Hayashida, R. Mori. 1982. Dissemination of herpes simplex virus in nude mice after intracutaneous inoculation and effect of antibody on the course of infection. J. Gen. Virol. **63:** 475-79.

(27) Klein, R. J. 1980. Effect of immune serum on the establishment of herpes simplex virus infection in trigeminal ganglia of hairless mice. J. Gen. Virol. **49:** 401-405.

(28) Kohl, S., D. L. Cahall, D. H. Walters, and V. E. Schaffner. 1979. Murine antibody-dependent cellular cytotoxicity to herpes simplex virus-infected target cells. J. Immunol. **123:** 25-30.

(29) Kohl, S. and L. S. Loo. 1982. Protection of neonatal mice against herpes simplex virus infection: probable in vivo antibody-dependent cellular cytotoxicity. J. Immunol. **129:** 370-376.

(30) Kuhn, J. E., G. Dunkler, K. Munk, and R. W. Braun. 1987. Analysis of the IgM and IgG antibody response against herpes simplex virus type 1 (HSV-1) structural and nonstructural proteins. J. Med. Virol. **23:** 135-50.

(31) Kumel, G., H. C. Kaerner, M. Levine, C. H. Schroder, and J. C. Glorioso. 1985. Passive immune protection by herpes simplex virus-specific monoclonal antibodies and monoclonal antibody-resistant mutants altered in pathogenicity. J. Virol. **56:** 930-937.

(32) Larsen, H. S., R. G. Russel, and B. T. Rouse. 1983. Recovery from lethal herpes simplex virus type 1 infection is mediated by cytotoxic T lymphocytes. Infect. Immun. **41:** 197-204.

(33) Little, S. P., J. T. Jofre, R. J. Courtney, and P. A. Schaffer. 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. Virology. **115:** 149-160.

(34) Maniatas, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. CSH. pp. 173-177.

(35) Marlin, S. D., T. C. Holland, M. Levine, and J. C. Glorioso. 1985. Epitopes of herpes simplex virus type 1 glycoprotein gC are clustered in two distinct antigenic sites. J. Virol. **53:** 128-136.

(36) Martin, S. and B. T. Rouse. 1987. The mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: clearance of local infection. J. Immunol. **138:** 3431-3437.

(37) McKendall, R. R. 1985. IgG-mediated viral clearance in experimental infection with herpes simplex virus type 1: role for neutralization and F_C-dependent functions but not C' cytolysis and C5 chemotaxis. J. Inf. Diseases. **151:** 464-470.

(38) McKendall, R. R., T. Klassen, and J. R. Baringer. 1979. Host defenses in herpes simplex infections of the nervous system: effect of antibody on disease and viral spread. Infect. Immun. **23:** 305-311.

(39) McKendall, R. R. and W. Woo. 1988. Murine IgG subclass responses to herpes simplex virus type 1 and polypeptides. J. Gen. Virol. **69:** 847-857.

(40) Mester, J. C., S. L. Highlander, A. Osmand, J. C. Glorioso, and B. T. Rouse. 1989. HSV-1-specific immunity induced by peptides corresponding to an antigenic site of glycoprotein B. Submitted.

(41) Metcalf, J. F., S. Chatterjee, J. Koga, and R. J. Whitley. 1988. Protection against herpetic ocular disease by immunotherapy with monoclonal antibodies to herpes simplex virus glycoproteins. Intervirol. **29:** 39-49.

(42) Muggeridge, M. I., V. J. Isola, R. A. Byrn, T. J. Tucker, A. C. Minson, J. C. Glorioso, G. H. Cohen, and R. J. Eisenberg. 1988. Antigenic analysis of a major neutralization site of herpes simplex virus glycoprotein D, using deletion mutants and monoclonal antibody-resistant mutants. J. Virol. **62:** 3274-3280.

(43) Nash, A. A., A. Jayasuriya, J. Phelan, S. P. Cobbold, H. Waldmann, and T. Prospero. 1987. Different roles for L3T4+ and Lyt 2+ T cell subsets in the control of an acute herpes simplex virus infection of the skin and nervous system. J. Gen. Virol. **68:** 825-833.

(44) Oakes, J. E. and R. N. Lausch. 1981. Role of F_C fragments in antibody-mediated recovery from ocular and subcutaneous herpes simplex virus infections. Infect. Immun. **33:** 109-114.

(45) Oakes, J. E. and R. N. Lausch. 1984. Monoclonal antibodies suppress replication of herpes simplex virus type 1 in trigeminal ganglia. J. Virol. **51:** 656-661.

(46) Openshaw, H., L.V. S. Asher, C. Wohlenberg, T. Sekizawa, and A. L. Notkins. 1979. Acute and latent infection of sensory ganglia with herpes simplex virus: immune control and virus reactivation. J. Gen. Virol. **44:** 205-215.

(47) Parham, P. 1986. Preparation and purification of active fragments from mouse monoclonal antibodies, pp. 14.1-14.23. In D. M. Weir (ed.). Handbook of experimental immunology, 4th ed., Vol. 1. Blackwell Scientific Publications, Ltd., Boston.

(48) Price, R. W., M. A. Walz, C. Wohlenberg, and A. L. Notkins. 1975. Latent infection of sensory ganglia with herpes simplex virus: efficacy of immunization. Science. **188:** 938-940.

(49) Rager-Zisman, B. and B. R. Bloom. 1974. Immunological destruction of herpes simplex virus 1 infected cells. Nature. **251:** 542-543.

(50) Rector, J. T., R. N. Lausch, J. E. Oakes. 1982. Use of monoclonal antibodies for analysis of antibody-dependent immunity to ocular herpes simplex virus type 1 infection. Infect. Immun. **38:** 168-174.

(51) Santiago, J. and B. Gomez. 1988. Antiviral response to herpes simplex virus type 1 in latent and active infection. J. Inf. Diseases. **157:** 817-819.

(52) Sarmiento, M., M. Haffey, and P. G. Spear. 1979. Membrane proteins specified by herpes simplex viruses III. Role of glycoprotein VP7(B_2) in virion infectivity. J. Virol. **29:** 1149-1158.

(53) Seto, M., T. Takahashi, S. Nakamura, Y. Matsudaira, and Y. Nishizuka. 1983. In vivo antitumor effects of monoclonal antibodies with different immunoglobulin classes. Cancer Res. **43:** 4768-4773.

(54) Simmons, A. and A. A. Nash. 1984. Zosteriform spread of herpes simplex virus as a model of recrudescence and its use to investigate the role of immune cells in prevention of recurrent disease. J. Virol. **52:** 816-821.

(55) Simmons, A. and A. A. Nash, 1985. Role of antibody in primary and recurrent herpes simplex virus infection. J. Virol. **53:** 944-48.

(56) Simmons, A. and A. A. Nash. 1987. Effect of B cell suppression on primary infection and reinfection of mice with herpes simplex virus. J. Inf. Diseases. **166:** 649-654.

(57) Sullivan, V. and G. L. Smith. 1987. Expression and characterization of herpes simplex virus type 1 (HSV-1) glycoprotein G (gG) by recombinant vaccinia virus: neutralization of HSV-1 infectivity with anti-gG antibody. J. Gen. Virol. **68:** 2587-98

(58) Tullo, A. B., C. Shimeld, W. A. Blyth, T. J. Hill, and D. L. Easty. 1982. Spread of virus and distribution of latent infection following ocular herpes simplex in the non-immune and immune mouse. J. Gen. Virol. **63:** 95-101.

(59) Zweerink, H. J. and L. Corey. 1982. Virus-specific antibodies in sera from patients with genital herpes simplex virus infection. Infect. Immun. **37:** 413-421.

(60) Zweerink, H. J. and L. W. Stanton. 1981. Immune response to herpes simplex virus infections: virus specific antibodies in sera from patients with recurrent facial infection. Infect. Immun. **31:** 624-630.

PART III :

HSV-1-SPECIFIC IMMUNITY INDUCED BY PEPTIDES CORRESPONDING TO AN ANTIGENIC SITE OF GLYCOPROTEIN B

CHAPTER 1

ABSTRACT

We have previously shown that a HSV-1-neutralizing anti-glycoprotein B-reactive monoclonal antibody (MAb) can protect mice against zosteriform spread. Three overlapping peptides, corresponding to the linear epitope recognized by this MAb, have been synthesized and used as immunogens in their free or carrier-bound form. Although not eliciting a strong T cell response with regard to HSV-1, one peptide-carrier conjugate elicited antibody able to neutralize HSV-1 *in vitro*. This antiserum was protective when passively transferred to naive mice before lethal HSV-1 zosteriform inoculation. Immunization with all of the peptides was able to enhance viral clearance of a low dose of HSV-1 in an ear challenge model.

CHAPTER 2

INTRODUCTION

Herpes simplex viruses are enveloped DNA viruses which infect a large portion of the human population. Investigation of the immune response to herpes simplex virus has indicated the importance of the viral envelope glycoproteins, especially gB, gC, and gD, in the generation of cellular and humoral immunity (2, 7, 14, 20, 21, 31, 33, 47, 55, 57). Glycoproteins B and D, essential for virus entry into host cells (5, 18), have been shown to be protective immunogens in animals when introduced in recombinant virus vectors, as purified proteins, or in transfected L cells (2, 6, 9, 12, 16, 29, 37, 43, 46, 50, 51). Additionally, a subunit of gD1, a 23 amino acid peptide corresponding to the first 23 residues of the amino-terminus of the glycoprotein (gD1-23mer) has been shown to be protective when administered in adjuvant preparations (16, 56).

Considering most antibody epitopes to be discontinuous or sensitive to perturbations of tertiary molecular structure, it would appear that the most appropriate method of generating anti-viral antibody with subunit immunogens would be through the use of anti-idiotypic reagents. However, a substantial number of reports have demonstrated that a significant number of linear antibody epitopes exist in the target proteins of human viral pathogens, and that these epitopes, when synthesized as peptides, are able to generate anti-viral antibody. These reports, in addition to those on the the HSV gD1-23mer, include the generation of neutralizing or hemagglutinating antibody to rhinovirus (17, 36), influenza virus (41), poliovirus (10), and human immunodeficiency virus (28), and the generation of viral cross-reactive antibody to hepatitis B virus (27, 40).

In earlier studies, we identified a gB1-specific monoclonal antibody (MAb B6) which was protective against a clinical isolate of HSV-1 (HSV-1_{BK}) in the murine zosteriform spread model (39). The epitope recognized by MAb B6 has been shown to be linear, and the mutation corresponding to the mar (monoclonal antibody resistant) mutant virus, which is resistant to neutralization by MAb B6, has been mapped to amino acid 84 of the mature glycoprotein (26). We have synthesized three overlapping peptides, corresponding to the wild type gB1 sequence, spanning this area, and analyzed their ability to stimulate immunity which cross-reacts with HSV-1. Our results show that all three peptides are effective, to certain extents, in stimulating immunity to HSV-1. Additionally, one peptide is able to generate HSV-1 neutralizing antibody which is protective in the zosteriform spread model.

CHAPTER 3

MATERIALS AND METHODS

A. VIRUSES

HSV-1 KOS 321, B6.1, a monoclonal antibody neutralization resistant (mar) mutant virus derived from KOS 321 (34), and a clinical isolate of HSV-1 : strain BK, were propagated in HEp-2 cells, maintained on McCoy's 5a medium with 10% donor calf serum (DCS) (Gibco, Grand Island, NY), as previously described (3). Infectious particles were recovered after 3 freeze/thaw cycles, aliquoted, and stored at -80°C. Titration on Vero cell monolayers (23) revealed titers of 1.8 X 10^9 $TCID_{50}$'s / ml for KOS 321 and B6.1, and 5.6 X 10^8 $TCID_{50}$'s / ml for $HSV\text{-}1_{BK}$.

B. PEPTIDES

Peptides were synthesized via solid phase t-Boc techniques on a SAM TWO Biosearch synthesizer using the standard Merrifield resin. After cleavage with HF (HF / anisole / peptide resin : 10 ml / 1 ml / 1 g), the peptides were extracted with 50% acetic acid, and the lower molecular weight contaminants removed by passage over a P-2 column. HPLC and amino acid analysis (Waters Picotag system) verified the purity (>90%) and composition of the peptides.

Three peptides corresponding to the mutation site of mar B6.1 {a single nucleotide substitution in the glycoprotein B gene corresponding to amino acid 84 of the mature gB

molecule, (26)} were synthesized as an overlapping series spanning amino acids 63 to 110 of the gB molecule (Figure 1). Three unrelated peptides were used as controls : gB4 (amino acid residues 520-543 of gB-1 : LGRVAIAWCELQNHELTLWNEARK), gC20mer (amino acid residues 98-117 of gC-1 : PTSTPDPKPKNNTTPAKSGR), and CSF 15mer (amino acid residues 118-132 of human granulocyte/macrophage colony stimulating factor : ITFESFKENLKDFLY).

C. CONJUGATION TO POLY-(L)-LYSINE (pL) AND BOVINE SERUM ALBUMIN (BSA)

EDC (1-Ethyl-3-(3-dimethyl-amino-propyl-carbodiimide•HCl, Pierce, Rockford IL) was used to couple the peptides to pL (MW 15,000-18,000, Sigma, St. Louis MO), and glutaraldehyde (Grade II, 25% aqueous, Sigma) to couple them to BSA (Calbiochem, La Jolla CA). Before conjugation to pL, the peptides were citraconylated to protect their amino terminal residues and avoid polymerization of the peptide molecules themselves {as recommended by Atassi & Habeeb (1)} when mixed with EDC. 30 mg's of each peptide was added to double-distilled (dd) H_2O and the pH and volume adjusted to 8.2 and 7.5 ml, respectively. With magnetic stirring at room temperature, 15 µl of citraconic anhydride (Sigma) was added every 30 minutes for 4 hours (120 µl in all), while maintaining the pH with µl additions of 5 or 2 N NaOH. After the four hour addition period, the mixture was stirred for an additional 2 hours. The citraconylated peptides were then transferred via glass pipettes to 1000MW cutoff dialysis tubing and placed in 4°C ddH_2O, adjusted to pH=8.6 with NH_4OH. After 24 hours, the peptides were lyophilized.

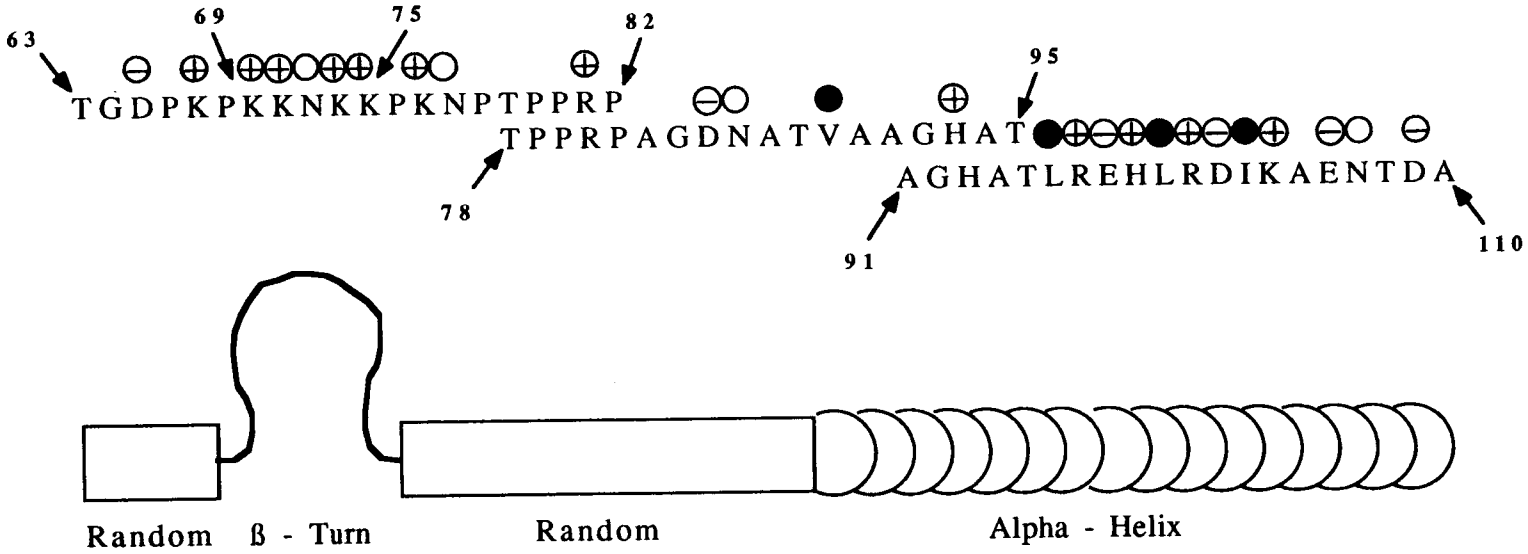


Figure 1 : Hydrophobicities & Secondary Structure : B6 Epitope Peptides.

The three overlapping peptides 20.1mer (amino acid residues 63-82), 18mer(78-95), and 20.2mer (91-110) used in this study (residues are numbered from the amino terminal methionine of the sequence of Bzik et al., 4). Hydrophobic and hydrophilic residues were determined by standardized weighted averaging of the standardized scales of Sweet and Eisenberg (53), Kyte and Doolittle (32), and Nozeki and Tanford (42). Residues with a numerical rank above 0.5 (on a scale ranging from -1.7 to +1.7) were designated hydrophobic (•) and those with a rank below -0.5 were designated hydrophilic (o). The plus or minus sign within the hydrophilic marker denote side chain charge (if any). The secondary structure of the gB-1 molecule in this region, modelled after Garnier et al.'s prediction (19), is shown below the peptide sequences. The 20.1mer contains a potential ß-turn (residues 69-75) flanked by random configurations, and the 20.2mer may be mainly alpha-helical. The region corresponding to the 18mer is mainly of random predicted configuration, with a possible short helical carboxyl tail.

For conjugation to pL, 5.5 mg of pL, approximately 30 mg of the citraconylated-peptides and 3.25 mg of sulfo-SMCC (Sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane-1-carboxylate, Pierce) were dissolved in 2.0ml of ddH_2O, and the pH of the mixture adjusted to 8, if necessary, by addition of a small amount of 2N NaOH. 19mg's of EDC, freshly dissolved in 0.4 ml of ddH_2O, was then added, and the mixture magnetically stirred for one hour at room temperature. The mixtures were then transferred to 6-8,000MW cutoff dialysis tubing and individually placed in 4°C ddH_2O, adjusted to pH=8.6 with NH_4OH. After 24 hours, the dialysis tubes containing the pL-conjugated peptides were transferred to 0.05M acetate buffer (pH=4.0) and gently stirred at 40°C for three hours. The individual peptide-conjugates were then lyophilized. Final weight yields for the conjugation procedure were 90% or better, and amino acid analysis of the conjugates indicated that for two peptides (gB 18mer & 20.2mer) every third lysine of pL was bound to one peptide molecule, and for gB 20.1mer every sixth lysine was peptide-bound.

For coupling to BSA, approximately 9 mg of each peptide was dissovled in 10 ml of a 1 mg/ml solution of BSA in PBS (for a final 30 : 1 molar ratio of peptide : BSA). With stirring at 4°C and pH=7.4, five 20 µl glutaraldehyde additions were performed over a 1 hour reaction time (final glutaraldehyde amount : 1% v/v). The coupling was terminated by the addition of sodium borohydride (Sigma) to a final concentration of 10 mg/ml, and stirring the mixture for an additional hour. The mixture was then transferred to 6-8,000MW cutoff dialysis tubing and dialyzed overnight against PBS.

D. MICE / INOCULATIONS

Female C3H/HEN (H-2K), Balb/c (H-2D), and C57BL (H-2B) mice were obtained from Harlan Sprague Dawley (Indianapolis IN). Primary immunization with 50 μg of either the free or pL-coupled forms of the peptide emulsified 1:1 in complete Freund's Adjuvant (FCA, Gibco) was performed via the footpad route when the mice were seven weeks old. Repeated 50 μg boosters were administered in incomplete Freund's Adjuvant (IFA) via the footpad at 3 to 4 week intervals.

E. MONOCLONAL ANTIBODY (MAb) / ELISAs

The production and characterization of MAb B6, which was used to isolate mar mutant B6.1, has been previously described (34). ELISAs were performed as described (35), using purified HSV-1 as antigen or 5 - 10 μg / well of the peptides or peptide conjugates dissolved in carbonate buffer (pH=9.6).

F. NEUTRALIZATION ASSAY

The ability of peptide-generated antisera to neutralize HSV-1 KOS was examined by preincubating 20 $TCID_{50}$'s of the virus with series of two-fold dilutions of each antiserum, both with and without complement (Low-Tox-M rabbit complement, Cedarlane Laboratories LTD, Hornby, Ontario). Each sample was run in triplicate in 96-well microtiter plates (Corning, Corning NY), and when complement was added its final

concentration was 1:10. After 3 hour preincubation, the mixture was transferred to a confluent monolayer of Vero cells {maintained in McCoy's 5A medium with 10% donor calf serum (DCS) (Gibco, Grand Island NY)}, and after a one hour adsorption period, three drops of Modified Eagle Medium (Gibco) with 2% DCS and 1.25% LMP agarose (BRL, Gaithersburg MD) (warmed to 37°C) were added to each well. After three days, the number of plaques was counted. The resulting neutralization titers are expressed as reciprocal 50% endpoints.

G. ANTIBODY-DEPENDENT COMPLEMENT-MEDIATED LYSIS (ADCMC) ASSAY

Vero cell targets were infected with HSV-1 KOS at a multiplicity of 5 and labeled by incubating 2 X 10^6 cells per ml of McCoy's 5A medium (5% DCS) with 150 µCi of ^{51}Cr (Amersham) per ml for five hours at 37°C. Two-fold dilution of antiserum (in triplicate) were prepared in 96-well round bottomed plates (Corning) and 4 X 10^4 targets added per well, for a final volume of 180 µl. After a two hour incubation at 4°C on a rotating platform, 20 µl of neat complement (Low-Tox-M, Cedarlane) was added per well, and the plates incubated at 37°C for one hour. The cells were then pelleted, 100 µl of the media supernant of each well collected, and ^{51}Cr release counted by a LKB-Wallac 1274 RiaGamma Counter (Wallac Oy, Turku Finland). Percent ^{51}Cr release was determined with the following formula : ^{51}Cr release = (experimental release - spontaneous release) / (total release - spontaneous release) X 100. The standard errors of the ^{51}Cr release sample means were 10% or less.

H. LYMPH NODE PROLIFERATION ASSAY

Viable cells were harvested from the popliteal lymph node 8-9 days after a 50 μg peptide footpad booster in IFA or 5 days after a live HSV booster (2 X 10^6 $TCID_{50}$s). Cell number was adjusted to 5 X 10^6 per ml in RPMI 1640-based proliferation medium {RPMI-P, containing 10% fetal bovine serum (Gibco) and supplemented with HEPES buffer (Research Organics, Inc., Cleveland Ohio), sodium bicarbonate, oxaloacetic acid, insulin, sodium pyruvate (Sigma), L-glutamine, PSN antibiotic mixture, essential and nonessential amino acids, and MEM vitamin solution (Gibco)}, and 100 μl added to each well of a round-bottomed 96-well microtiter plate (Corning). A ten-fold dilution series of each peptide (in RPMI-P) was added to the plates in quadruplicate, the final amount ranging from 0.04 to 40 μgs per well. The plates were incubated for 96 hours at 37°C, pulsed with 2 μCi [^{3}H]-thymidine (ICN Biomedicals, Irvine CA) per well for the final 6 hours, and the cells harvested on glass fiber filter strips with a PHD cell harvester (Cambridge Technology Inc., Watertown MA). Individual disks were immersed in Ecolume scintillation cocktail (ICN), and harvested counts per minute (cpm) determined on a Beckman model LS 7000 scintillation counter (Beckman Instruments, Inc., Palo Alto CA). Tartrate gradient-purified HSV-1 (KOS 321) was UV-inactivated and added to certain wells at a multiplicity of one. The proliferation of normal or adjuvant alone elicited lymph node cells to any of the peptide concentrations or the HSV preparation was similar to the proliferation of these cells in medium alone (no inherent mitogenic activity was found for any of the antigen preparations).

I. PROTECTION STUDIES

The ability of the peptides to induce a HSV-1 protective response *in vivo* was assessed by ear clearance experiments (35), using HSV-1 KOS as the challenge virus, and zosteriform spread analysis (48), using HSV-1_{BK}. The IgG fraction of select sera was collected using HiPAC protein G LTQ columns (ChromatoChem, Inc., Missoula, Montana) as recommended by the manufacturer.

CHAPTER 4

RESULTS

A. PEPTIDE REACTIVITY OF MAB B6 AND ANTI-HSV POLYCLONAL SERA

Recognition of the three B6 epitope peptides (which are depicted in Figure 1) by MAb B6 and polyclonal HSV-immune sera from C3H, Balb/c, and C57/Bl mice was determined by ELISA, and the results shown in Table 1. The recognition shown was consistent using either the BSA or pL-conjugated form of the peptide, or the free peptide itself, demonstrating that the 18mer and 20.1mer peptides were able to mimic viral epitopes when presented as solid phase antigens, and that conjugation to the BSA and pL carriers did not alter this. MAb B6 reacted strongly with the 18mer and 20.1mer, and the reactivity of polyclonal anti-HSV serum from the three strains of mice was strongest with the 20.1mer. Of the three polyclonal immune sera tested, the C3H serum reacted most strongly to the 20.1mer.

B. PEPTIDE REACTIVITY OF HSV-IMMUNE LYMPH NODE CELLS

The B6 epitope peptides were added to cultures of HSV-immune lymph node cells to assess their ability to stimulate cellular proliferation. The results, shown in Table 2, demonstrate that in both the C3H and Balb/c mouse a significant amount of recognition is evident for both the 18mer and 20.1mer. The 20.2mer, however, does not appear to be

Table 1 : Peptide Reactivity of HSV - Immune Sera & MAb B6

Serum	Peptide 18mer	20.1mer	20.2mer	gB4
MAb B6	++++	++++	+/-	ND
C3H anti-HSV	+	+++	+/-	-
Balb/c anti-HSV	+/-	+	-	-
C57/Bl anti-HSV	-	+	-	-

The peptide reactivity of MAb B6 and HSV-immune sera from C3H, Balb/c, and C57/Bl mice was determined via ELISA. The pluses denote ABS 490 readings above background (negative control antigens : pL, BSA, and unrelated peptides) as follows : ++++ : ≥ 1.0, +++ : 0.8-0.99, ++ : 0.5-0.79, + : 0.3-0.49, +/- : 0.1-0.29, - : < 0.1. MAb B6 was added at a concentration of 5µg/ml, and the polyclonal sera at a 1 : 200 dilution. Peptide gB4 is a 24mer corresponding to amino acids 520-543 of gB1.

ND : not done.

Table 2 : Proliferation of HSV-Immune Cells to B6 Epitope Peptides

Antigen	(Amt.)	Mouse Strain C3H	Balb/c	C57/Bl
HSV		12,873±2,218	16,430±2,403	8,809±1,335
18mer	4 µg	4,411±402	*	*
	0.4 µg	6,407±919	4,568±566	6,155±1,875
	0.04 µg	ND	3,675±681	ND
20.1mer	4 µg	3,618±514	*	*
	0.4 µg	5,359±630	4,814±277	6,107±551
	0.04 µg	ND	3,773±530	ND
20.2mer	4 µg	2,624±461	*	*
	0.4 µg	2,624±461	2,787±974	6,254±730
	0.04 µg	ND	2,649±806	ND
Media		2,584±587	2,422±334	4,849±770

Proliferation of acute HSV immune lymph node cells is shown as mean counts per minute (cpm), ± the standard error, resulting from [^{3}H]-thymidine uptake. A series of ten-fold dilutions of each peptide in RPMI-P was added to the cells at the initiation of culture, and the final amount of each peptide addition is shown. Peptide amounts above 4 µg / well were inhibitory and are not shown.

* : sample mean cpm < media control cpm.

ND : not done.

recognized. The proliferation of HSV-immune C57/Bl lymph node cells is difficult to interprete due to the high background (media alone) proliferation, but if positive, the recognition of all three peptides is weak.

C. VIRAL REACTIVITY OF PEPTIDE-IMMUNE SERA : ELISA AND ADCMC

The three B6 peptides, in both the free and pL-conjugated forms, were repeatedly injected into mice to generate high titer antiserum. All of the peptides, in either form, were able to generate antibody reactive with HSV in ELISA assays; however, most of this reactivity was nonspecific in that a substantial background recognition of unrelated peptides (gB4-pL and CSF 15mer) usually occurred for sera generated against the 18mer and 20.1mer (Appendix D, Figures 1-6). The strongest HSV-specific ELISA reactivity was found in the sera raised against the 20.2mer (Figure 2). ADCMC assays were performed to further evaluate the HSV-reactivity of peptide-immune sera. Figure 3 shows that all of the peptide antiserum generated in C3H mice could mediate HSV-specific ADCMC to an extent significantly above that of normal serum. For the Balb/c antiserum, only that generated by the conjugated form of the three peptides could mediate ADCMC of HSV-infected cells to a significant level (above the lysis seen with complement alone). The C57/Bl antisera also mediated ADCMC of HSV-infected cells, although at a level generally lower than that of the C3H and Balb/c sera.

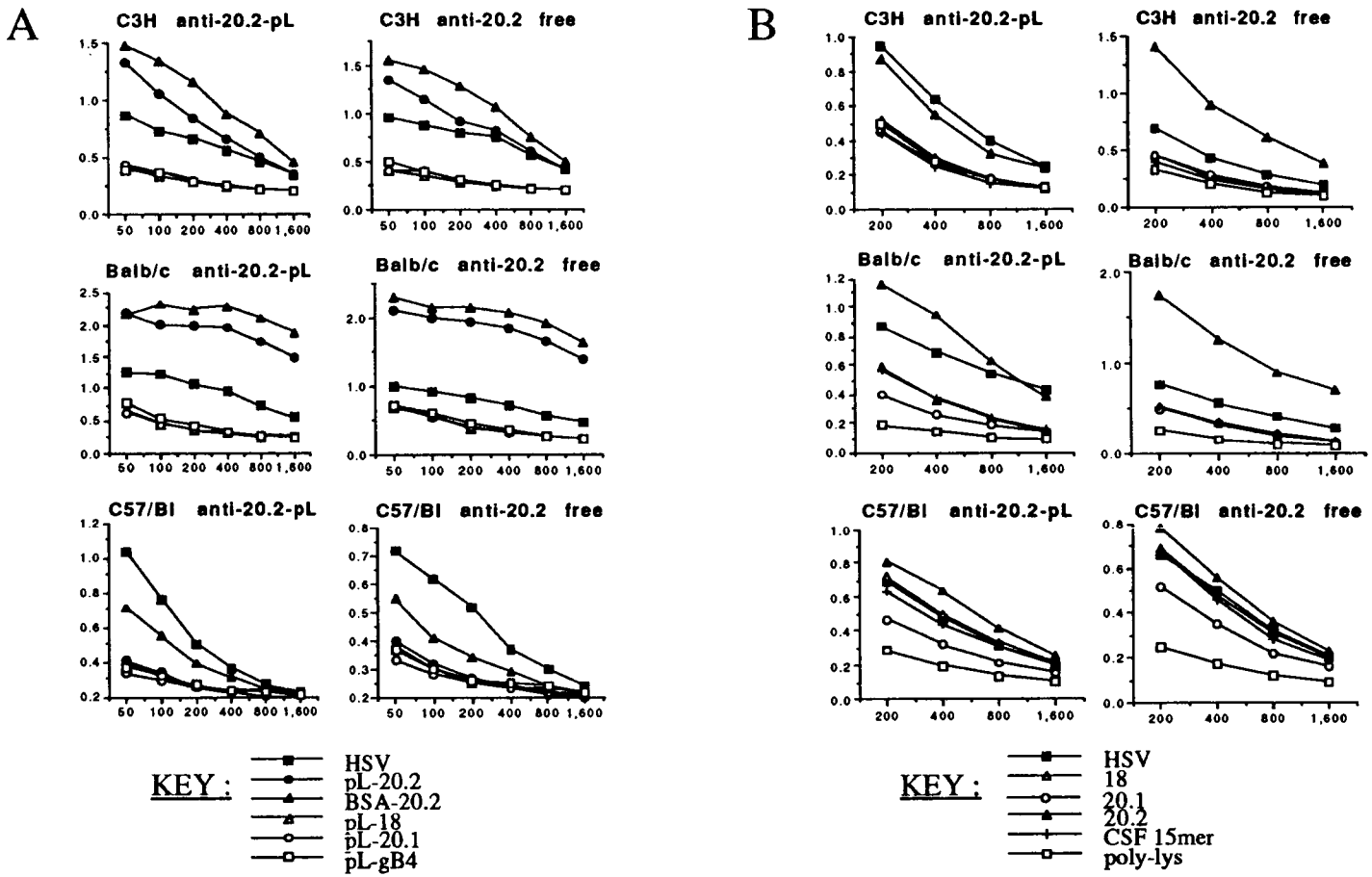


Figure 2 : ELISA Reactivity of 20.2mer--Generated Antiserum.

Serial two-fold dilutions of the anti-20.2mer sera were screened, via ELISA, against conjugated and free forms of the peptides (A.) results using conjugated ELISA antigens, (B.) results using free ELISA antigens. The ordinate represents serum dilution factors, and the abscissa ABS 490. The subtitle of each graph denotes the specific antiserum used. Standard errors of the sample means were less than 10%.

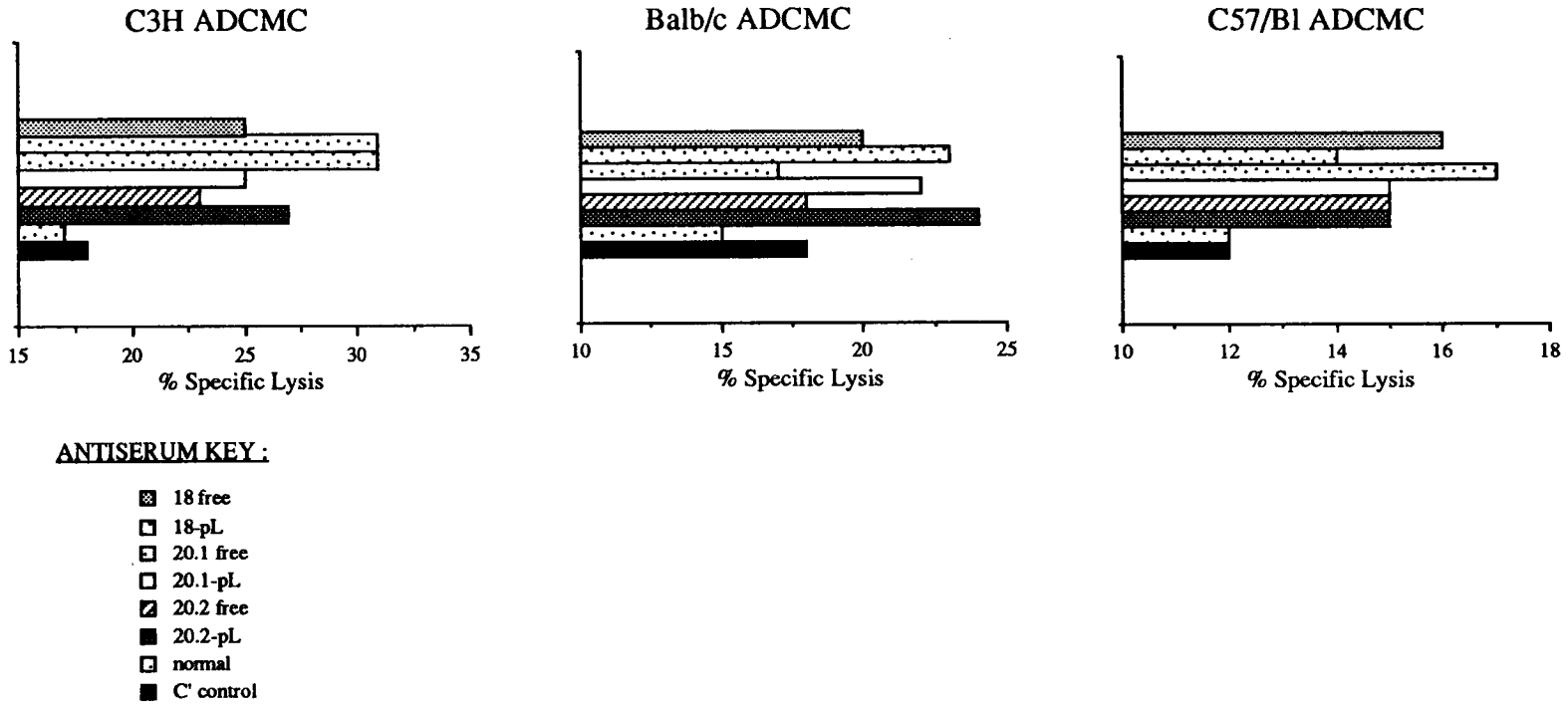


Figure 3 : Peptide Sera Anti--HSV ADCMC.

Antibody-dependent complement-mediated lysis (ADCMC) of HSV-infected Vero cells. The results shown correspond to final serum dilutions of 1:40. No mouse serum was added to the complement (C') control wells. Positive controls, polyclonal anti-HSV C3H serum and 5 μg / well of MAb B6, gave % specific release values of 73 and 58, respectively. The standard errors of the sample means were 10% or less.

D. NEUTRALIZATION AND ADCC MEDIATED BY THE B6 PEPTIDE ANTISERA

The anti-peptide sera were screened for HSV-1 (KOS) neutralizing activity, and only the 20.2mer elicited positive titers in the C3H and Balb/c mice (Table 3). The coupled form of the 20.2mer generated the highest neutralization titers, being four and ten-fold higher than that generated by the free peptide in C3H and Balb/c mice, respectively. The anti-20.2mer Balb/c sera was also tested for its ability to neutralize mar mutant B6.1, and a ten-fold reduction in activity, relative to wild-type KOS, was seen.

All anti-peptide sera generated in the C3H strain were examined for HSV-specific ADCC activity, and none was found for any antiserum. Several anti-peptide sera generated in the Balb/c and C57/Bl strains were also examined for their ability to mediate ADCC, and only the Balb/c anti-18mer (free) demonstrated a specific release significantly above that of normal serum.

E. VIRAL REACTIVITY OF PEPTIDE-IMMUNE LYMPH NODE CELLS

Acute lymph node cells from peptide-immune mice were cultured with the peptides or HSV to assess their proliferative response. As shown in Tables 4, 5, and 6, a substantial amount of background proliferation occurred for almost every lymph node population -- so much so, that proliferation to the peptide which elicited the immune cells, and any possible HSV-specific or overlapping-peptide cross-reactive response, was obscured. The highest peptide and HSV-specific response was found for the 18mer-pL and 20.2mer free elicited lymph node cells of Balb/c mice (Tables 4 and 6).

Table 3 : Neutralization Titer of Anti-20.2mer Sera

Serum		Reciprocal 50% Endpoint KOS.321	B6.1
C3H	anti-20.2 free	10	ND
	anti-20.2 - pL	40	ND
Balb/c	anti-20.2 free	20	-
	anti-20.2 - pL	200	20
C57/Bl	anti-20.2 free	-	ND
	anti-20.2 - pL	-	ND

Neutralization titers of the anti-peptide sera was determined by incubating serial dilutions of the sera with HSV-1 KOS or mar mutant B6.1 in the presence of complement, and determining plaque reduction on Vero cell monolayers, as described in **Materials and Methods G. Neutralization Assay**. All anti-peptide sera were assayed, but only the results from the 20.2mer-generated sera are shown. Reciprocal 50% endpoint refers to the serum dilution factor which caused a 50% reduction in the number of viral plaques. The data shown is representative of several experiments.

ND : not done.

Table 4 : Proliferation of 18mer-Generated Immune Lymph Node Cells

		C3H		Balb/c		C57/Bl	
Antigen	(Amt.)	Free	pL-Conj.	Free	pL-Conj.	Free	pL-Conj.
HSV		14,653±398	6,758±193	14,792±814	15,545±4,238	3,752±526	11,182±1,648
18mer	4 µg	14,097±1,629	*	8,824±1,333	10,702±1,152	2,996±86	*
	0.4 µg	15,829±4,098	6,835±129	9,033±2,123	15,182±2,799	3,383±550	11,621±1,082
	0.04 µg	16,693±1,183	7,229±1,423	12,349±1,303	17,583±1,120	3,599±1,160	11,921±673
20.1mer	4 µg	16,228±1,240	*	*	12,906±1.554	*	*
	0.4 µg	16,015±1,647	*	8,389±552	12,892±1,219	3,345±263	8,700±133
	0.04 µg	13,918±2,320	*	*	14,303±2,302	4,321±496	9,372±2,218
20.2mer	4 µg	15,003±1,075	*	*	12,114±3,303	*	*
	0.4 µg	15,661±640	*	*	11,470±348	*	*
	0.04 µg	13,918±2,320	*	9,063±1,118	13,121±2,766	*	*
gC20mer	0.4 µg	*	*	8,765±786	8,516±2,454	3,943±778	9,221±1,483
	0.04 µg	*	*	*	*	*	*
Media		13,726±1,102	6,738±646	7,462±1,448	7,769±3,060	2,440±739	8,564±147

Proliferation of acute anti-18mer lymph node cells is shown as mean counts per minute (cpm), ± the standard error, resulting from [^{3}H]-thymidine uptake. Free refers to immune cells generated by the free form of the 18mer, and pL-Conj. refers to those generated by the poly-(L)-lysine-conjugated form in the C3H, Balb/c, and C57/Bl strains of mice. A series of ten-fold dilutions of each peptide in RPMI-P was added to the cells at the initiation of culture, and the final amount of each peptide addition is shown. B6-peptide amounts above 4 µg / well were inhibitory and are not shown. Negative control peptide gC20mer was toxic to the cells above 0.4 µg / well.

* : sample mean cpm < media control cpm.

ND : not done.

Table 5 : Proliferation of 20.1mer-Generated Immune Lymph Node Cells

		C3H		Balb/c		C57/Bl	
Antigen	(Amt.)	Free	pL-Conj.	Free	pL-Conj.	Free	pL-Conj.
HSV		*	*	25,461±1,731	24,096±2,929	8,887±1,485	8,643±744
18mer	4 µg	22,045±1,101	*	20,079±2,334	ND	5,447±1,207	6,100±1,070
	0.4 µg	23,421±2,412	*	20,072±4,544	ND	5,301±1,014	6,066±191
	0.04 µg	*	17,552±615	19,407±562	ND	5,920±537	6,857±317
20.1mer	4 µg	24,342±5,136	*	21,788±1,844	22,732±2,156	7,127±914	5,507±830
	0.4 µg	20,520±1,282	17,112±1,620	20,260±2,254	22,689±1,060	7,450±1,842	7,397±1,774
	0.04 µg	22,881±3,893	*	20,222±2,245	22,234±3,602	7,029±1,469	6,211±1,535
20.2mer	4 µg	19,327±2,267	*	20,151±3,879	ND	5,819±1,574	6,528±1,418
	0.4 µg	*	*	18,999±2,871	ND	6,963±1,954	6,269±1,437
	0.04 µg	*	*	20,520±4,420	ND	7,086±1,463	4,920±1,520
gC20mer	0.4 µg	22,792±368	16,157±3,257	24,379±2,202	ND	7,360±1,018	7,582±1,071
	0.04 µg	*	*	*	ND	*	*
Media		17,857±1,326	16,707±2,424	17,998±2,363	19,044±4,165	3,965±709	4,564±368

Proliferation of acute anti-20.1mer lymph node cells is shown as mean counts per minute (cpm), ± the standard error, resulting from [^{3}H]-thymidine uptake. Free refers to immune cells generated by the free form of the 20.1mer, and pL-Conj. refers to those generated by the poly-(L)-lysine-conjugated form in the C3H, Balb/c, and C57/Bl strains of mice. A series of ten-fold dilutions of each peptide in RPMI-P was added to the cells at the initiation of culture, and the final amount of each peptide addition is shown. B6-peptide amounts above 4 µg / well were inhibitory and are not shown. Negative control peptide gC20mer was toxic to the cells above 0.4 µg / well.

* : sample mean cpm < media control cpm.

ND : not done.

Table 6 : Proliferation of 20.2mer-Generated Immune Lymph Node Cells

		C3H		Balb/c		C57/Bl	
Antigen	(Amt.)	Free	pL-Conj.	Free	pL-Conj.	Free	pL-Conj.
HSV		*	12,815±1,727	15,999±963	*	11,673±2,958	11,724±1,221
18mer	4 μg	*	*	*	ND	*	*
	0.4 μg	23,531±909	14,227±1,064	*	ND	*	6,644±598
	0.04 μg	21,335±1,336	13,136±874	*	ND	*	7,915±1,117
20.1mer	4 μg	*	*	*	ND	*	*
	0.4 μg	*	*	*	ND	*	6,778±1,414
	0.04 μg	22,335±1,336	13,469±1,572	*	ND	*	*
20.2mer	4 μg	*	*	14,207±509	14,075±2,749	*	*
	0.4 μg	*	14,377±1,397	*	*	*	7,410±1,707
	0.04 μg	*	13,804±1,108	*	*	7,763±1,850	*
gC20mer	0.4 μg	*	12,813±625	*	ND	*	6,961±1,057
	0.04 μg	*	*	*	ND	*	*
Media		20,877±2,834	12,109±794	10,233±621	13,679±1,417	6,322±1,796	6,021±1,335

Proliferation of acute anti-20.2mer lymph node cells is shown as mean counts per minute (cpm), ± the standard error, resulting from [^{3}H]-thymidine uptake. Free refers to immune cells generated by the free form of the 20.2mer, and pL-Conj. refers to those generated by the poly-(L)-lysine-conjugated form in the C3H, Balb/c, and C57/Bl strains of mice. A series of ten-fold dilutions of each peptide in RPMI-P was added to the cells at the initiation of culture, and the final amount of each peptide addition is shown. B6-peptide amounts above 4 μg / well were inhibitory and are not shown. Negative control peptide gC20mer was toxic to the cells above 0.4 μg / well.

* : sample mean cpm < media control cpm.

ND : not done.

F. THE B6 PEPTIDES AS PROTECTIVE IMMUNOGENS

In vivo challenge experiments were performed to assess the ability of the peptides to act as protective immunogens. Ear clearance experiments, using a low challenge dose (2.5×10^4 $TCID_{50}$s) of KOS 321, demonstrated that all of the peptides, in either the free or pL-conjugated form, could confer some degree of HSV-specific immunity (Figure 4 and Table 7). Statistical comparison (using the Student t-test) of the ear titers of the peptide-immunized groups to that of the normal or adjuvant controls showed that the conjugated forms of the peptides were better able to prime for a live HSV challenge (Table 7). Considering the distribution of the ear titers for all of the immunization groups (Figure 4), it is apparent that mice primed with the B6 peptides did not eliminate the virus as thoroughly as the HSV-immune mice, but they effectively controlled the extent of local viral replication. Most of the peptide-immune mice had ear virus titers below 10^3 $TCID_{50}$s, the exceptions being one of six mice in the 20.1mer free and 20.2mer free immunization groups. Dissimilarly, approximately forty percent of the mice in the normal and adjuvant control groups had ear titers above 10^3 $TCID_{50}$s.

A more rigorous test of peptide-induced immunity was performed by zosteriform spread analysis using the virulent BK strain of HSV-1. Since zosteriform spread occurs only when mice are challenged before eight weeks of age, the protocal for direct immunization with the peptides was limited to a period of approximately three weeks, during which a primary immunization and one booster were administered. Using this method, no protection against zosteriform spread and death was seen for any of the peptide-immunized mice. When polyclonal peptide-immune sera, generated over three months by repeated immunization, was passively transferred before zosteriform challenge, however, protection was seen in a portion of the mice (Table 8).

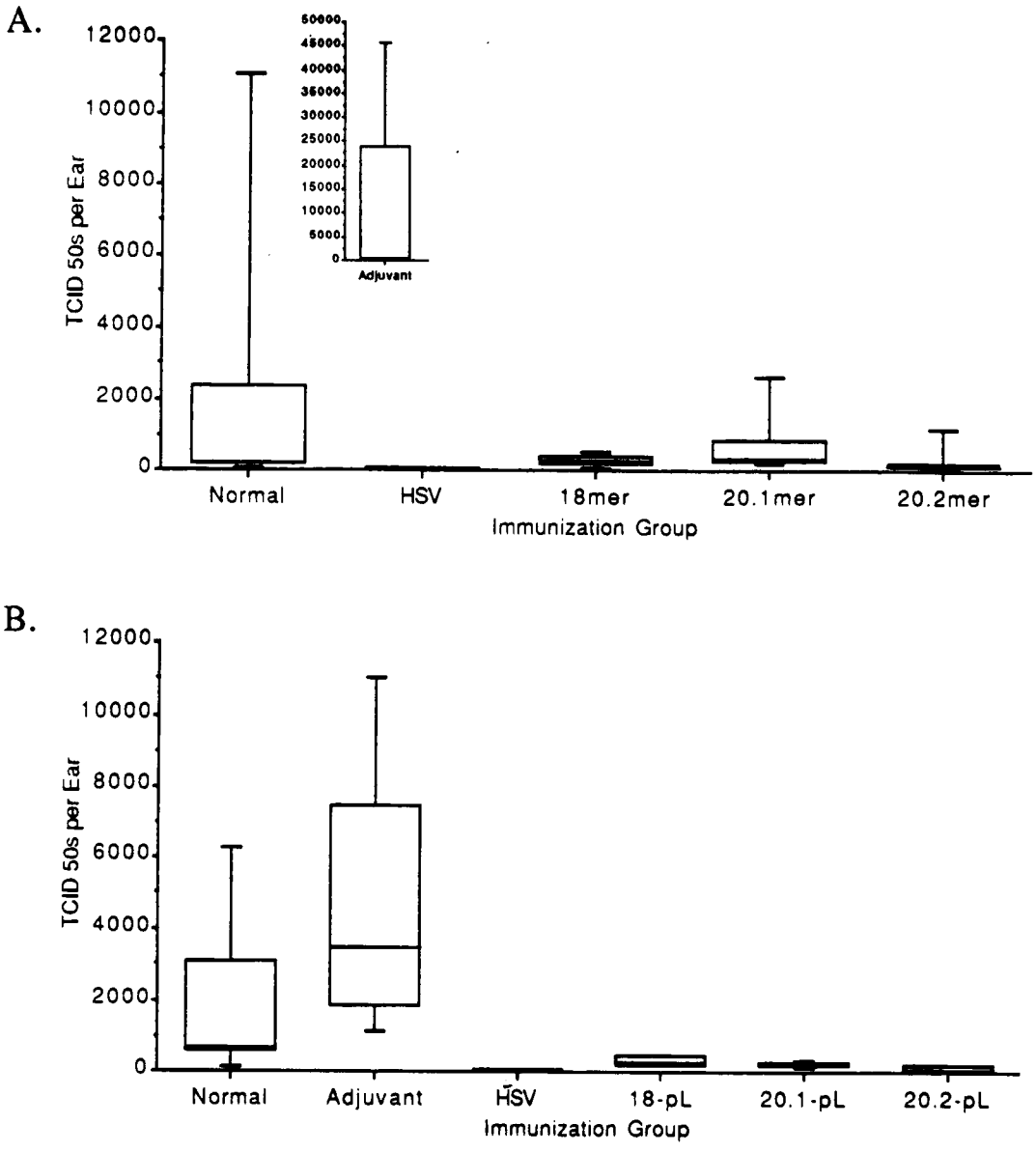


Figure 4 : Ear Clearance of HSV-1 by Peptide-Immune Mice.

Ear clearance results depicted as box and whisker plots of the amount of virus found in the ear flaps three days after challenge in each immunization group : (A.) immunization with free peptides, (B.) immunization with pL-conjugates. The boxes represent the titers found in the second and third quartiles of each group (the line dividing these quartiles represents the group mode), and the whiskers the first and fourth quartiles. Each immunization group contained from four to eight mice.

Table 7 : HSV-1 Ear Clearance by Peptide-Immune Mice

	Immunogen	Mean Titer	P-Values
Exp. 1 :	Normal	2.4×10^3	
	Adjuvant	1.2×10^4	
	HSV	2.2×10^1	
	18mer free	2.8×10^2	> 0.1 / < 0.1
	20.1mer free	7.7×10^2	> 0.1 / < 0.1
	20.2mer free	3.2×10^2	> 0.1 / < 0.1
Exp. 2 :	Normal	1.9×10^3	
	Adjuvant	4.8×10^3	
	HSV	6.7×10^0	
	18mer-pL	3.0×10^2	> 0.1 / < 0.05
	20.1mer-pL	1.9×10^2	< 0.1 / < 0.03
	20.2mer-pL	1.0×10^2	< 0.1 / < 0.03

Ear clearance of HSV-1 KOS was assessed by injecting 2.5×10^4 $TCID_{50}$s of virus into the right ear flap of adjuvant, HSV, and peptide-immunized mice, or nonimmunized normal controls, and titering the remaining infectious virus after 72 hours. Experiment 1 utilized the free form of the peptides (50 µgs administered 4 to 5 times in adjuvant over the course of three months) and experiment 2 the pL-conjugated form (similarly administered). Mean titer refers to the average amount of virus in each group of from 4 to 8 mice. P-values are derived by two group, one-way t-tests of the peptide immune groups with the normal or adjuvant control groups (shown as : vs. normal / vs. adjuvant).

Table 8 : C3H & Balb/c Anti-Peptide Sera Zosteriform Protection

Antiserum :	% Positive Zosteriform Spread C3H	Balb/c
pG-20.1free	75	ND
20.1free	50	100
20.1-pL	75	75
pG-20.2free	75	ND
20.2free	ND	50
20.2-pL	100	25
pG-HSV-1	25	ND
HSV-1	50	0
Adjuvant Control	100	ND
HBSS Control	75	100

Protection against zosteriform spread of HSV-1_{BK} mediated by peptide immune sera generated in C3H and Balb/c mice. The first experiment utilized C3H sera and the second Balb/c sera, and the results are shown in the second and third columns, respectively, as the percentage of four mice which demonstarated zosteriform spread. For both experiments, 300 µl of whole serum or the protein G (pG) isolated IgG fraction equivalent were injected intravenously into six week old naive C3H mice two hours before challenge. Negative control animals received 300 µl of serum from Freund's Adjuvant injected animals or 300 µl of HBSS. Positive "protection" controls received polyclonal HSV-immune serum or 0.5 mgs of either of two glycoprotein D reactive monoclonal antibodies (MAbs D7 and D8) shown previously to be protective (39). For both experiments, the mice receiving MAbs D7 or D8 demonstrated 0% positive zosteriform spread.

ND : not done.

This is most evident in the experiment utilizing Balb/c antiserum (Table 8, column 3), where the anti-20.2mer sera significantly protected the mice from zosteriform spread. The anti-20.1mer Balb/c sera was not significantly protective, and the slight protection by the C3H anti-20.1mer sera is nonsignificant due to the 75% positive outcome in the HBSS positive control group. Serum generated against the 18mer was not tested in this model.

CHAPTER 5

DISCUSSION

Our initial analysis of the protective capacity of a panel of MAbs reactive with discrete antigenic sites on gB-1 in the murine zosteriform spread model of HSV-1 infection identified one (MAb B6) which conferred a high degree of passive protection (39). Earlier analysis has defined this antigenic site as linear and HSV-type-1-specific and has localized the epitopic region to amino acid 84 of the mature gB-1 protein (26).

In this study, three overlapping peptides spanning this site were synthesized and used as immunogens in their free and pL carrier-bound form. Initial screening of these peptides with polyclonal anti-HSV sera and lymph node cell populations demonstrated recognition of two of them, the 18mer and 20.1mer. As immunogens, all of the peptides were able to prime mice for enhanced ear clearance of a low virus dose. Additionally, the 20.2mer peptide elicited HSV-1 neutralizing antibody in C3H and Balb/c mice, and the Balb/c anti-20.2mer serum was passively protective in the zosteriform spread model.

The epitope recognized by MAb B6 can not yet be precisely localized to a discrete six to eight amino acid stretch within any of the three B6 epitope peptides, but is affected by regions contained in each of the three peptides. The single mutation found in the MAb B6-selected mutant virus mar B6.1 at amino acid position 84 of gB1 causes a glycine to aspartate substitution (26). Since this amino acid position is represented only in the MAb B6-reactive 18mer, and is not present in the MAb B6-reactive 20.1mer, the mutation may map outside of the actual site of antibody recognition. These data indicate that the region of overlap between the 18mer and 20.1mer may be the relevant epitope. Serum generated by the MAb B6-nonreactive 20.2mer in Balb/c mice, however, was less effective in

neutralizing mar B6.1 than wild type KOS, suggesting that the configuration of amino acid residues 91-110 are affected by the mutation at amino acid 84, and that serum generated by a downstream region of the glycoprotein may behave somewhat similarly to the MAb. Considering the fact that polyclonal HSV-immune sera reacts predominantly with the 20.1mer, the whole area represented by the three peptides appears to be an immunoreactive site potentially composed of several overlapping epitopes. Since the gB-1 molecule in the region of the 18mer and 20.1mer is predicted to be of highly random configuration, neighboring and less localized interactions may be important in determining the conformation of any discrete area within those sequences. The linear MAb B6 epitope may thereby have an underlying discontinuous nature.

The 20.2mer may have represented the best immunogen in terms of eliciting neutralizing and protective serum due to its assuming a more restricted configuration *in vivo*, as a consequence of its repetitive hydrophobic residues, than the other, more hydrophilic, peptides. Alternatively, the potential alpha-helical arrangement of the 20.2mer may have generated a more vigorous T helper cell response and thereby more potent humoral immunity. Proliferation analysis of 20.2mer-immune lymph node cells did not lend evidence for this idea, although the background proliferation of the assays was too high for discriminatory analysis. No proliferation of HSV-immune cells occurred in response to the 20.2mer, and polyclonal anti-HSV sera as well as MAb B6 did not react with the peptide. It is fascinating that this "silent" epitope provoked suitable virus-reactive immunity : generated against the HSV-1 laboratory strain KOS gB sequence, the anti-20.2mer sera was effective *in vivo* against both KOS and the virulent HSV-1 clinical isolate BK. Furthermore, this region of KOS gB is identical to that of the Patton and F strains of HSV-1 (4, 45, 52), and 80% homologous to the corresponding gB-2 region of strain 333 (52). The region corresponding to the 18mer also shares a high degree of

homology between the KOS, Patton, and 333 strains. The region corresponding to the 20.1mer is highly homologous among the HSV-1 strains mentioned, but is only fifty percent homologous with HSV-2 strain 333.

While previous reports of peptide-induced protective immunity to HSV have indicated that the HSV immunodominant gD1-23mer may be a successful immunogen (24), immunological investigations utilizing whole gD and gB have shown that either glycoprotein can stimulate protective immunity (39). Our initial MAb-mediated protection experiments indicated that several antigenic sites exist on gD which are the targets of protective antibody, whereas only one antigenic site recognized by the gB-reactive MAbs appeared to be the target of protective immunity. Here it is further shown that within that antigenic site, a peptide apparently not recognized by HSV-generated immune mechanisms stimulated effective anti-HSV immunity. With this as a point of reference, it can be inferred that gB does possess several antigenic sites, but only a few of which may elicit protective antibody *in vivo*. Additionally, immunity generated against the whole virus may overlook potentially protective epitopes. A synthetic immunogen representing a "silent" epitope may unlock the immunity essential for controlling herpes simplex virus infection. Furthermore, since gB of HSV shares significant amino acid homology and immunological cross-reactivity with related glycoproteins of EBV (8, 22, 44), VZV (15, 30), and HCMV (11), it may be possible, using a single conserved region, to stimulate immunity to several human herpesviruses with a gB-based subunit immunogen.

The most important question regarding these findings is their relevancy to human HSV infection. The B6 peptides are able to generate anti-viral immunity in the mouse, but are these epitopes degenerate and recognizable in a unique outbred population ? Evidence exists for the recognition of gB by HSV-immune humans (14, 31, 55, 57), and both degenerate and nondegenerate recognition of peptides between the murine and human

immune systems (13, 25, 38, 49, 54). Additionally, to generate a response in the murine system, the peptides had to be repeatedly administered in adjuvant preparations which would not be acceptable for use in humans. Clearly, the development of alternative adjuvants and carriers, such as lymphokines, muramyl-peptides, proteosomes, and liposomes, is necessary to potentiate the anti-peptide / anti-viral response.

REFERENCES

(1) Atassi, M. Z. and A. F. S. A. Habeeb. 1972. Reaction of proteins with citraconic anhydride. Meth. Enzymology. **25:** 546-553.

(2) Blacklaws, B. A., A. A. Nash, and G. Darby. 1987. Specificity of the immune response of mice to herpes simplex virus glycoproteins B and D constituitively expressed on L cell lines. J. Gen. Virol. **68:** 1103-1114.

(3) Bone, D. R. and R. J. Courtney. 1974. A temperature sensitive mutant of herpes simplex virus type 1 defective in the synthesis of the major capsid polypeptide. J. Gen. Virol. **24:** 17.

(4) Bzik, D. J., B. A. Fox, N. A. Deluca, and S. Person. 1984. Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. Virology. **133:** 301-314.

(5) Cai, W., B. Gu, and S. Person. 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. **62:** 2596-2604.

(6) Cantin, E. M. , R. Eberle, J. L. Baldick, B. Moss, D. E. Willey, A. L. Notkins, and H. Openshaw. 1987. Expression of herpes simplex virus 1 glycoprotein B by a recombinant vaccinia virus and protection of mice against lethal herpes simplex virus 1 infection. PNAS (USA). **84:** 5908-5912.

(7) Carter, V. C., P. C. Schaffer, and S. S. Tevethia. 1981. The involvement of herpes simplex virus type 1 glycoproteins in cell-mediated immunity. J. Immunol. **126:** 1655-1660.

(8) Chan, W. L. 1989. Functional cross-reactivity between the glycoprotein B of herpes simplex virus type 1 and Epstein-Barr virus. Immunology. **66:** 14-19.

(9) Chan, W. L., M. L. Lukig, and F. Y. Liew. 1985. Helper T cells induced by an immunopurified herpes simplex virus type 1 (HSV 1) 115 kilodalton glycoprotein (gB) protect mice against HSV-1 infection. J. Exp. Med. **162:** 1304-18.

(10) Chow, M., R. Yabrov, J. Bittle, J. Hogle, and D. Baltimore. 1985. Synthetic peptides from four separate regions of the poliovirus type 1 capsid protein VP1 induce neutralizing antibodies. PNAS (USA). **82:** 910-914.

(11) Cranage, M. P., T. Kouzarides, A. T. Bankier, S. Satchwell, K. Weston, P. Tomlinson, B. Barrell, H. Hart, S. E. Bell, A. C. Minson, and G. L. Smith. 1986. Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. EMBO J. **5:** 3057-3063.

(12) Cremer, K. J., M. MacKett, C. Wohlenberg, A. L. Notkins, and B. Moss. 1985. Vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D prevents latent herpes in mice. Science. **228:** 737-740.

(13) DeFreitas, E. C., B. Dietzschold, and H. Koprowski. 1985. Human T-lymphocyte response in vitro to synthetic peptides of herpes simplex virus glycoprotein D. PNAS (USA). **82:** 3425-3429.

(14) Eberle, R. and S. W. Mou. 1983. Relative titers of antibodies to individual polypeptide antigens of herpes simplex virus type 1 in human sera. J. Inf. Diseases. **148:** 436-444.

(15) Edson, C. M., B. A. Hosler, R. A. Respess, D. J. Waters, and D. A. Thorley-Lawson. 1985. Cross-reactivity between herpes simplex virus glycoprotein B and a 63,000 dalton varicella-zoster virus envelope glycoprotein. J. Virol. **56:** 333-336.

(16) Eisenberg, R. J, C. P. Cerini, C. J. Heilman, A. D. Joseph, B. Dietzschold, E. Golu, D. Long, M. P. Leon, and G. H. Cohen. 1985. Synthetic glycoprotein D - related peptides protect mice against herpes simplex virus challenge. J. Virol. **56:** 1014-1017.

(17) Francis, M. J., G. Z. Hastings, D. V. Sangar, R. P. Clark, A. Syred, B. E. Clarke, D. J. Rowlands, and F. Brown. 1987. A synthetic peptide which elicits neutralizing antibody against human rhinovirus type 2. J. Gen. Virol. **68:** 2687-2691.

(18) Fuller, A. O. and P. G. Spear. 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. PNAS (USA). **84:** 5454-5458.

(19) Garnier, J., D. J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120:** 97-120.

(20) Glorioso, J. C., U. Kees, G. Kumel, H. Kirchner, and P. H. Krammer. 1985. Indentification of herpes simplex virus type 1 (HSV-1) glycoprotein gC as the immunodominant antigen for HSV-1-specific memory cytotoxic T lymphocytes. J. Immunol. **135:** 575-582.

(21) Glorioso, J., C. H. Schroder, G. Kumel, M. Szczesiul, and M. Levine. 1984. Immunogenicity of herpes simplex virus glycoproteins gC and gB and their role in protective immunity. J. Virol. **50:** 805-812.

(22) Gong, M., T. Ooka, T. Matsuo, and E. Kieff. 1987. Epstein-Barr virus glycoprotein homologous to herpes simplex virus gB. J. Virol. **61:** 499-508.

(23) Grist, N. R., E. J. Bell, E. A. C. Follett, and G. E. D. Urquhart. 1979. Neutralization tests, pp. 84-85. In N. R. Grist and G. E. D. Urquhart (eds.). Diagnostic methods in clinical virology, 3rd ed. Blackwell Scientific Publications, Ltd., Oxford.

(24) Heber-Katz, E. and B. Dietzschold. 1986. Immune response to synthetic herpes simplex virus peptides: the feasibility of a synthetic vaccine. Curr. Topics Microbiol. Immunol. **130:** 51-64.

(25) Heber-Katz, E., S. Valentine, B. Dietzschold, and C. Burns-Purzycki. 1988. Overlapping T cell antigenic sites on a synthetic peptide fragment from herpes simplex virus glycoprotein D, the degenerate MHC restriction elicited, and functional evidence for antigen-Ia interaction. J. Exp. Med. **167:** 275-287.

(26) Highlander, S. L., D. J. Dorney, P. J. Gage, T. C. Holland, W. Cai, S. Person, M. Levine, and J. C. Glorioso. 1989. Identificantion of 'mar' mutations in herpes simplex virus type 1 glycoprotein B which alter antigenic structure and function in virus penetration. J. Virol. **63:** 730-738.

(27) Howard, C. R., J. Allan, S-H. Chen, S. E. Brown, and M. W. Steward. 1986. Progress toward a synthetic hepatitis B vaccine, pp. 133-136. In H. Peeters (ed.). Protides of the biological fluids, Vol. 34.

(28) Kennedy, R.C., T. C. Chanh, P. Kanda, J. W. Eichberg, J. S. Allen, T. H. Lee, M. Essex, and G. R. Dreesman. 1986. Synthetic peptides identify native antigenic determinants associated with human T-lymphotropic virus type III, pp. 107-110. In H. Peeters (ed.). Protides of the biological fluids, Vol. 34.

(29) Kino, Y., T. Eto, K. Nishiyama, N. Ohtomo, and R. Mori. 1986. Immunogenicity of purified glycoprotein gB of herpes simplex virus. Arch. Virol. **89:** 69-80.

(30) Kitamura, K., J. Namazue, H. Campo-Vera, T. Ogina, and K. Yamanishi. 1986. Induction of neutralizing antibody against varicella-zoster virus (VZV) by VZV gp3 and cross-reactivity between VZV gp3 and herpes simplex viruses gB. Virology. **149:** 74-82.

(31) Kuhn, J. E., G. Dunkler, K. Munk, and R. W. Braun. 1987. Analysis of the IgM and IgG antibody response against herpes simplex virus type 1 (HSV-1) structural and nonstructural protein J. Med. Virol. **23:** 135-150.

(32) Kyte, J. and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:** 105-132.

(33) Lawman, M. J. P., R. J. Courtney, R. Eberle, P. A. Schaffer, M. K. O'Hara, and B. T. Rouse. 1980. Cell-mediated immunity to herpes simplex virus: specificity of cytotoxic T cells. Infect. Immun. **30:**451-461.

(34) Marlin, S. D., S. L. Highlander, T. C. Holland, M. Levine, and J. C. Glorioso. 1986. Antigenic variation (mar mutations) in herpes simplex virus glycoprotein B can induce temperature-dependent alterations in gB processing and virus production. J. Virol. **59:** 142-153.

(35) Martin, S. and B. T. Rouse. 1987. The mechanisms of antiviral immunity induced by a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: clearance of local infection. J. Immunol. **138:** 3431-3437.

(36) McCray, J. and G. Werner. 1987. Different rhinovirus serotypes neutralized by antipeptide antibodies. Nature. **329:** 736-738.

(37) McDermott, M. R., F. L. Graham, T. Hanke, and D. C. Johnson. 1989. Protection of mice against lethal challenge with herpes simplex virus by vaccination with an adenovirus vector expressing HSV glycoprotein B. Virology. **169:** 244-247.

(38) McMichael, A. J., F. M. Gotch, and J. Rothbard. 1986. HLA B37 determines an influenza A virus nucleoprotein epitope recognized by cytotoxic T lymphocytes. J. Exp. Med. **164:** 1397.

(39) Mester, J. C., J. C. Glorioso, and B. T. Rouse. 1989. Neutralization, antibody-dependent cellular cytotoxicity, and epitope recognition in monoclonal antibody-mediated protection against the zosteriform spread of herpes simplex virus 1. Submitted.

(40) Milich, D. R., A. McLachlan, A. Moriarty, and G. B. Thornton. 1987. A single 10-residue pre-S(1) peptide can prime T cell help for antibody production to multiple epitopes within the pre-S(1), pre-S(2), and S regions of HBsAg. J. Immunol. **138:** 4457-4465.

(41) Muller, G. M., M. Shapira, and R. Arnon. 1982. Anti-influenza response achieved by immunization with a synthetic conjugate. PNAS USA. **79:** 569-573.

(42) Nozaki, Y. and C. Tanford. The solubility of amino acids and two glycine peptides in aqueous ethanol and dioxane solutions. Establishment of a hydrophobicity scale. 1971. J. Biol. Chem. **246:** 2211-2217.

(43) Pachl, C., R. L. Burke, L. L. Stuve, L. Sanchez-Pescador, G. V. Nest, F. Masiarz, and D. Dina. 1987. Expression of cell-associated and secreted forms of herpes simplex virus type 1 glycoprotein gB in mammalian cells. J. Virol. **61:** 315-325.

(44) Pellet, P. E., M. D. Biggin, B. Barrell, and B. Roizman. 1985. Epstein-Barr virus genome may encode a protein showing significant amino acid and predicted secondary structure homology with glycoprotein B of herpes simplex virus 1. J. Virol. **56:** 807-813.

(45) Pellett, P. E., K. G. Kousoulas, L. Pereira, and B. Roizman. 1985. Anatomy of the herpes simplex virus 1 strain F glycoprotein B gene: primary sequence and predicted protein structure of the wild type and monoclonal antibody-resistant mutants. J. Virol. **53:** 243-253.

(46) Rooney, J. F., C. Wohlenberg, K. J. Cremer, B. Moss, and S. L. Notkins. 1988. Immunization with a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: long-term protection and effect of revaccination. J. Virol. **62:** 1530-1534.

(47) Schrier, R. D., L. I. Pizer, and J. W. Moorhead. 1983. Type-specific delayed hypersensitivity and protective immunity induced by isolated herpes simplex virus glycoprotein. J. Immunol. **130:** 1413-1418.

(48) Simmons, A. and A. A. Nash. 1984. Zosteriform spread of herpes simplex virus as a model of recrudescence and its use to investigate the role of immune cells in prevention of recurrent disease. J. Virol. **52:** 816-821.

(49) Sinigaglia, F., M. Guttinger, J. Kilgus, D. M. Doran, H. Matile, H. Etlinger, A. Trzeciak, D. Gillessen, and J. R. L. Pink. 1988. A malaria T-cell epitope recognized in association with most mouse and human MHC class II molecules. Nature. **336:** 778-780.

(50) Stanberry, L. R., D. I. Bernstein, R. L. Burke, C. Pachl, and M. G. Myers. 1987. Vaccination with recombinant herpes simplex virus glycoproteins: protection against initial and recurrent genital herpes. J. Inf. Diseases. **155:** 914-919.

(51) Stanberry, L. R., R. L. Burke, and M. G. Myers. 1988. Herpes simplex virus glycoprotein treatment of recurrent genital herpes. J. Inf. Diseases. **157:** 156-163.

(52) Stuve, L. L., S. Brown-Shimer, C. Pachl, R. Najarian, D. Dina, and R. L. Burke. 1987. Structure and expression of the herpes simplex virus type 2 glycoprotein gB gene. J. Virol. **61:** 326-335.

(53) Sweet, R. M. and D. Eisenberg. 1983. Correlation of sequence hydrophobicities measures similarity in three-dimensional protein structure. J. Mol. Biol. **171:** 479-488.

(54) Townsend, A. R. M., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael. 1986. The epitopes of influenza nucleoprotein recognised by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell. **44:** 959.

(55) Vestergaard, B. F. 1980. Herpes simplex virus antigens and antibodies: a survey of studies based on quantitative immunoelectrophoresis. Rev. Inf. Diseases. **2:** 899-913.

(56) Watari, E., B. Dietzschold, G. Szokan, and E. Heber-Katz. 1987. A synthetic peptide induces long-term protection from lethal infection with herpes simplex virus 2. J. Exp. Med. **165:** 459-470.

(57) Zarling, J. M., P. A. Moran, R. L. Burke, C. Pachl, P. W. Berman, and L. A. Lasky. 1986. Human cytotoxic T cell clones directed against herpes simplex virus-infected cells IV. Recognition and activation by cloned glycoproteins gB and gD. J. Immunol. **136:** 4669-4672.

PART IV :

CONCLUSIONS AND SPECULATIONS

CHAPTER 1

CONCLUSIONS AND SPECULATIONS

The abundance of reports detailing the immune response to HSV have clearly shown that both humoral and cellular mechanisms may protect against infection, and that their protective capacity is potentiated by the further involvement of nonspecific aspects of immunity. Although neutralizing polyclonal antibody was initially demonstrated to be ineffective against virus spread, neutralizing monoclonal antibodies (MAbs) have recently been shown to be effective *in vivo*. The role of both neutralization and antibody-dependent cellular cytotoxicity (ADCC) *in vivo* is unclear, and although many researchers have correlated the *in vitro* activities of their MAbs with *in vivo* protection, the actual protective mechanism remained a subject of speculation.

The initial studies performed in Part II extensively analyzed the role of assayable *in vitro* activities and epitope and glycoprotein recognition in the ability of a panel of MAbs to protect against *in vivo* infection with HSV. This marks the first time that a study of such depth has been performed, and the range of the results indicates that all of the examined MAb properties may be influential in determining a protective from a nonprotective MAb.

All of the MAbs directed against glycoproteins C and D (gC and gD) were protective. The gD-reactive MAbs exhibited the full range of anti-viral activities *in vitro*, while the gC-reactive MAbs all demonstrated strong ADCC and weak neutralizing abilities *in vitro*. The gB-reactive MAbs, of which only one was protective, were all stongly neutralizing and did not effectively mediate ADCC. Thereby, neutralization, ADCC, and glycoprotein and epitope recognition may all be important in the ability of antibody to protect *in vivo*.

Additionally, the ability of the MAbs to inhibit the cell-to-cell spread of HSV (demonstrated by the protective gB and gD-reactive MAbs) may also be an important mechanism of antibody mediated protection. The capacity of antibody to directly or indirectly inhibit viral replication in infected cells deserves further investigation, and may shed insight into the mechanism of viral latency and reactivation.

The ability of short synthetic peptides to stimulate a protective anti-viral response *in vivo* is just beginning to be appreciated. Recent work with the gD1-23mer has extended the original demonstration of *in vivo* protection with the VP1 141-160 peptide of foot-and-mouth disease virus, and has proven the power of the peptide technique in HSV immunology. The work detailed here in Part II is the first demonstration of the protective capacity of a gB related peptide.

Although gB is a highly conserved protein among the herpesviruses, the region corresponding to the protective peptide is significantly homologous only among the sequenced HSV 1 and 2 strains. The significance of the location of the epitope on gB and its predicted secondary structure are unclear. No detailed crystallographic or NMR analyses have been performed on the HSV glycoproteins, and the overall structural predictions have been based on computer algorithms. Additional complications in predicting the tertiary structure of gB and the precise three dimensional location of the B6 epitope are encountered when considering that gB may exist as a monomer, dimer, or trimer.

The B6 epitope peptide most effective in generating antiviral immunity had a periodic repeat of hydrophobic residues within a stretch of hydrophilic residues, and the corresponding region of the native molecule was predicted to be in an alpha-helical configuration. The other B6 epitope peptides were mostly hydrophilic, and corresponded to regions predicted to be of random configuration. As immunogens, these "random"

peptides elicited a highly nonspecific response, and proved that the "peptide approach" may not be as specific as one would hope. The importance of structural constraint for "proper" immunogenicity is evident. Modification of the sequences designed to restrict the many possible conformations adoptable in solution may render these peptides potent and specific immunogens.

The importance of the alpha helical configuration in immunogenicity was examined through the use of additional gB peptides (Appendix E) predicted to correspond to unique helical regions of gB. While immune sera generated against two of these peptides demonstrated complement lysis of HSV infected cells, and HSV-immune sera reacted well with an additional one in ELISAs, no neutralizing activity was found for any of the sera generated by these peptides. Still, initial *in vivo* protection experiments hinted that serum generated to one of the peptides may be able to protect from zosteriform spread. These results indicate that peptides corresponding to computer predicted alpha-helical sites are not necessarily able to generate effective antiviral immunity, and that epitope mapping by the sequencing of MAb neutralization resistant mutant viruses (which led to the identification of the epitopic region recognized by MAb B6) is a powerful method for identifying regions of viral glycoproteins able to generate a protective response.

Vaccinia virus has been widely used as an expression vector for foreign viral genes. As immunogens, recombinant vaccinia-HSV constructs have been demonstrated to be effective stimulants of protective immunity. Experiments described here (Appendix A), however, indicate that cellular immunity generated by vaccinia-gB or vaccinia-gD is not completely protective *in vivo*. This is most likely due to a detraction or suppression of an essential component of HSV immunity caused by HSV gene expression in a vector which synthesizes a great variety of its own proteins. Since the peptide approach may be more specific, one would expect greater interest in identifying T and B cell epitopes on HSV

glycoproteins. However, the phenomenon of major histocompatibility complex (MHC) restriction of peptide recognition and the lack of potent adjuvants suitable for human use has hindered widespread interest in the dissection of the HSV glycoprotein-specific immune response with synthetic peptides. Recent documentation of the degeneracy of certain viral epitopes (*i.e.*, their ability to be recognized in the context of more than one MHC molecule), and the development of lymphokines and other potent , nontoxic adjuvants, should encourage the further exploration of the ability of peptides to act as effective subunit vaccines.

APPENDICES

APPENDIX A :

ZOSTERIFORM ANALYSIS OF IMMUNE LYMPH NODE CELLS GENERATED BY VACCINIA-gB AND VACCINIA-gD

Adoptive Transfer Procedure for the Evaluation of Protection Against the Zosteriform Spread of HSV by Lymph Node Cells Generated by HSV-Vaccinia Recombinants

(1.) Mice are immunized with 10^5 to 10^6 PFU of the recombinant vaccinia viruses (in saline) in each footpad. Positive control mice receive a similar dose of whole live HSV.

(2.) After one week, the popliteal nodes of the immunized mice are aseptically removed, washed and counted. Cell numbers are adjusted (in saline) so that 2-6 X 10^7 cells can be transferred in a 300-500 µl IV injection to six week old naive mice.

(3.) Naive mice are shaved and depilated with Nair the day before percutaneous flank inoculation with HSV. On the day of inoculation, the mice are divided into treatment groups and given the prescribed amount of the appropriate population of immune lymph node cells. Negative control mice receive saline without cells. Subsequently, each mouse is scratched on the mid-flank region of the skin through a 10 µl drop containing 10^5 PFU of the BK strain of HSV-1. This inoculation is done in a blind fashion so that each group's identity is not know during inoculation.

(4.) Each group is monitored for the following two weeks and the observed zosteriform banding percentages used to determine the protective ability of each of the immune cell populations.

Table 1 : Protection Mediated by Vaccinia-Vector Stimulated Immune Cells in the Zosteriform Model

Immunogen	# of Cells Transferred	Zosterification # (+)	Zosterification # (-)	% Positive	p - value
Exp. 1					
Vac-gB	3×10^7	2	2	50	NS
	2×10^7	4	0	100	NS
Vac-gD	3×10^7	4	1	80	NS
	2×10^7	5	0	100	NS
	1×10^7	5	0	100	NS
Vac-tk$^-$	3×10^7	4	0	100	
	2×10^7	7	0	100	
	1×10^7	5	0	100	
HBSS Control	0	4	0	100	
Exp. 2					
Vac-gB	2×10^7	4	1	80	NS
Vac-gD	2.5×10^7	2	2	50	NS
HSV-1	3×10^7	2	2	50	NS
	2×10^7	1	3	25	.07
	1×10^7	2	4	33	.08
Vac-tk$^-$	2.5×10^7	4	0	100	
HBSS Control	0	5	1	83	
Exp. 3					
Vac-gB	6×10^7	2	2	50	NS
	5×10^7	5	5	50	NS
Vac-gD	6×10^7	2	2	50	NS
	5×10^7	2	3	40	.10
	4×10^7	2	0	100	NS
HSV	2.5×10^7	1	15	6	.0001
	2×10^7	0	11	0	.0001
Vac-HA	4.5×10^7	3	0	100	
HBSS Control	0	14	4	78	

Protection against zosteriform spread mediated by the adoptive transfer of immune lymph node (LN) cells. The LN cell preparation and transfer procedure is listed on the previous page (131). C3H mice were used for all experiments. Percent positive refers to the percentage of mice in each treatment group which formed zosteriform bands after challenge with HSV-1 BK. P-values were determined by Chi-Square analysis of each treatment group with the HBSS (Hank's Balanced Salt Solution) control group.

NS : not significant ($p>0.05$).

APPENDIX B :

COMPLEMENT LYSIS OF HSV-1-INFECTED CELLS MEDIATED BY THE PANEL OF MONOCLONAL ANTIBODIES

Table 1: MAb-Mediated ADCMC.

MAb	% Specific Cr-release
Exp. 1	
B3	24
B6	35
C3	18
D8	38
HSV Immune	60
Exp. 2	
B3	5
B4	5
B6	7
B8	4
C3	7
C4	4
C8	6
D2	12
D7	24
D8	22
HSV Immune	33

Vero cell targets were infected with HSV-1 KOS at a multiplicity of 5 and labeled by incubating 2 X 10^6 cells per ml of McCoy's 5a medium (5% DCS) with 150 µCi of ^{51}Cr (Amersham) per ml for five hours at 37°C. Dilutions of antibody (in triplicate) were prepared in 96-well round bottom plates (Corning) and targets added for a final volume of 180 µl. After a two hour incubation at 4°C on a rotating platform, 20 µl of neat complement (Low-Tox-M, Cedarlane) was added per well, and the plates incubated at 37°C for one hour. The cells were then pelleted, 100 µl of the media supernant of each well collected, and percent ^{51}Cr release determined with the following formula : ^{51}Cr release = (experimental release - spontaneous release) / (total release - spontaneous release) X 100. The standard errors of the ^{51}Cr release sample means were 10% or less. For experiment 1, 50 µg of each MAb and 1 X 10^5 targets were added per well. Polyclonal HSV immune serum was used at a 1:50 final dilution, and the specific release percentages shown are reduced by the 18% lysis achieved with complement alone. For experiment 2, 10 µg of each MAb and1 X 10^4 targets were added per well. Polyclonal HSV immune serum was used at a 1:50 final dilution, and the specific release percentages shown are reduced by the 12% lysis achieved with complement alone.

APPENDIX C :

MAB PROTECTION AGAINST GANGLIAL COLONIZATION

Table 1 : MAb Prevention of Latent Ganglial Infection.

MAb	# of Mice	Zosteriform Protection	# With Latent HSV-1
B6	10	(+)	0
C3	9	(+)	3
C4	2	(+)	2
C8	1	(+)	0
D2	3	(+)	0
D7	5	(+)	0
D8	5	(+)	0
B3	1	(-)	1
B4	1	(-)	1
B8	1	(-)	1
D2	1	(-)	0
D8	2	(-)	1
HBSS Control	10	(-)	5

Two to three months after infection, the dorsal root ganglia innervating the site of inoculation were explanted onto Vero cell monolayers, which were subsequently examined for HSV-induced cytopathic effect (cpe). Mice were recorded as with latent HSV if the initial or twice repeated passage over fresh Vero cells yielded HSV-induced cpe.

APPENDIX D :

ELISAs OF THE 18MER AND 20.1MER-GENERATED SERA

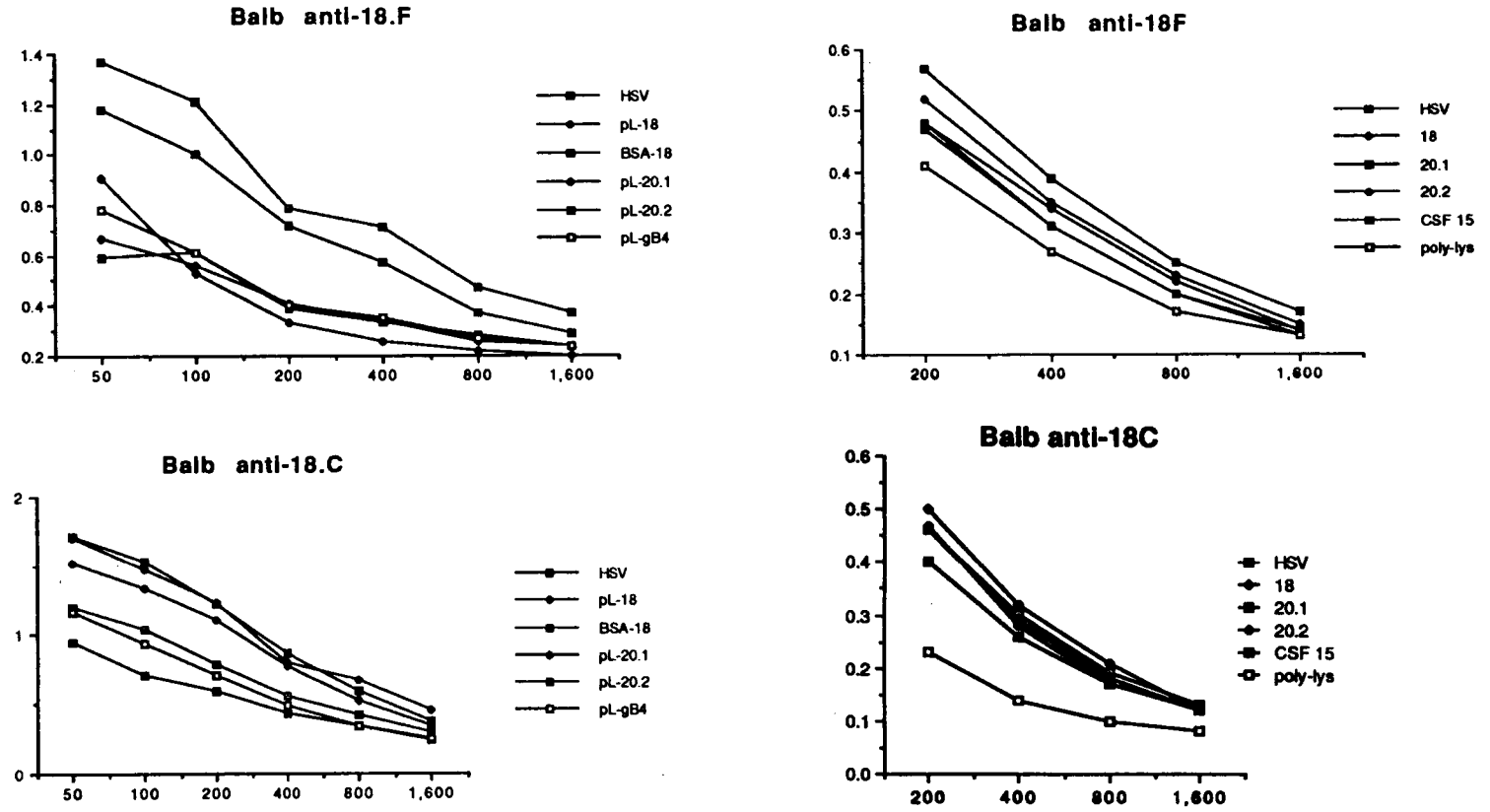


Figure 1 : Balb/c Anti-18mer Sera.

Serial two-fold dilutions of the sera generated by the free (18F) or poly-lysine conjugated (18C) form of the 18mer peptide were screened via ELISA against HSV, poly-lysine (pL), and pL and BSA-conjugated and free forms of the B6 epitope peptides (18mer, 20.1mer, and 20.2mer) and unrelated control peptides (gBIV, CSF 15). The ordinate represents serum dilution factors, and the abscissa ABS 490. The subtitle of each graph denotes the specific antiserum used. Standard errors of the sample means were less than 10%.

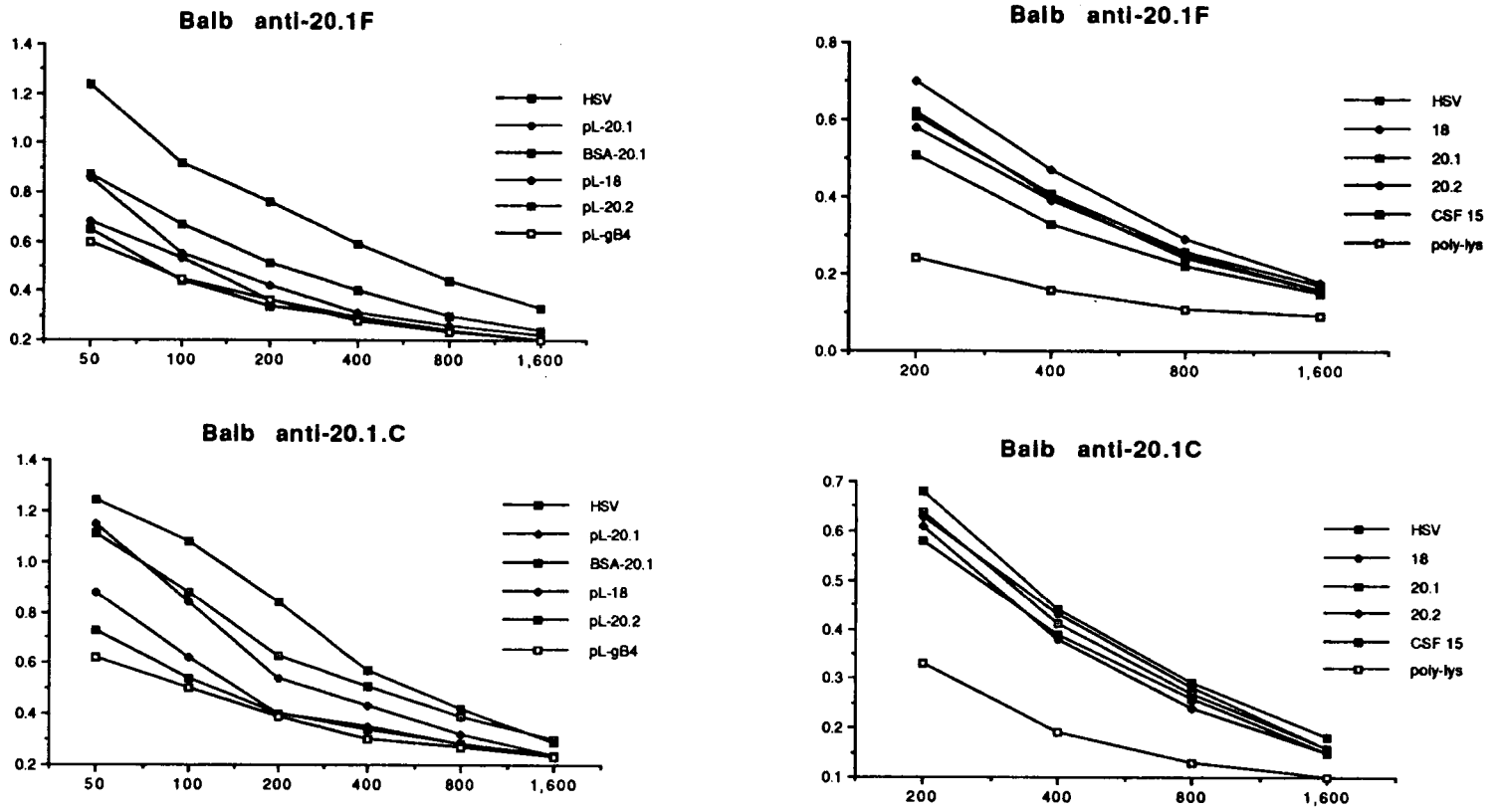


Figure 2 : Balb/c Anti-20.1mer Sera.

Serial two-fold dilutions of the sera generated by the free (20.1F) or poly-lysine conjugated (20.1C) form of the 20.1mer peptide were screened via ELISA against HSV, poly-lysine (pL), and pL and BSA-conjugated and free forms of the B6 epitope peptides (18mer, 20.1mer, and 20.2mer) and unrelated control peptides (gBIV, CSF 15). The ordinate represents serum dilution factors, and the abscissa ABS 490. The subtitle of each graph denotes the specific antiserum used. Standard errors of the sample means were less than 10%.

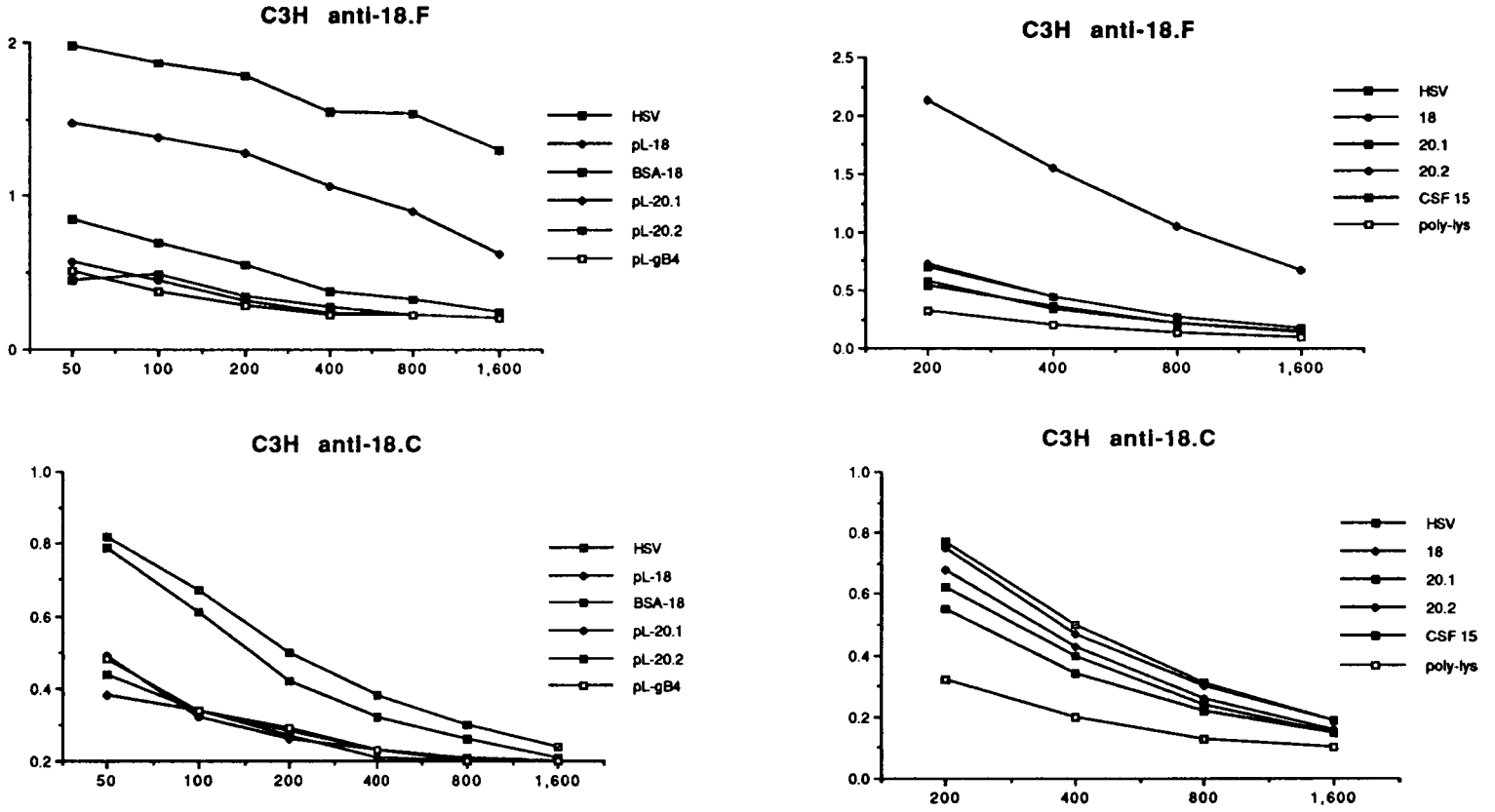


Figure 3 : C3H Anti-18mer Sera.

Serial two-fold dilutions of the sera generated by the free (18F) or poly-lysine conjugated (18C) form of the 18mer peptide were screened via ELISA against HSV, poly-lysine (pL), and pL and BSA-conjugated and free forms of the B6 epitope peptides (18mer, 20.1mer, and 20.2mer) and unrelated control peptides (gBIV, CSF 15). The ordinate represents serum dilution factors, and the abscissa ABS 490. The subtitle of each graph denotes the specific antiserum used. Standard errors of the sample means were less than 10%.

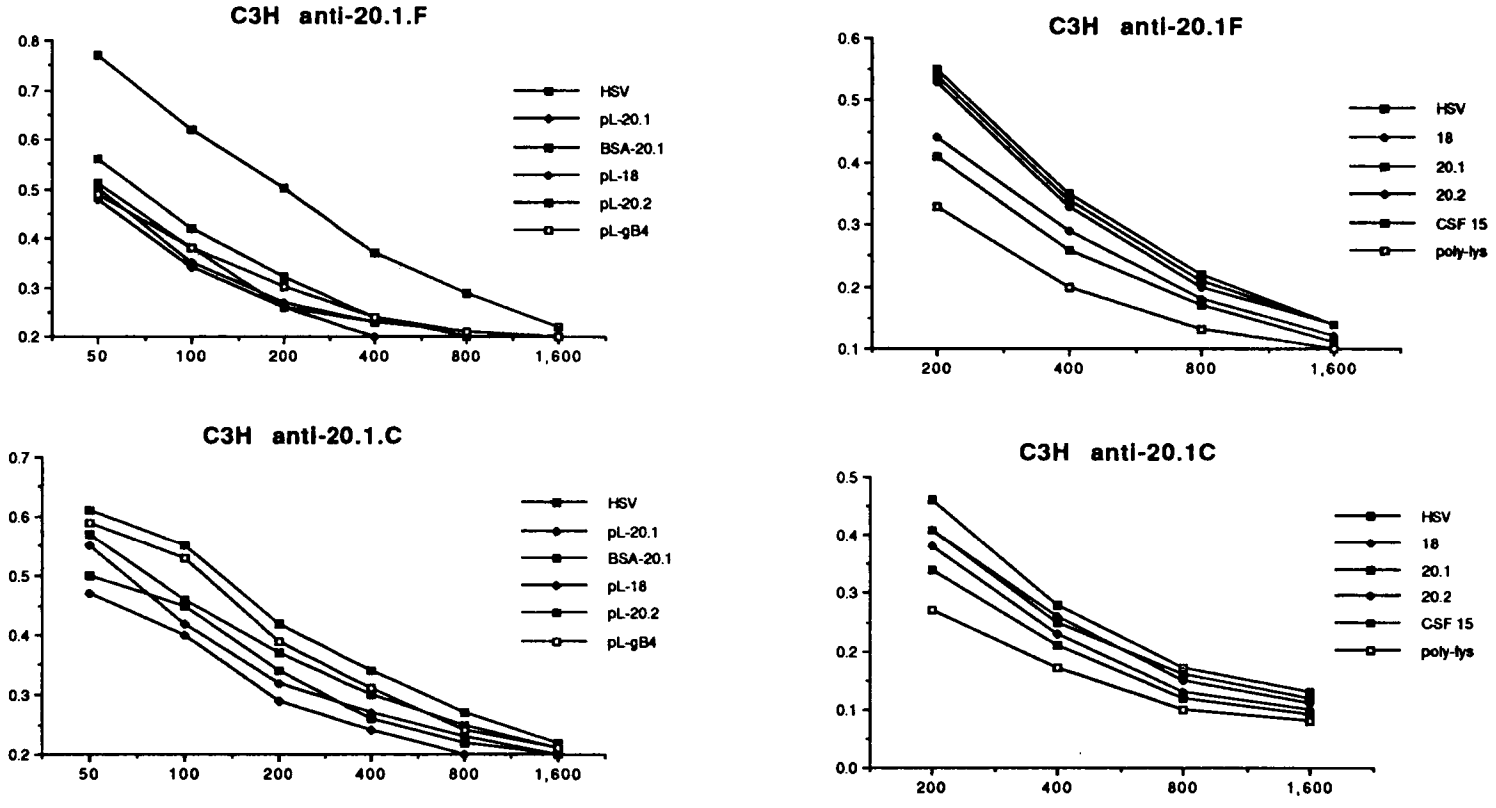


Figure 4 : C3H Anti-20.1mer Sera.

Serial two-fold dilutions of the sera generated by the free (20.1F) or poly-lysine conjugated (20.1C) form of the 20.1mer peptide were screened via ELISA against HSV, poly-lysine (pL), and pL and BSA-conjugated and free forms of the B6 epitope peptides (18mer, 20.1mer, and 20.2mer) and unrelated control peptides (gBIV, CSF 15). The ordinate represents serum dilution factors, and the abscissa ABS 490. The subtitle of each graph denotes the specific antiserum used. Standard errors of the sample means were less than 10%.

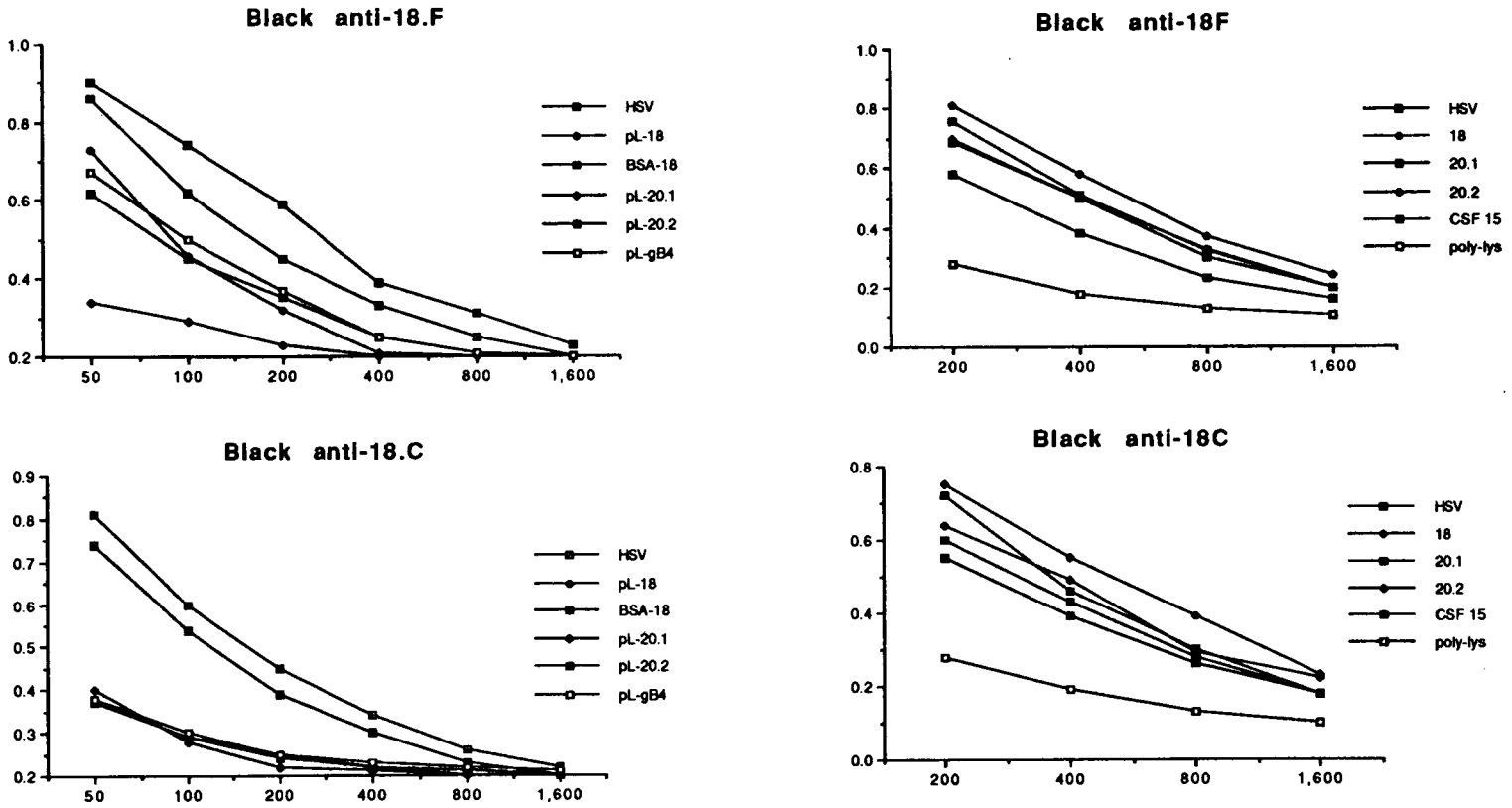


Figure 5 : C57/Bl Anti-18mer Sera.

Serial two-fold dilutions of the sera generated by the free (18F) or poly-lysine conjugated (18C) form of the 18mer peptide were screened via ELISA against HSV, poly-lysine (pL), and pL and BSA-conjugated and free forms of the B6 epitope peptides (18mer, 20.1mer, and 20.2mer) and unrelated control peptides (gBIV, CSF 15). The ordinate represents serum dilution factors, and the abscissa ABS 490. The subtitle of each graph denotes the specific antiserum used. Standard errors of the sample means were less than 10%.

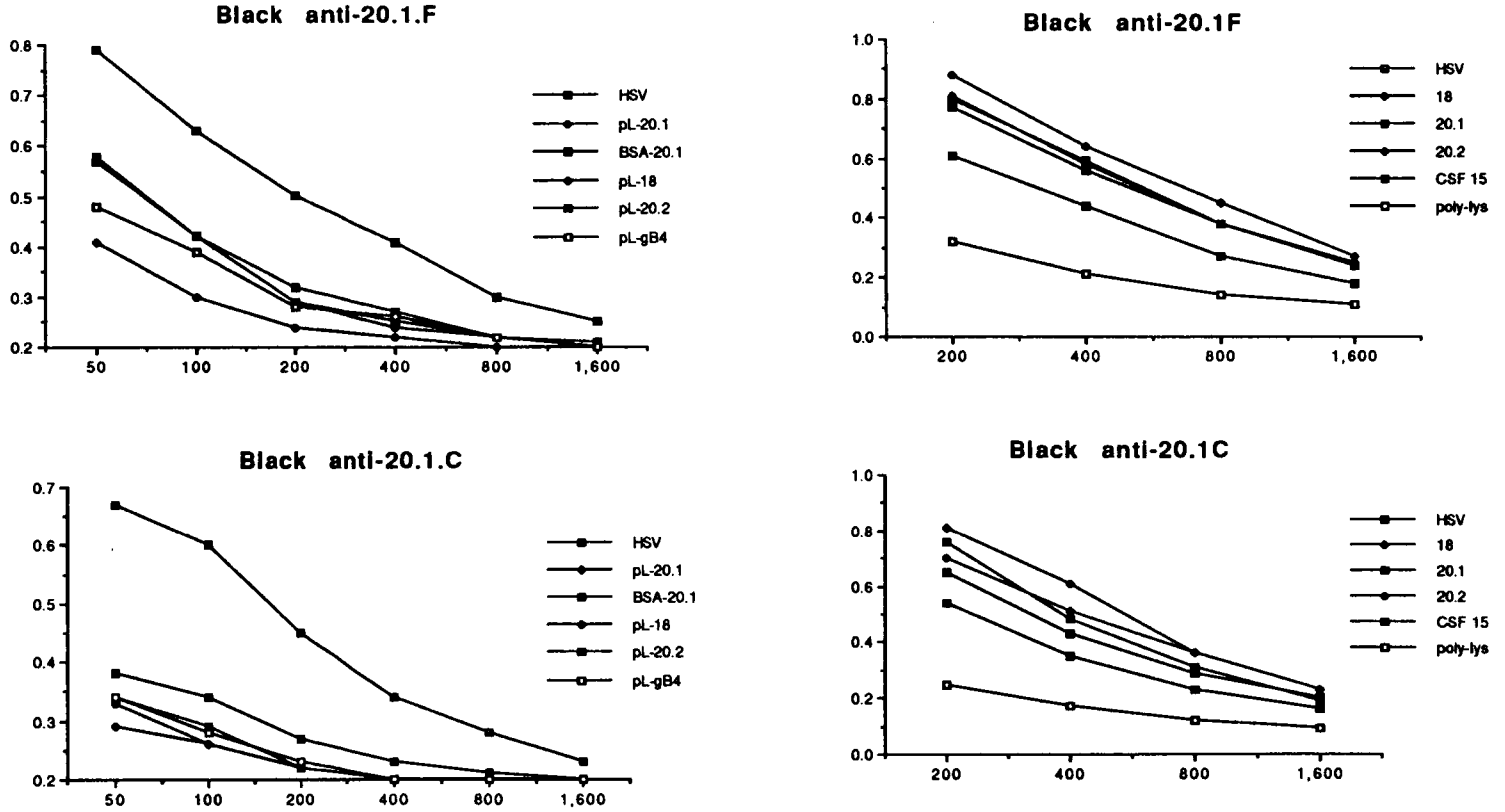


Figure 6 : C57/Bl Anti-20.1mer Sera.

Serial two-fold dilutions of the sera generated by the free (20.1F) or poly-lysine conjugated (20.1C) form of the 20.1mer peptide were screened via ELISA against HSV, poly-lysine (pL), and pL and BSA-conjugated and free forms of the B6 epitope peptides (18mer, 20.1mer, and 20.2mer) and unrelated control peptides (gBIV, CSF 15). The ordinate represents serum dilution factors, and the abscissa ABS 490. The subtitle of each graph denotes the specific antiserum used. Standard errors of the sample means were less than 10%.

APPENDIX E :

ANALYSIS OF THE SERA GENERATED BY THE PEPTIDES gB I, gB II, gB III, AND gB IV

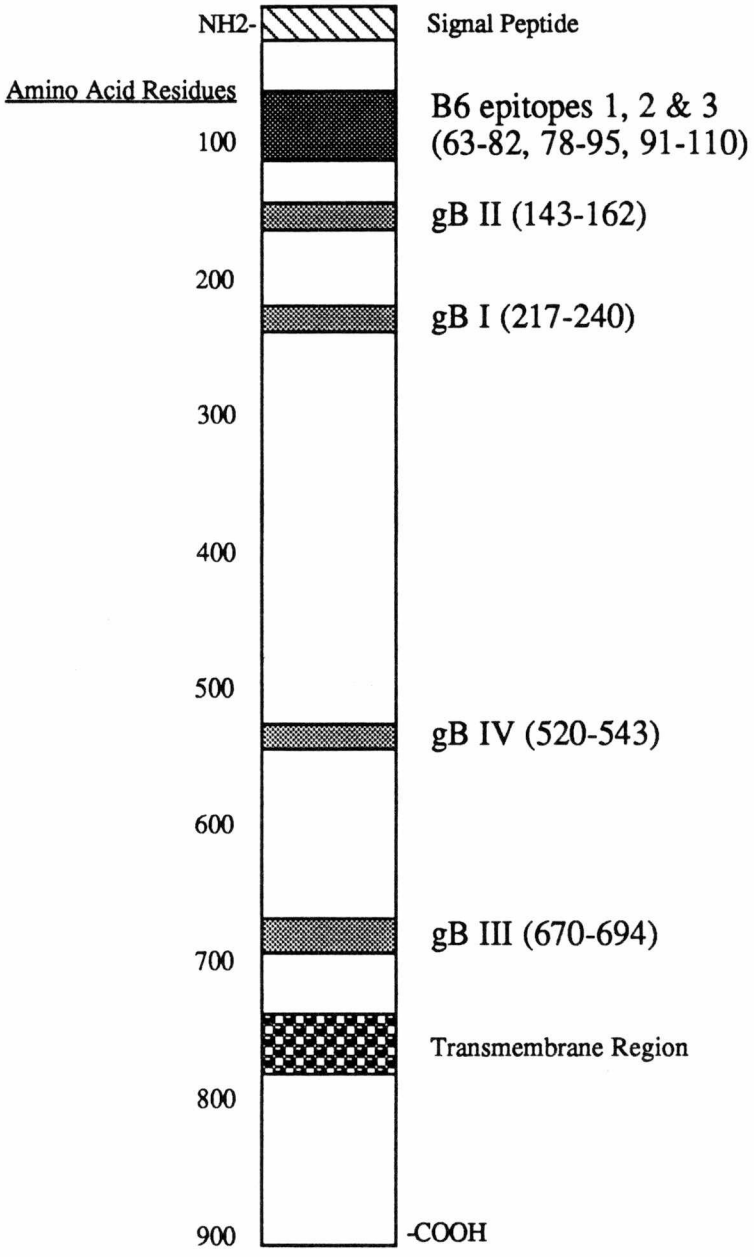


Figure 1 : HSV-1 Glycoprotein B Peptide Map.

Location of the gB peptides used in this study on HSV gB-1.

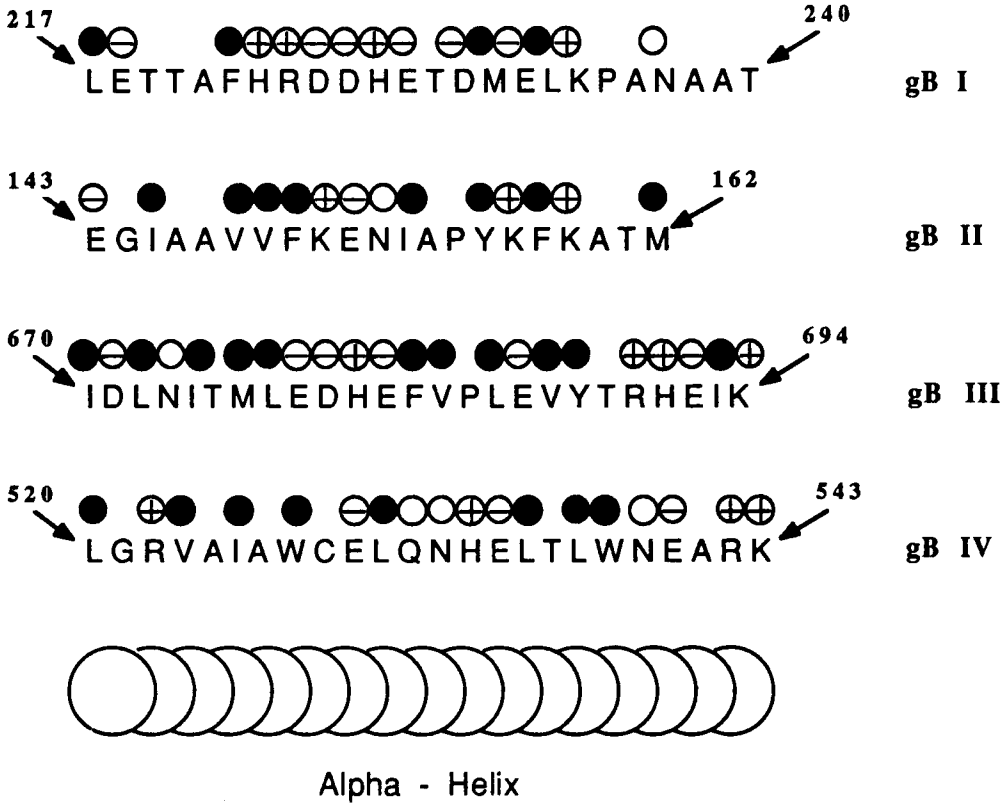


Figure 2 : Hydrophobicities & Secondary Structure : gB's I - IV.

Hydrophobic amino acids (as determined by Osmand's Universal Hydrophobicity Scale, p. 147) are represented as filled circles, and hydrophilic amino acids as open circles with or without a side chain charge. The numbers refer to the amino acid "address" of each of the peptides on HSV gB-1. All four peptides are predicted to correspond to alpha-helical regions of gB-1.

Table 1 : Osmand's Universal Hydrophobicity Scale.

A.A.	Code	Number	Designation	R-Charge
Phe	F	1.68	•	
Ile	I	1.42	•	
Leu	L	1.23	•	
Val	V	1.08	•	
Tyr	Y	1.04	•	
Met	M	0.89	•	
Trp	W	0.71	•	
Cys	C	0.25	-	
Ala	A	0.12	-	
Gly	G	-0.11	-	
Thr	T	-0.18	-	
Pro	P	-0.2	-	
Ser	S	-0.4	-	
His	H	-0.56	°	(+)
Asn	N	-0.91	°	
Gln	Q	-0.95	°	
Glu	E	-1.05	°	(-)
Lys	K	-1.17	°	(+)
Asp	D	-1.19	°	(-)
Arg	R	-1.68	°	(+)

The twenty amino acids (A.A.'s) arranged hydrophobically. Hydrophobic and hydrophilic residues were determined by standardized weighted averaging of the standardized scales of Sweet and Eisenberg, Kyte and Doolittle, and Nozeki and Tanford. Residues with a numerical rank above 0.5 (on a scale ranging from -1.7 to +1.7) were designated hydrophobic (•) and those with a rank below -0.5 were designated hydrophilic (o). The plus or minus signs denote side chain charge (if any).

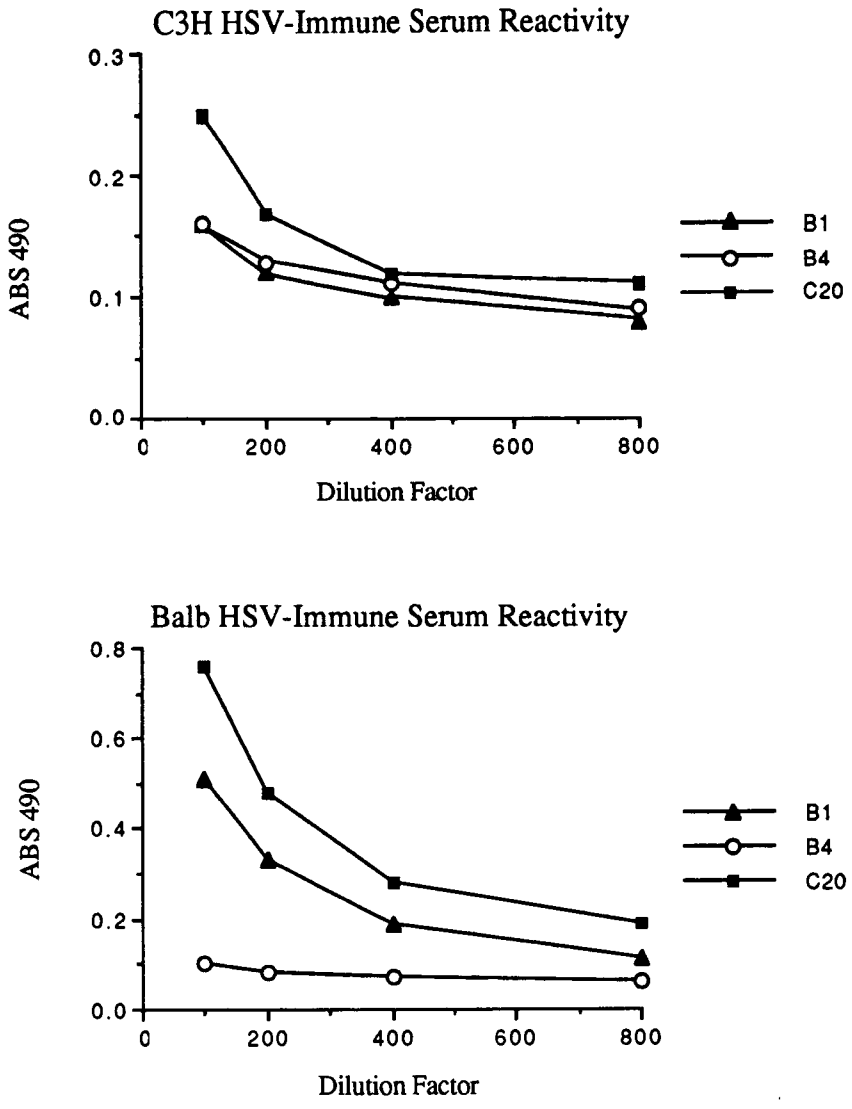


Figure 3 : ELISA Reactivity of HSV-Immune Sera With Peptides gB I & IV & gC20mer.

Serial two-fold dilutions of HSV polyclonal immune sera were screened via ELISA for gB I, gB IV, and gC20mer (as a control) peptide reactivity. The subtitle of each graph denotes the specific antiserum used. Standard errors of the sample means were less than 10%.

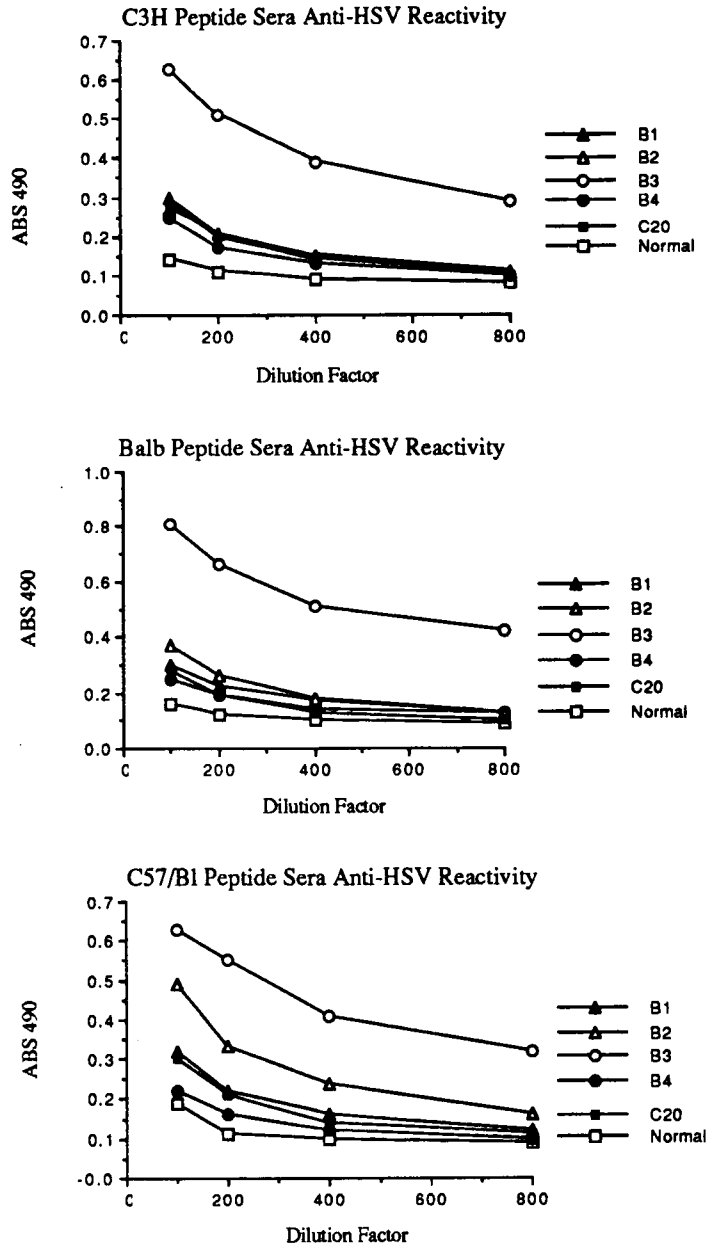


Figure 4 : ELISA Reactivity of Peptide-Immune Sera with HSV-1.

Serial two-fold dilutions of the sera generated by the peptides gB I-IV and gC20mer (as a control) were screened via ELISA for HSV reactivity. The subtitle of each graph denotes the specific antiserum used. Standard errors of the sample means were less than 10%.

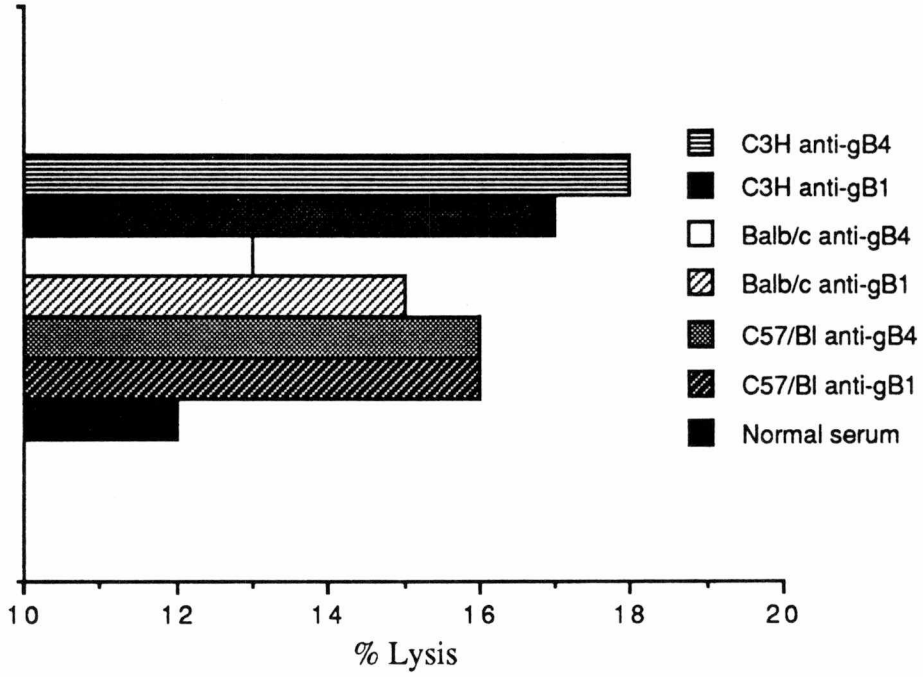


Figure 5 : ADCMC Mediated by gBI & gB IV Immune Sera.

Antibody-dependent complement-mediated lysis (ADCMC) of HSV-infected Vero cells mediated by gB I and gB IV immune sera. The results shown correspond to final serum dilutions of 1:40. Polyclonal HSV immune serum from C3H mice (at 1:40) gave 53% lysis, and 12% lysis occurred in wells containing complement alone or with normal C3H serum The standard errors of the sample means were 10% or less.

Table 2 : C3H & Balb/c Anti-gB I & gB IV Sera Zosteriform Protection.

Serum :	C3H Zosterification		Balb/c Zosterification	
	# (+)	# (-)	# (+)	# (-)
Adj. Control	4	0	-	-
HBSS Control	3	1	4	0
gB I	1	3	3	1
gB IV	4	0	3	1
pG-HSV	1	3	-	-
HSV 1	2	2	0	4

Protection against zosteriform spread of HSV-1_{BK} mediated by gB I and gB IV peptide immune sera generated in C3H and Balb/c mice. The first experiment utilized C3H sera and the second Balb/c sera, and the results are shown as the number of mice which demonstrated zosteriform spread. For both experiments, 300 µl of whole serum or the protein G (pG) isolated IgG fraction equivalent were injected intravenously into six week old naive C3H mice two hours before challenge. Negative control animals received 300 µl of serum from Freund's Adjuvant injected animals (Adj. control) or 300 µl of HBSS. Positive "protection" controls received polyclonal HSV-immune serum or 0.5 mgs of either of two glycoprotein D reactive monoclonal antibodies (MAbs D7 and D8) shown previously to be protective. For both experiments, the mice receiving MAbs D7 or D8 demonstrated 0% positive zosteriform spread.

VITA

Joseph Mester was born on January 1, 1962, in Lancaster Pennsylvania, where he attended Lancaster Catholic High School and Franklin and Marshall College. He received a BA degree in Biology from the University of Rochester, Rochester New York, in 1983. While at the University of Tennessee, he taught a variety of biology and microbiology courses, and collaborated on research projects with Ann Draughon in the UT Agriculture School's Department of Food Technology, and Maureen Warwick of the Biological Response Modifiers Division of Cetus Corporation (Emeryville California). His postdoctoral studies, involving genetic manipulation of herpes simplex virus to enhance its efficiency as an expression vector, will be performed at the University of Pittsburgh in the laboratory of Joseph Glorioso.