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Novel Vaccine Strategies to Prevent Herpes Simplex Virus Infection

Christopher D. Pack
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Christopher D. Pack entitled "Novel Vaccine Strategies to Prevent Herpes Simplex Virus Infection." I have examined the final electronic 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 Comparative and Experimental Medicine.

Barry T. Rouse, Major Professor

We have read this dissertation and recommend its acceptance:

Robert Moore, Stephen Kennel, Al Ichiki

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

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Stephen J. Kemp

Robert L. Tsai

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Vice Chancellor and Dean of
Graduate Studies

NOVEL VACCINE STRATEGIES TO PREVENT HERPES SIMPLEX VIRUS INFECTION

A Dissertation Presented for the

Doctor of Philosophy Degree

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Christopher D. Pack

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Abstract

Herpes simplex virus, which infects a majority of the population, remains a significant human pathogen for which no effective vaccine exists. Despite decades of intensive research, this virus has resisted numerous classical and cutting edge approaches. We can only hope that additional research into new vaccines ideas as well as the fundamentals of how the virus interacts with the immune system will someday lead to the design of an effective vaccine. The first part of this dissertation focuses on the virus and how it interacts with various immune cells in the body. HSV-1 exerts a number of influences on a number of key immune cells, especially dendritic cells. The overview goes on to fully describe these interactions, the nature of the immune response generated to the virus, as well as past and possible future vaccine strategies. The second part explores the use of hsp70 and peptide as an effective vaccine candidate in the neonate, in which infection can lead to life threatening situations. Results suggest that hsp70 is an effective adjuvant in neonates. The third section examines the use of hsp70 and peptide as a mucosal adjuvant in adult mice. These data also demonstrate that hsp70 can act as an potent mucosal adjuvant. The final section explores utilizing an anti-DNA antibody to enhance targeting of plasmid DNA to antigen presenting cells in order to improve immune responses. Results indicate that anti-DNA antibody enhances immune responses to plasmid encoded antigen. In all, we hope that these novel vaccine approaches may one day be used in humans to induce protective immune responses to HSV-1.

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Abbreviations

APC.....	Antigen presenting cell
CD.....	Cluster of differentiation
CFA.....	Complete Freund's adjuvant
CTL.....	Cytotoxic T lymphocyte
DC.....	Dendritic cell
ELISA.....	Enzyme linked immunosorbent assay
ELISPOT.....	Enzyme linked immunospot
gB.....	Glycoprotein B
Hsp70.....	Heat-shock protein 70
HSV-1.....	Herpes simplex virus-1
IFN-α.....	Interferon alpha
IFN-γ.....	Interferon gamma
IL-2, 4, 6, 7, 10, 12, 15	Interleukins 2, 4, 6, 7, 10, 12, 15
i.m.....	Intramuscular
i.n.....	Intranasal
i.p.....	Intraperitoneal
MHC Class I, II.....	Major histocompatibility complex class I, II
NK cell.....	Natural killer cell
NKT cell.....	Natural killer T cell
OVA.....	Ovalbumin

PFU……..	Plaque forming units
s.c……..	Subcutaneous
SFU……..	Spot forming units
Th……..	T helper
TNF–α……..	Tumor necrosis factor alpha
VV-gB……..	Vaccinia virus expressing glycoprotein B

Part I.

Background and Overview

Introduction

Herpes simplex virus types 1 and 2 both represent significant human pathogens that infect a majority of the population. They should be controllable by vaccines, but despite notable effort, satisfactory vaccines remain unavailable. One hopes that fundamental studies of both the virus and how it interacts with the host will uncover clues useful for the design of effective prophylactic and therapeutic vaccines. This review focuses on host responses to the virus revealed in both animal models and human patient studies.

Initial immune events and their consequences

Initial invasion by pathogens is met by numerous components of innate defense. These may serve to curtail infection, but this outcome is probably uncommon with HSV unless the infecting dose is minimal. The caveat of such failure is that virus can gain access to sensory nerve endings, is transported to sensory ganglia and establishes latency or perhaps persistence. These events occur well before effector T cells or antibodies are induced. Hence, if sterile immunity is to be achieved against primary infection it must rely solely on innate immune defenses. Many components participate in innate immunity, but their respective roles against HSV have received little attention. This may be because until recently we had few if any therapeutic options to manipulate them. However, there is now an expanding array of approaches that can activate aspects of innate immunity which could affect the course of infection and the pattern of immunity. Some of these have been tested against HSV infections in humans and animal models [1]. We also

realize that manipulating innate immune function may impact on the subsequent magnitude and quality of the adaptive immune response. This review discusses the major components of innate immunity that can be targeted perhaps for future modulation.

Upon entry into the host infectious virus is exposed to numerous innate defenses. These include several humoral molecules as well as a number of cellular defenses. Recently, most mechanistic studies have focused on cellular components of defense, especially dendritic cells (DC) and to a lesser extent natural killer (NK) cells, macrophages, and neutrophils [2-4]. However, humoral factors may well be pivotal and likely curtail infection with low doses. The humoral factors involved include complement factors, lactoferrin, natural antibody, and of course interferons. Most studies involve the latter and are discussed later. A few key studies have evaluated the role of complement, of which the most compelling are the observations of the Friedman group [5]. They showed that the major glycoprotein gC of HSV bound to C3b, an important molecule in the complement cascade. Additionly, gC binding to C3b prevented the otherwise destructive effects of complement activation. In support of this idea, gC minus mutants were only pathogenic in C3 depleted animals [5]. Nevertheless, whether or not variations in C3 levels, which can occur during certain chronic diseases, are associated with increased susceptibility to HSV infection is not clear. However, it is known that mice unable to make C3 are notably more susceptible to infection than control animals [6].

Natural antibody is another humoral innate component that acts as a hindrance to certain infections which include HSV [7]. This antibody either acts to neutralize and so prevent infection or perhaps mediate antibody dependent cell mediated cytotoxicity (ADCC) [8].

Support for a possible role for natural antibody mediated defense was reported by Deshpande et al [9]. They showed that $\mu M^{-/-}$ mice, which lack B cell-mediated immunity, were considerably more susceptible to HSV, but their resistance could be partially reconstituted by transfer of naïve serum containing natural antibody.

With regard to interferons, the story is complex as is the interferon system itself [10]. Herpes simplex virus infection does induce various interferon species and viral replication is inhibited by interferons. Plasmacytoid DC, upon exposure to HSV, produce an abundance of type I interferons, and this may be a major source of IFN-α [11]. Moreover, knockout mice lacking either the IFN-α or γ receptor are extremely susceptible to infection [12,13]. Interferons are further discussed in a later section.

Dendritic cells and HSV

Dendritic cells (DC) are one of the first immune cells to interact with pathogens, including HSV-1. These specialized antigen presenting cells (APC) play a critical role in acquiring antigen, processing it, and presenting foreign epitopes on MHC Class I and II molecules in order to efficiently prime T cells. In addition, they also must serve as "sentinels" of the body, determining what type of immune response, if any, is needed to combat a potential pathogen. These "danger" signals are primarily orchestrated through Toll-like receptors, which possess the ability to recognize pathogen-associated molecular patterns. Examples of such patterns (and the TLR that they bind) include peptidoglycan (TLR2), dsRNA (TLR3), LPS (TLR4), flagellin (TLR5), ssRNA (TLR7), and CpG methylated dsDNA (TLR9) [14]. Recent studies have emphasized that herpes viruses may provide ligands for certain TLRs and implied that interaction of such ligands with

TLRs on DC and perhaps other cells represents a pivotal event in viral pathogenesis. However, most observations have been made in vitro with purified and likely unphysiological concentrations of ligands. Studies have shown that gB protein of human CMV binds TLR2 [15], as does an unknown protein of HSV [16]. HSV DNA is also known to bind TLR9 [17], although whether or not this is a significant event in vivo remains in doubt. Recently, plasmacytoid DC were shown in vitro to react to HSV-2 DNA and secrete IFN-α, but in vivo studies were not performed [18]. Similarly, HSV-1 DNA stimulated plasmacytoid DC to produce IFN-α upon exposure to HSV-1 via TLR9, but, surprisingly, the majority of IFN-α came from other cell types, such as macrophages, in a TLR9 independent manner [17].

The outcome of initial interaction between viruses and DC influences the nature, magnitude, and quality of the adaptive immune response. If DC are impaired in their ability to mature or properly prime T cells, a chronic infection or death of the host may ensue for some viruses [19]. We do not know, however, if HSV interaction with DC represents a pivotal event, particularly in vivo. Some have suggested that HSV infection impairs DC function by preventing maturation, thus serving to retard or even prevent immune induction [20]. The Lanzavecchia group also showed that infected DCs failed to upregulate costimulatory molecules, produce cytokines, and were not responsive to chemokines, a process crucial for migration to secondary lymphoid organs. Similarly, mature DCs infected in vitro with HSV-1 changed both their phenotype and their ability to stimulate T cells [21]. However, these in vitro data may have been overinterpreted, since DC themselves seem not to be a major target for HSV infection in vivo, and in the mouse may avoid direct infection [22]. Nevertheless, the type of interaction made

between HSV and DC may impact on the quality of the adaptive immune response made, but if and how this occurs in vivo needs to be proven.

Some recent studies in the mouse have focused on DC, and shed light on early events in T cell immune induction. For example, it was assumed that Langerhans cells, a predominant sub-type of DC in the skin, are one of the first cells to contact HSV-1. This is followed by transport of virus by a now mature DC to the draining lymph node where T cell priming occurs [2]. However, the Carbone group, using footpad infection of mice, demonstrated that neither Langerhans cells ($CD11c^{+}CD205^{high}CD8\alpha^{low}$) nor plasmacytoid DC ($CD11b^{+}CD11c^{+}CD8\alpha^{low}$ were responsible for priming of $CD8^{+}$ T cells. Instead, a different and uncommon DC sub-population was responsible, the $CD11c^{+}CD8\alpha^{+}$ DC [23,24]. It appears that these cells may not be directly infected by HSV, but instead acquire viral antigens from other cell types by a process referred to as cross-priming [25]. Cross-priming currently enjoys popularity among mouse immunologists, but whether or not the process is a major event in human T cell immune induction remains to be established. Moreover, whereas $CD8^{+}$ DC may be the most effective T cell priming cells, it is likely that other cell types, especially abundant ones, can also participate. The situation could also be different at mucosal entry sites from that reported in the skin. For instance, the Iwasaki lab reported that vaginal submucosal $CD11b^{+}CD8\alpha^{-}$ DC were responsible for the generation of protective Th1 responses to HSV-2 infection [26]. Intravaginal infection led to rapid recruitment of these submucosal DC to the infected epithelium. Careful analysis of various DC populations from the draining lymph nodes revealed that only the $CD11b^{+}$ submucosal DCs, but not Langerhans cell-derived or

$CD8\alpha^+$ DCs, presented viral antigens to $CD4^+$ T cells and induced IFN-γ secretion, an unanticipated role for this DC subset [26] . Moreover, no evidence of direct infection of the DC could be found [22].

In contrast, a recent study from the Katz group indicated that human myeloid DC in the skin do become infected by HSV [27]. Curiously, this was followed by both infected and uninfected cells maturing and presumably migrating to the draining lymph node. It also seemed that the viral component from infected cells responsible for the bystander activation of uninfected DC was gD. This also resulted in the release of type I interferon. They suggested that the stage is set for a race between the maturation and priming capacity of the DC and the ability of HSV to disrupt such events [27].

We may conclude that one or more subsets of DC do indeed play an important role in immunity to HSV. DC seem responsible for mediating some innate defense but are perhaps even more important for the influence they exert on the subsequent adaptive immune response. Manipulating the function of DC does represent a logical target for vaccine design. Some initial studies have shown promise in this regard. Thus, the Spraunce group and others demonstrated that the TLR 7/8 agonists imiquimod and resiquimod showed some efficacy against the severity and frequency of herpetic disease in humans [28-30]. In mice as well, manipulating DC function with TLR ligands was shown to raise the barrier to infection. Thus, at least two groups have shown that the mucosal delivery of CpG motifs provides temporary protection against vaginal challenge with HSV [31,32]. These TLR9 activating compounds may also act as excellent adjuvants permitting mice at least to respond and make $CD8^+$ T cell responses to peptide antigens [33].

Assuming HSV under in vivo conditions does indeed infect DC, there are additional effects which the virus can exert on DC function. These relate to effects on the antigen-presenting machinery, the expression of costimulatory molecules involved in antigen presentation, as well as the production of immunomodulatory cytokines [34]. Indeed, the DC has become almost over-exposed by its avid followers. Comprehensive reviews on DC in relation to their influence on infectious disease have been published and should be consulted for the full story [2,34].

Role of NK and NKT cells in HSV infection

These cell types are both considered to participate in the initial response to an infection. They can both act to recognize infected cells and kill them or release numerous cytokines that participate directly or indirectly in immune defense [3]. We have known since the pioneering studies of Lopez that NK cells influence susceptibility to HSV infection [35]. Most notably, susceptibility to severe herpes infection occurs in humans with genetic defects in the NK cell response [36], and in mice genetic susceptibility differences may be explained by NK cell functional differences [37], but details of this genetic control remain confused [38]. We know that the NK cell response, and perhaps also NKT cells, helps prevent initial infection, but for HSV it is not clear if the virus-NK cell interaction affects the pattern of subsequent adaptive immune response, as it clearly does in mouse CMV infection [39]. It has become quite evident that the NK cell system is diverse and that several phenotypic and some functional subsets exist [40]. The basis of the phenotypic diversity is explained by the multiple receptors expressed on NK cells, some of which are expressed on all NK cells and others limited to different subsets [40].

The NK cell receptors fall into two major functional groups: inhibitory receptors and activating. The inhibitory receptors mainly recognize MHC proteins, and when such ligands are diminished, such as can occur on a virus infected or tumor cell, the inferior signal stimulation results in NK cell activation [40,41]. This "missing self" hypothesis could explain why HSV infected cells, which do down regulate MHC molecules, result in NK cell activation [42]. NK cells additionally express many activating receptors although little is currently known about the ligands for such receptors. One exciting story, however, involves the herpesvirus MCMV. Here it was shown that the CMV M157 protein bound to the activating receptor Ly-49H and mice strains encoding Ly-49H were resistant to infection [43]. It is not known if other herpesviruses such as HSV encode proteins that engage NK activating receptors, but doubtless the search for such molecules is ongoing. Conceivably, HSV could also interact with NK cells by acting as a ligand for TLR2 or TLR9, both of which are present on NK cells [44]. Thus, HSV is reported to present ligands for both TLRs [16,18].

It seems likely that the major function that NK cells play during the initial response is to act as a source of cytokines that impact on antiviral defense and immune induction. Thus, NK cells act as a major source of IFN-γ and a minor source of IL-12, and abundant production of each are an early event in HSV infection [45]. The release of IFN-γ activates other cells, such as macrophages, and possesses potent antiviral properties. IL-12 is a critical cytokine involved in shaping adaptive immunity, tipping the response to Th1 [46]. NK cells express other molecules involved in antiviral defense and immune induction, but they do not directly participate in antigen presentation.

Although the relevance of NK cells in HSV immunobiology has been somewhat neglected, recent studies addressed the contribution of NKT cells. These cells share identity markers (NK1.1+) and function much like NK cells, but they additionally express a highly limited T cell receptor repertoire. Most cells in the mouse recognize the non-peptide antigen α-galactosylceramide presented by the nonclassical MHC molecule CD1d. Mice deficient in CD1d have few if any NKT cells and show delayed clearance and increased mortality upon infection with HSV [47]. Other data also indicate that NKT cells contribute to defense against HSV. Rosenthal showed that mice deficient in IL-15, a critical factor for NKT cells, were highly susceptible to infection [48].

We can conclude that NK cells are important cells in HSV immunity but it is not yet evident how we can influence the function of these cells with ligands that target them. However, we anticipate that the topic will be receiving attention in the future, likely coming primarily from observations in the MCMV field.

Neutrophils and Macrophages in HSV infection

Both these cell types are prominent early participants in inflammatory reactions to HSV infection [49]. Older studies focused heavily on macrophages and showed that depleting such cells or impeding their function enhanced susceptibility to infection [50]. The macrophage has become a somewhat neglected cell in immunology and many of them have been renamed as a subset of DC. In the mouse, if not in man, stimulated macrophages can exert powerful antiviral effects by producing reactive radicals such as NO [51]. Accordingly, iNOS knockout mice showed enhanced susceptibility to infection with HSV-1 [52].

Neutrophils also exert antiviral innate defense, and several groups have shown this in mouse systems [4,53]. Accordingly, if neutrophils are depleted by specific antibody mice show significantly enhanced susceptibility to infection [54,55]. How neutrophils exert their defense is not fully understood, but reactive radical production and the secretion of antiviral cytokines are suspected mechanisms.

The role of adaptive immunity

Observations in humans and studies in mouse model systems leave little doubt that acquired immunity is crucial to contain infection [56]. Thus, as mentioned, innate immunity can handicap the chance of infection, but if it proves unsuccessful removing the virus becomes primarily the task of the adaptive immune response. With regard to resolving primary infection, most would agree that T cells are crucial for this to occur. In fact, T cell defects, either acquired (as occurs in AIDS), congenital, or constructed (as in some mouse models), result in prolonged and severe infections [57]. In T cell deficient mice the outcome is usually lethal encephalitis [58]. The role of antibody in the control of primary infection is usually considered as minor, especially since this response occurs later in comparison to T cell induction and antibody-mediated immunity is largely directed at extracellular virus. However, older studies did emphasize that perhaps ADCC was an important participant in anti-viral defense [59]. More recently, however, the Friedman group has questioned this, since HSV, via gE and gI proteins (which act as viral Fcγ receptors), seems to have an evasive maneuver against antibody-mediated immunity [5].

Antibody, however, is an important component to prevent infection. This was best illustrated in humans by the observation that vaginally delivered infants exposed to HSV-2 were protected if they received maternal antibody [60]. Furthermore, there is good evidence that the presence of type common antibody induced by HSV-2 protects against HSV-1 infection, although the reverse situation seems not to have a prominent effect [61]. It is also well known that once infected with an HSV-1 strain, patients generally do not become infected with additional strains of the same type. This situation is most likely mediated by antibody. Protection of susceptible mucosal surfaces from reinfection appears to be performed most efficiently by antibody as was shown by mouse studies [62,63]. However, mucosal memory tends to be of less duration than systemic, so mucosal surfaces tend to lose antibody –mediated protection at a faster rate in comparison to systemic immunity [62]. Nevertheless, also in support of a role for antibody is the evidence that the currently used GlaxoSmithKline (GSK) vaccine seems to protect females from infection, but not males [64]. Any vaccine designed to protect against HSV must be capable of inducing neutralizing antibody, preferably including mucosal antibody.

Most of the focus on immunity mechanisms against HSV has been directed at T cell immunity. Since it has considerable implication vaccine design, effort over the years has gone into defining what aspects of T cell immunity dominate antiviral defense and how protection proceeds mechanistically. Several studies have been done in mice, a mixed blessing since the mouse can often convey a misleading message, particularly since this non-mouse pathogen shows several differences in pathogenesis. Mice are more likely to suffer from herpetic encephalitis upon infection, and curiously almost any form

of immunization can be contrived to protect them. In contrast, humans, fortunately, seldom succumb to encephalitis, but so far no vaccine seems able to protect them. Our understanding of human adaptive immunity to HSV comes largely from groups led by Corey in Seattle and Cunningham in Sydney. Both have written recent valuable reviews which cover the topic more comprehensively than the following summary saga [61,65].

Primary infection by HSV, if not successfully dampened by innate defenses, becomes clinical with lesions persisting up to 3 weeks and viral shedding lasting for 10 days or so. During this time an adaptive immune response is being generated, but it may be retarded by HSV infecting DC, making such cells inferior APC [65]. Lesion cessation appears to be a consequence of both $CD4^+$ and $CD8^+$ T cell function. In the recurrent situation, which is better investigated since it is easier to study, things happen more quickly. Lesions last up to 10 days and viral shedding for around 4 days [66]. The issue has been why clinical lesions occur at all, since, invariably, latently infected patients retain circulating antibody and have memory $CD4^+$ and $CD8^+$ T cell immunity. Moreover, the extent of these immune parameters do not show significant differences in magnitude nor pattern from latently infected patients who never suffer recurrences [66]. However, when following the sequence of immunological events studied by serial biopsies of recurrent lesions a pattern emerges. It seems that $CD4^+$ T cells, along with neutralizing antibody through leaky vessels, are the first to invade [56]. $CD4^+$ T cells can be induced to produce an abundance of IFN-γ and other cytokines.

$CD8^+$ T cells invade later, but their arrival seems to coincide with infection control [56]. This has given rise to the notion that perhaps $CD8^+$ T cells are principally responsible for resolution, an idea supported by the observation that the severity of

genital herpes in AIDS patients seemed to correlate best with the magnitude of the $CD8^+$ T cell response [67]. However, it is by no means certain how the $CD8^+$ T cells subserve their protective function. Thus, HSV infection of cells may cause them to downregulate MHC restricting molecules by a variety of mechanisms [42]. Moreover, the Jerome laboratory just described another hazard for $CD8^+$ T cell-mediated immunity. They showed that human HSV infected cells somehow deliver an off function signal to $CD8^+$ CTL when they come in contact [68]. Hence, the $CD8^+$ T cells theoretically should not be able to kill the virus infected epithelial cells. Presumably this negative interaction is counteracted by other molecules in the milieu, most notably IFN-γ. The latter is present in high concentration in fever blisters as are a number of other cytokines and chemokines. Most notably these include TNF-α, other interferons, and IL-12. These molecules help orchestrate the inflammatory response with its arsenal of antiviral defenders. Amongst the protective molecules antibody must also be considered since this can neutralize extracellular virus even if retarded by the mechanisms described by the Friedman group [69].

Defense against HSV is usually advocated to largely result from the action of cytotoxic $CD8^+$ and $CD4^+$ T cells and the products of Th1 $CD4^+$ T cells [61,65]. However, formal proof for this opinion is lacking. Mouse studies clearly show that IFN-γ and perhaps TNF-α play a major role in antiviral defense [70]. Curiously, IFN-γ may be directed at infected cells and cause them to purge themselves of viral products. Some elegant studies from the Hendrick's lab showed that antigen-specific $CD8^+$ T cells in the infected mouse trigeminal ganglion appeared to purge cells of HSV and prevent

productive infection [71]. This process was even advocated as the true means by which HSV sustains latency- a hard pill to swallow for the countless souls who have worked on molecular mechanisms of HSV latency!

What the mouse tells us about HSV infection

Many groups have evaluated the role of the adaptive immune components against HSV in the mouse. Such studies, more or less confirm many of the concepts that have emerged from human investigations, but some discrepancies do exist. The biggest problem with mouse studies is that almost all of them use primary infection models, while the main problem in humans is recrudescence. Moreover, virus can readily spread to the CNS and cause lethal encephalitis in the mouse. Protection against such encephalitis appears to be the primary domain of $CD8^+$ T cell mediated immunity [72]. This was shown more than twenty years ago and studies done since that time have merely confirmed and extended these initial observations. A problem has always been to explain how $CD8^+$ T cells act to protect the CNS, since neurons have little or no MHC class I molecules and HSV infection is supposed to downregulate whatever they have [73]. . However, adoptive transfer experiments comparing the protective efficacy of $CD8^+$ T cells with other cell types along with observations in models which $CD8^+$ T cell function was compromised all point to them as neuroprotectors [74,75].

It might be that the neuroprotection proceeds other than by cytotoxicity, certainly an unwanted effect on neurons since such cells are not replaced. Thus, as mentioned previously, the Hendricks group demonstrated that in the mouse PNS, neuron protection

appeared to be mediated by IFN-γ secreted from HSV immune $CD8^+$ T cells [76]. The IFN-γ was proposed to purge the neurons of viral products, a concept first described by Frank Chisari and colleagues for the protective effects of CD8+ T cells against Hepatitis B infected liver cells [77].

Control of infection at peripheral sites in the mouse can be accomplished by both $CD4^+$ and $CD8^+$ T cells as well as by antibody. On a cell to cell basis $CD4^+$ T cells appear to be more effective, but this may vary between mouse strains [78]. Moreover, the protective effects of both $CD4^+$ and $CD8^+$ T cells appears to depend on the IFN-γ response [79]. In accord with this, STAT1 $^{-/-}$ and IFN-γ receptor $^{-/-}$ mice are more susceptible to infection, as are IFN-γ $^{-/-}$ mice [12,80]. Curiously, the effect of IFN-γ may act via NO induction, primarily released by IFN-γ activated macrophages [50].

Another aspect of HSV immunity that the mouse has helped us understand is how various aspects of immunity are induced and regulated. Focus has been directed at a variety of dendritic cells and their role as APC as mentioned previously. Surprisingly, of the large number of DC subtypes, $CD8^+$ lymphoid DC appear to be the most successful at inducing T cell immunity [23,24]. Moreover, such DC appear to receive their antigen from other cell types by a process termed cross-priming [25]. Currently, it is not clear if cross-priming forms part of a significant immune induction event in humans.

The other series of events which the mouse has helped elucidate is the influence of regulatory cells on immune induction and effector function. Suvas et al recently demonstrated that both the magnitude and function of $CD4^+$ and $CD8^+$ T cell responses following either infection or vaccination was hampered by a concomitant regulatory T

cell response [81]. Curiously, mice were more protected from HSV challenge if their Treg response was abrogated. Subsequent studies also showed that recall vaccination responses as well as humoral responses at mucosal sites were similarly compromised by the Treg response [82]. The relevance of T regulation to HSV pathogenesis in the mouse as well as in human systems continues to be investigated. One might wonder why such a process exists since it hampers the efficacy of host defense against the virus. The answer to this seems to lie in the fact that Tregs also serve a beneficial role and act to minimize collateral tissue damage associated with the effector function of T cell mediated immunity [83]. Such concepts were recently reviewed [84]. It could be that vaccines that fail to activate the Treg response along with effector function might be more efficacious. Such issues are under investigation.

The memory T cell response to HSV: Why is it unable to prevent reactivation?

A major issue for vaccinology is how to maximally induce and retain memory responses to pathogens so that re-infection, or in the case of HSV, reactivation, is kept at a sub-clinical level. Recently, a number of fundamental studies have established ground rules governing the generation and maintenance of $CD8^+$ T cell memory [85,86]. These studies were performed primarily using acute infection models (LCMV) and model antigen systems (ovalbumin). Far less is known about $CD4^+$ T cell memory, although it appears that this component is usually reduced in magnitude and less sustained than $CD8^+$ T cell memory. Moreover, alteration in the functional subsets of $CD4^+$ Th memory

cells has been observed over time in some chronic viral infections. It appears that Th cells lose the ability to produce critical cytokines over the course of the disease, starting with a decline in IL-2 and then followed with reduced levels of TNF-α. Surprisingly, IFN-γ levels appear to remain constant [87].

The focus of memory studies has been on $CD8^+$ T cell memory, a component that may be most critical for clearance of HSV infection, at least in the natural host. In consequence, the application of a fundamental understanding of $CD8^+$ T cell memory will likely have application to vaccine design, especially the long sought for therapeutic vaccines. It is evident that short-term stimulation of naïve antigen-specific $CD8^+$ T cells is sufficient to trigger a program of proliferation and differentiation into effector cells that can react directly against antigen expressing cells [88]. These cells are generally both cytotoxic and able to generate a range of cytokines critical for antiviral control, especially IFN-γ. After the peak of the effector response, which occurs at approximately 1 week after infection, the vast majority of these effectors die of apoptosis. A small percentage of this population, about 5-10%, survive the contraction phase (days 10-15) and go on to form the long-lived memory pool. These cells produce elevated levels of anti-apoptotic proteins, such as bcl-2, and express (or re-express), important receptors for survival, such as IL-7 receptor [86]. It has been noted that cells which are destined for memory also express the homodimeric CD8αα molecule on their surface, but the role for this surface protein is not clear at present [89]. Some of these cells remain in the lymphoid tissues (so-called central memory cells), due to re-expression of CCR7. However, the effector memory subset loses CCR7 on their surface and migrates to peripheral sites, retaining the

capacity to make cytokines and kill infected cells upon contact. These effector memory cells then patrol the peripheral tissues indefinitely.

The central memory cells remain more or less static in number over prolonged periods, but the small fraction that do die are replaced by a slow turnover of such cells. This so-called homeostatic division is not driven by antigen, but is believed to be a response to endogenous cytokines such as IL-15 and IL-7 [90]. Antigen re-exposure results in a rapid recall of the central memory cells into proliferation and differentiation into effector cells. Such cells also tend to move into tissues, the usual site of new antigen introduction. The details of such events are thought to differ depending on the different types of infection and perhaps other factors. For instance, in an infection model of LCMV Clone 13, which results in a persistent viral infection, functional exhaustion of at least some antigen specificities appears to occur. It appears that constant availability of antigen tends to prevent the development of a stable central effector memory pool [86]. A similar event may exist in HSV infection in the sensory ganglia, but the details of events in the memory response to HSV infection have yet to be worked out.

Curiously, whereas the initial expansion and effector response by $CD8^+$ T cells appears to occur in response to a variety of antigen preparations and can occur without signals from other lymphocytes, the long-term memory response appears to require signals from other helper T cells at the time of immune induction [91]. It was shown, for example, that when $CD4^+$ helper T cells are absent or not stimulated during the time of $CD8^+$ induction, a defective long-term memory response ensues [92-94]. In the case of murine responses to HSV, we observed that antigen formulations that engage naïve $CD8^+$ specific T cells, but not antigen specific $CD4^+$ T helper cells, resulted in normal acute

$CD8^+$ acute responses of the $CD8^+$ T cells, but a poor memory response [95]. In addition to the size of the memory pool, the function of these cells was also diminished. Upon antigen re-exposure the recalled population demonstrated lower overall avidity in comparison to $CD8^+$ T cells that had received help during priming [95]. Currently, the actual mechanism by which helper T cells communicate signals resulting in optimal memory responses is not fully understood. One idea, however, is that signals from the helper T cells influence the maturation status and function of the DC that is presenting antigen. Most likely this "signal" is linked to CD40 on the DC binding to CD40L present on $CD4^+$ T cells. This interaction, termed licensing by the Matzinger group, results in a number of effects on DC, including enhanced costimulatory molecule expression (some of which may be critical for memory development) as well as the secretion of key cytokines such as IL-12 [96].

There seems to be general agreement that helper cell costimulation is generally required for optimal memory $CD8^+$ T cell responses, although with some agents the pathogens themselves promote a range of nonspecific signals, primarily via the strong stimulation of various TLRs that may substitute for helper effects. Less is known about the necessity for helper cell stimulation during other phases of the $CD8^+$ T cell response profile, such as the contraction, maintenance, and recall phases. Furthermore, it is important to understand if in conditions of defective $CD8^+$ T cell memory some form of rescue allowing normal function can be achieved. Thus in HSV immunity, it could be that those subject to frequent or severe recurrences may have some deficiency in central or tissue residing effector memory $CD8^+$ T cells. In mouse systems with a defective $CD8^+$ T cell memory response we have achieved some level of rescue in various ways. Toka et al

co-administered IL-15 plasmid DNA at the time of priming with plasmid DNA encoding gB as well as during the memory phase. This treatment led to enhanced memory pool numbers as well as increased functional capacity of the responding $CD8^+$ T cells [97]. Efforts might also be directed at increasing IL-7 receptor levels on either effector cells of existing memory cells in order to enhance or maintain adequate memory responses.

It will be important to develop and test vaccines in humans that express IL-15 or other such molecules that may serve to "top up" the efficacy of $CD8^+$ T cell memory responses against HSV. Other forms of activation may also be successful including immunization with persistent cross-reacting vehicles, use of TLR agonists, and possibly the use of cytokine activators.

The immune response to HSV and immunopathology

Effector T cell recognition of virus infected or viral antigen expressing cells can result in target cell destruction, but usually an inflammatory reaction is also elicited that causes collateral tissue damage. This outcome, which is usually set off by $CD4^+$ effector T cells recognizing their cognate antigens, is mediated in large part by inflammatory cytokines [98]. Such inflammatory reactions of varying intensity invariably occur at tissue sites of virus infection. Although often painful and tissue damaging, they are a small price to pay for control of infection. Moreover, the tissue at most sites is usually repaired and thus functions normally. Alas, there are exceptions to this generality. At some sites inflammatory reactions can cause profound and often permanent damage to function. Sites such as the eye and brain represent examples. With respect to HSV, we understand best the consequences of chronic inflammatory reactions in the eye [98,99].

Thus, HSV infection commonly affects the eye, but typically only the epithelium is affected. The painful event resolves, especially if treated with antiviral drugs. However, in about 20% of cases the corneal stroma becomes involved and a chronic inflammatory reaction occurs whose management requires long-term treatment with powerful anti-inflammatory drugs. This stromal keratitis lesion (SK) is mainly a $CD4^+$ T cell-mediated immunopathological response to virus. In support of this, $CD4^+$ T cells with antiviral specificity can be recovered from corneal buttons long after primary infection [100].

Understanding of the details of SK pathogenesis comes mainly from experimental infections in mice models. In this model, SK results from primary ocular infection with HSV and, as in humans, lesions are immunopathological and orchestrated principally by $CD4^+$ T cells [98,101]. Multiple other events are involved that include additional cell types, cytokines, chemokines, angiogenic factors, and stress proteins. The pathogenesis of SK has been reviewed elsewhere [98,102]. Curiously, the virus which initiates the lesion is only briefly present in the eye and then usually not as infectious virions in the inflamed stromal tissues. Despite this, the lesion is usually progressive and certainly increases in severity for some time after virus has been controlled by the immune response. This observation, which parallels what usually occurs in human SK, supports the notion that the lesion may be an immunopathogical reaction against viral antigens, but subsequently becomes sustained by an autoinflammatory process [102].

An additional site where HSV sets off an immunopathological reaction is in the sensory ganglia where HSV sets up latency. In the mouse, and perhaps in humans too, a long-term inflammatory reaction in the sensory ganglia occurs after virus is transmitted from peripheral sites of infection [71]. This reaction is dominated by $CD8^+$ T cells, many

of which, at least in the C57BL/6 mouse, are viral antigen-specific. Some suggest that these $CD8^+$ T cells somehow repress virus in neurons from replicating abundantly, which would result in the destruction of these irreplaceable cells [103]. This process, which has been called viral purging, may be independent of true HSV latency [71]. This fascinating topic was recently reviewed [103].

Finally, HSV is an occasional and usually tragic cause of encephalitis. Most would consider the human syndrome to largely represent a viral-driven inflammatory event with little or no role for immunopathology [57]. However, recent studies in a mouse model have indicated that HSV-induced encephalitis may represent largely an immunopathogical reaction reminiscent of the situation in the cornea [16]. The brain, like the eye, cannot sustain long-term immunopathological reactions without compromising its function.

HSV vaccines

HSV 1 and 2 stand out as two of mankind's more prevalent and significant viral pathogens that so far have not been satisfactorily controlled by vaccines. It is not for lack of trying, and many candidate preparations have been put on the market place. Many, in fact, have appeared efficacious when tested by their developer, but none have stood up to long term evaluation by disinterested parties. Currently, a vaccine is in the final phases of testing and may soon be available in the USA. This GSK vaccine is an adjuvanted protein vaccine that upon initial testing by its developers was shown to provide significant protection of seropositive women against HSV-2 infection from their seropositive partners [64]. The obverse situation provided no such protection. This GSK

vaccine appears to be showing protection even though the approach differs little from a previous vaccine discarded because of discouraging results. However, the adjuvant being used for the apparently successful GSK vaccine (alum and deacylated monophoshorylipid A) is different and may be able to better emphasize the $CD4^+$ type one response pattern considered important for protection [64]. Another possibility is that the vaccine may be conferring some protection to women since it induces a good protective antibody response in the genital tract. These issues require investigation as does the reasons for the female-only protection.

We would, from our previous discussions, speculate that vaccines need to that additionally induce notable $CD8^+$ T cell responses to be optimally protective. Although not formally tested, the GSK vaccine is unlikely to provide epitopes for all MHC types and the adjuvant may not represent an ideal one to induce $CD8^+$ T cell responses. The induction of optimal HSV specific CD8 T cell response will likely to require different technology than the GSK vaccine approach. For example, attenuated viral vaccines would be more likely to induce $CD8^+$ T cells. Such vaccines have been tested in the past and none have been adopted for widespread use. One candidate looked particularly appealing at least from an armchair perspective. This was the discontinuous replicating vaccine (DISC) that was a glycoprotein H deletion mutant that, except in complementary cells, would only undergo a single cycle of replication [104]. This DISC vaccine did well in animal testing and had at least a phase II trial in humans. Its discard by the industry probably means it is not sufficiently immunogenic or too complex to develop commercially.

HSV vaccinologists are left therefore in a state of frustration since currently there is no ideal solution on the horizon. It means that more fundamental research on HSV immunobiology is required in model systems but more particularly in humans themselves. We emphasize the latter since animal models tend to be the bearers of good news at least when it comes to prophylactic vaccine candidates. Were we rodents, in fact, we would already have a pharmacy full of valuable vaccines. Thus killed vaccines, attenuated mutants, recombinant vaccines of numerous types, DNA vaccines, adjuvanted proteins, and peptides may all provide useful immunity, at least when used prophylactically. Alas we are primates, but fortunately with brains and imagination. The challenge remains to use our creative skills and overcome the vaccine road block. New clues from those optimists who think outside the box are encouraged to take up the challenge.

Conclusions

Additional work is clearly needed to further understand how HSV accommodates itself so well to the perils of both innate and adaptive immunity. We need to devise prophylactic vaccines which, even if inadequate to preclude infection, can dampen such to subclinical levels. These future vaccines will likely need to take advantage of our ever expanding knowledge of adjuvants and the mechanisms by which they shape and enhance defenses. We anticipate that successful prophylactic vaccines may be only just over the horizon. Therapeutic vaccines, which in the Western world may provide more rewards to the marketplace, could prove more problematic to develop. We shall be challenged to improve upon nature's vaccine since the virus itself, forever resident in those subject to

recurrent disease, appears unable to confer adequate protective immunity. Modern medicine has solved many of mankind's problems. One hopes therapeutic anti-HSV vaccines become added to the list.

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Part II.

Heat-Shock Protein 70 Acts as an Effective Adjuvant in Neonatal Mice and Confers Protection Against Challenge With Herpes Simplex Virus

Abstract

Immunization of the neonate is a highly desirable goal for vaccine developers, since the neonate is profoundly susceptible to a number of viral and bacterial pathogens. The neonatal immune system tends to generate Th2 recall responses, known as neonatal tolerance, which may not protect against viral challenge later in life. In this study we demonstrate that a potent immune proinflammatory stimulator, heat-shock protein 70 (hsp70), can act as an effective and safe adjuvant in neonates. Priming of neonates with hsp70 coupled to a viral MHC Class I-restricted epitope (gB498-505) and injection with recombinant gB generated strong cytotoxic T lymphocyte (CTL) responses and a Th1 primary T helper cell response during the neonatal period. In addition, enhanced CTL and predominant Th1 recall responses to viral antigens were observed following secondary challenge as adults. These responses were sufficient to allow protection against a lethal challenge with Herpes Simplex Virus Type-1 (HSV-1). Therefore, hsp70 in conjunction with viral epitopes and recombinant viral protein can perhaps prime protective immune responses to herpes viruses early in life when infection, which can be life-threatening, and the establishment of latency frequently occur.

Introduction

Exposure to antigens in utero or shortly after birth usually leads to antigen-specific tolerance, but exceptions to this rule do exist and vaccination is possible. Antigenic exposure during the neonatal period is believed to lead to the development of Th2 responses [1,2], rather than Th1 responses needed to prevent or control viral infections

such as Herpes Simplex Virus-1 (HSV-1) that frequently occur during this time. The Matzinger group suggested that the neonatal system, though not fully mature, may be able to respond if antigens were presented by adult dendritic cells (DC), suggesting that neonatal DC may be handicapped in their ability to prime Th1 responses [3]. In addition, other requirements for an effective response in the neonate are strong stimulation of the innate immune response as well as repeated immunizations [4]. Upregulation and engagement of costimulatory molecules (CD80, CD86, and CD40) on antigen presenting cells, especially dendritic cells (DC), appears to be of extreme importance. In addition, due to the maturity level of the immune system it may be more difficult to induce such costimulatory molecules on neonatal DC in comparison to adult DC [1]. Curiously, a recent report suggests that neonatal DC may be able to upregulate these key costimulatory molecules following stimulation as efficiently as adult DC [5]. Production of critical proinflammatory mediators, such as IL-12, may also be limited in neonatal DC [6].

A number of groups have devised strategies to overcome the perceived limitations of neonates. Some examples include immunization with antigen emulsified in Complete Freund's Adjuvant (CFA) [7], immunization with a low level of replicating retrovirus [8], use of a herpes virus capable of only one round of replication [9], use of plasmid DNA vaccines [10-14], plasmid DNA vaccines in conjunction with genetic adjuvants [15], and injection with antigen along with CpG motifs [16,17]. These approaches were all successful in generating Th1 responses as well as effective cell-mediated immunity, but additional novel as well as safe approaches are desirable [18]. Each of the

aforementioned strategies possesses possible safety limitations for use in humans for various reasons.

HSV-1 or HSV-2 infections of neonates can lead to life-threatening situations [19]. Therefore, a vaccine that could be administered during this period would be of particular value, since the neonatal response may not be able to control viral infection, spread, and replication. Such events may ultimately lead to infection of the CNS, encephalitis, and possibly death. Typically the neonate does control the infection and the virus then most likely retreats into sensory ganglia and sets up latency, setting the stage for future recrudescent lesions and painful outbreaks throughout the life of the individual.

Several investigators, particularly the Srivastrava group, have investigated the use of heat-shock proteins (hsps) in the generation of both humoral and cell-mediated immunity [20]. Hsps are capable of mediating cross-presentation of antigen (presentation of exogenous antigen on MHC Class I) and are able to generate efficient $CD8^+$ T cell responses to both tumors and virus infected cells [21,22]. More importantly, hsps are self-proteins that possess strong adjuvant properties that lead to the production of proinflammatory mediators [23,24]. Binding of hsps has been shown to induce production of IL-1β, IL-12, IL-6, and TNF-α from antigen presenting cells. In addition, hsp binding to the surface of APC leads to the secretion of chemokines such as MIP-1α, MIP-1β, and RANTES [25]. Exposure of DC to hsps also induces maturation and migration to the draining lymph node [26]. Also, hsp immunization has been demonstrated to prevent TNF-associated toxic shock [27]. These characteristics make hsp immunization an attractive candidate in the neonate.

Recent work from Schoenberger, Chen, and Bevan has demonstrated the importance for $CD4^+$ T cell help at the time of priming of $CD8^+$ T cells [28-30]. Therefore, it would be likely that $CD4^+$ help would also be critical during the priming of $CD8^+$ T cell responses in the neonate for the development of long-term memory.

In this study we addressed whether priming of neonatal mice with hsp70 coupled to the immunodominant peptide epitope from glycoprotein B (gB) (gB498-505) in C57BL/6 mice in conjunction with recombinant gB protein would be able to prevent neonatal tolerance, generate protective Th1 and $CD8^+$ CTL memory recall responses, and lead to the production of gB-specific antibody responses. Such immunized mice were then subjected to a lethal zosteriform challenge with HSV-1 to ascertain if protective immunity had been established.

Materials and Methods

Mice

Four- to five-week-old C57BL/6 (*H-2*^{b}) mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and bred in our animal facility to generate neonates. In conducting the research described in this work we adhered to the *Guide for the Care and Use of Laboratory Animals* as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences of the National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Peptides

The HSV gB (amino acids 498 to 505) peptide SSIEFARL and the chicken ovalbumin (aa 257 to 264) peptide SIINFEKL were synthesized and supplied by Research Genetics (Huntsville, AL)

Viruses

HSV-1-17 was grown on Vero cells (ATCC catalog no. CCL81), titrated, and stored in aliquots at -80°C until use. The recombinant vaccinia virus encoding the minigene SSIEFARL (VV-gB) was kindly provided by S. S. Tevethia [31].

Recombinant hsp70 protein preparation

The murine *hsp70* gene was amplified using high-fidelity PCR from the *hsp70.1* gene from a plasmid kindly provided by S. Calderwood [32]. It was then inserted into the pET-22b$^+$ expression vector (Invitrogen, Carlsbad, CA) using an engineered 5' NdeI site and a 3' BamHI site. The insert was then sequenced for confirmation. Recombinant protein was then produced in transformed BL21 (DE3) *E. coli* (Invitrogen) and purified as previously described [33]. In brief, the bacteria were lysed using a french press following a 4 h IPTG induction and then dialyzed in binding buffer. The dialyzed lysate was then purified using ATP-agarose affinity chromatography, dialyzed in binding buffer, and then further

purified by ion exchange chromatography to remove any contaminating DnaK (Q sepharose was used rather than POROS HQ). The final elute was then dialyzed in PBS. Western blot analysis demonstrated a single band at approximately 70 kD. Limulus amebocyte lysate testing (BioWhittaker, Walkersville, MD) revealed that the final protein product contained only trace levels of endotoxin (3 EU/ml).

Cell lines

Vero (African green monkey kidney cell line) was used for growing of viral stocks and MC38 was used as a target cell (C57BL/6, *H-2*^{b}). All cell lines were cultured in Dulbecco's Modification of Eagle's medium (Mediatech, VA) supplemented with 10% heat-inactivated fetal bovine serum, 5 mg/L of gentamicin sulfate, and 2 mM L-glutamine. T cell stimulation assays were carried out in 25 mM Hepes buffered RPMI-1640 media (Sigma, ST. Louis, MO) containing 10% heat-inactivated fetal bovine serum, 5 mg/L of gentamicin sulfate, 0.05 mM 2-ME, and 2 mM L-glutamine.

Hsp70 and peptide binding

SSIEFARL and SIINFEKL peptides were loaded onto hsp70 according to a previously described procedure [21]. Briefly, peptides were incubated with hsp70 in binding buffer (phosphate-buffered saline with 2 mM $MgCl_2$) at 37°C for 60 min (5 μg each in 50 μl total vol, 75:1 molar ratio). ADP was then added to 0.5 mM (Sigma) and the incubation

was continued for another 60 min. Preparations were then added to 50 μl of Alhydrogel aluminum hydroxide (Superfos Biosector, Denmark) and incubated at 4°C for 2 h.

Neonatal immunizations

Neonatal C57BL/6 mice were immunized i.p. on day 7 after birth with either 5 μg of SSIEFARL or SIINFEKL coupled to 5 μg of hsp70 in binding buffer with alum added in a 1:1 ratio for a total vol of 100 μl/ mouse. Some groups received 10 μg/mouse recombinant full-length gB protein (<5 EU LPS/ml) (a kind gift from Chiron, Emeryville, CA) in their immunization either in conjunction with hsp70 and peptide or alone. Neonatal spleen responses were then assessed 7 d post immunization or additional immunized mice were allowed to mature to 7 weeks old and challenged i.p. with 10^6 pfu VV-gB. T cell responses in the spleen were then analyzed 5 days later.

CTL assay

The CTL assay was performed as described earlier [34]. Briefly, effector cells generated after a 5 d in vitro expansion (with SSIEFARL-loaded, irradiated splenocytes) were analyzed for their ability to kill major histocompatibility complex (MHC)-matched antigen-presenting targets. The cells were mixed with the target at various ratios and incubated for 4 h. The targets included ^{51}Cr-pulsed MHC-matched SSIEFARL-pulsed as

well as control SIINFEKL-pulsed MC38 target cells. Percent specific lysis was then calculated according to the following formula:

100 x [(experimental release - spontaneous release)/ (maximum release - spontaneous release)].

Intracellular IFN-γ staining

To enumerate the number of IFN-γ producing cells intracellular cytokine staining was performed as previously described [35]. In brief, 10^6 freshly explanted splenocytes were cultured in U-bottom 96-well plates. Cells were either left untreated or stimulated with SSIEFARL peptide (1 μg/ml) for 6 h at 37°C in 5% CO_2. Brefeldin A was added for the duration of the culture period to facilitate intracellular cytokine accumulation. Cell surface staining was then performed followed by intracellular cytokine staining using the Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA) in accordance with the manufacturer's recommendations. For intracellular cytokine staining the antibodies used were FITC-anti-CD8α and PE-anti-IFN-γ (clone XMG1.2). All antibodies were purchased from BD Pharmingen and samples were collected on a BD FACScan and analyzed using CellQuest software.

ELISPOT and ELISA

ELISPOT assay for IFN-γ secreting cells was performed as previously described [35], except for slight modifications. Briefly, 96-well filter plates (Millipore HA) were coated with capture antibody for IFN-γ (RA-6A2)(BD Pharmingen) ON @ 4°C (BD Pharmingen) (2 μg/ml) in PBS. Plates were then washed with sterile PBS and blocked with culture media containing 10% FCS. Splenocytes from a single cell suspension were incubated at various effector:stimulator ratios. 10^5 irradiated, SIINFEKL or SSIEFARL-pulsed irradiated (3000 rads), syngeneic splenocytes were used as stimulators. Serial 2-fold dilutions of effectors were then incubated with stimulators starting with 5 x 10^5 and ending with 6.25 x 10^4. 20 U/well of IL-2 was also added to the culture. Cells were then incubated for 48 h at 37°C, 5% CO_2. Wells were then washed with PBS, followed by PBS-Tween. Biotin anti-mouse IFN-γ (XMG1.2) (BD Pharmingen) (1 μg/ml in PBS 3% BSA) was then added to each well and incubated ON @ 4°C. Wells were then washed with PBS-Tween and streptavidin-conjugated peroxidase (Jackson Laboratories) in PBS 3% BSA was added to each well (1 μg/ml) for 30 min @ 37°C. Wells were then washed with PBS-Tween and developed using 3-amino-9-ethylcarbazole (AEC) (Sigma) in 0.1 M acetate buffer pH 5.0, containing 0.05% H_2O_2. Reactions were allowed to proceed for 10 min and were ended by extensive washing with dH_2O. Plates were allowed to dry and then counted on a stereo microscope.

ELISA was performed as previously described [36] to determine cytokine levels (IFN-γ and IL-4) in supernatants of stimulated $CD8^+$ and $CD4^+$ T cell cultures stimulated

with peptide or rgB protein. Antibody pairs and concentrations were identical to those used for ELISPOT. The substrate ABTS was used for development. Determination of gB-specific total IgG, IgG2a, and IgG1 antibody in the serum was performed with appropriate HRP-goat-anti-mouse detection antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL). Briefly, plates were coated with rgB (1 μg/ml in PBS) and various dilutions of serum were incubated ON @ 4°C. The appropriate HRP-conjugated goat anti-mouse detection antibody (1 μg/ml) was then used for detection and the plates were developed with ABTS as previously stated.

Virus challenge

A zosteriform challenge experiment was performed as previously described [37] .In brief, the left flank area was depilated prior to challenge by a combination of hair clipping and use of the depilatory chemical (Nair; Carter-Wallace, Inc., New York, NY). The animals were anesthetized with Metofane (Pitman-Moore, Inc., Mundelein, IL), and 20 scarifications were made in a ~4-mm^2 area. To such scarifications 10 μl containing 2 $\times 10^5$ PFU of HSV-1 (strain 17) was added and the area was gently massaged. Animals were inspected daily for the development of zosteriform ipsilateral lesions, general behavior changes, encephalitis, and mortality. The severity of the lesions were scored as follows: 1+, vesicle formation; 2+, local erosion and ulceration of the local lesion; 3+, mild to moderate ulceration with spreading of lesion; 4+, severe ulceration, hind limb paralysis, and encephalitis; and 5, death (mice that were moribund and hence euthanized).

Statistical analysis

The data were analyzed by using a 2-tailed student's *t* test, and p values less than .05 were deemed significant.

Results

Hsp70 immunization regimens induce strong $CD8^+$ T cell responses during the neonatal period

We first set out to determine if hsp70 + peptide immunization strategies, which have previously been demonstrated to be an effective strategy in adult mice [38], would generate efficient $CD8^+$ cytokine and CTL responses in neonates. Preliminary experiments in our lab showed that hsp70+SSIEFARL immunization of neonates without alum enhanced adult recall responses to HSV-1 and conferred protection from a lethal zosteriform challenge with HSV-1-17 at 7 weeks of age (data not shown). Developmental studies of mice suggest that 7 days after birth most accurately correlates to the immune maturity level of a newborn human [39]. Therefore, 7 day old mice were used in this study and given an intraperitoneal (i.p.) injection of recombinant murine hsp70 (5 μg) non-covalently complexed to gB498-505 (SSIEFARL) (5 μg) and adsorbed to alum (1:1 ratio, 100 μl total) prior to injection. Additional groups also received recombinant gB protein (10 μg) in addition to hsp70 and peptide. Control groups were injected with either

hsp70 and an irrelevant peptide (OVA 257-264 or SIINFEKL) in alum, hsp70 + SIINFEKL + recombinant gB in alum, SSIEFARL alone in alum, SSIEFARL + gB in alum, or gB in alum alone. Neonatal mice were then assessed 7 days later for CTL and cytokine responses in the spleen. As seen in figure 1A (see appendix), mice that received hsp70, SSIEFARL, and gB generated significantly higher levels of IFN-γ producing cells against both MHC Class I and Class II-restricted epitopes. Mice that received hsp70, SIINFEKL, and rgB demonstrated a modest response, while all other groups failed to generate detectable responses. Following a 5 day in vitro expansion with irradiated syngeneic APC loaded with gB498-505, stimulated cultures were tested for their ability to lyse Cr^{51}-labelled, peptide-pulsed target cells (MC38). Neonatal derived CTL demonstrated the capacity to kill gB498-505 loaded target cells, provided that the neonate had been primed with hsp70 and gB498-505 (Figure 1B). Neonates that received hsp70, gB498-505, and rgB mounted the most effective primary CTL responses, leading to greater than 50% lysis (Figure 1B). Mice receiving hsp70 and SSIEFARL also mounted significant cytotoxic responses, albeit slightly less than groups which received hsp70, SSIEFARL, and rgB. The group that received hsp70, SIINKEKL, and rgB consistently produced low levels of CTL, suggesting that gB may possess some immunogenic potential of its own. Groups that received SSIEFARL only, or SSIEFARL and rgB failed to produce any detectable ELISPOT or CTL activity (data not shown).

Neonatal hsp70 immunization approaches enhance adult recall responses to antigen

In order to evaluate the relative numbers of responding memory $CD8^+$ T cells specific for gB 498-505 following i.p challenge with VV-gB, both ELISPOT (Figure 2A) and

intracellular staining for IFN-γ (Figure 2B) were carried out. For recall cytokine response evaluation adult mice were immunized as neonates with various regimens, infected with VV-gB, and five days later the spleens were removed and stimulated for 48 hours with irradiated syngeneic splenocytes loaded with gB498-505. Mice that received hsp70 in conjunction with gB498-505 and rgB produced a statistically significant ($p<0.05$) (75% greater) recall response in comparison to control mice receiving only hsp70 and OVA257-264. Mice receiving hsp70 and gB498-505 as neonates also showed enhanced responses in comparison to the control mice that received hsp70 and the irrelevant peptide (50% greater). However, these differences were not significant, since the primary response to VV-gB is extremely strong in C57BL/6 mice and may overshadow small differences. Intracellular staining for IFN-γ was also performed on adult mice from the same set of groups. Again, the most significant elevation in response in comparison to the negative control group was found in the group that had received hsp70, gB498-505, and rgB (~6-fold). Approximately a 4-fold increase was observed in the group which received hsp70 and gB498-505 only. This again supports the need for concurrent $CD4^+$ T cell priming during $CD8^+$ T cell priming for optimal memory responses. Cytotoxicity assays (Figure 2C) supported data from ELISPOT and intracellular staining experiments, with the group receiving hsp70, SSIEFARL, and gB yielding the highest level of killing. It was interesting to note the enhanced recall CTL killing in all groups that had received rgB as neonates, again suggesting that rgB may have inherent immunogenic properties. Groups that received both SSIEFARL and rgB generated similar responses to mice receiving rgB alone in all assays, while control groups that received SSIEFARL alone failed to show any enhanced recall response (data not shown).

$CD4^+$ T cell recall responses from mice immunized as neonates with hsp70-based preparations are biased towards Th1

Since $CD4^+$ T cell responses are critical for controlling HSV-1 infections, we assayed the recall response to determine if tolerance was occurring to the rgB administered to the neonates. Development of a Th1 response is therefore a necessary goal to prevent or control HSV-1 infection. Adult recall $CD4^+$ T cell responses were accordingly measured in mice that had been immunized as neonates with various hsp70 regimens and then challenged as adults with VV-gB (Figure 3). Briefly, supernatant from splenocytes that had been stimulated for 48 h with either gB498-505 (Figure 3A) (for $CD8^+$ T cell response) or rgB (for $CD4^+$ T cell response) was examined by ELISA for the presence of IFN-γ or IL-4 (Figure 3B). Groups receiving a combination of hsp70 and rgB, regardless of the peptide conjugated to hsp70, produced strong Th1 recall responses to gB. Each group produced approximately 3 ng/ml of IFN-γ, but secreted only 1 ng/ml or less of IL-4. In contrast, groups that did not receive rgB failed to generate large amounts of either IFN-γ or IL-4. As expected, groups that received rgB without hsp70 to act as a strong adjuvant primarily demonstrated a Th2 recall response dominated by IL-4, as expected with alum (Figure 3B).

Neonatal immunization approaches with hsp70 and rgB yield IgG1 gB-specific antibody responses

Antibody is also a critical factor in limiting or preventing initial infection with HSV-1, especially at the site of infection (typically the mucosa). Therefore, we analyzed sera

from adult mice that had been immunized as neonates with the various hsp70/antigen combinations and challenged with VV-gB five days prior to collection (Figure 4). Surprisingly, only IgG1 gB-specific antibody responses were observed, despite the development of Th1 $CD4^+$ T cell responses. It appeared that inclusion of hsp70 yielded slightly enhanced levels of gB-specific antibody when one compares groups which received gB alone versus those that received hsp70 and rgB. However, these differences were not statistically significant. Therefore, it is interesting to speculate that neonates may have a profound bias to the generation of IgG1 responses.

Neonatal vaccination strategies utilizing hsp70 decrease HSV-1 associated lesions and prevent mortality

In an effort to assess the protective capacity of a neonatal hsp70 based vaccine design, the zosteriform challenge model was utilized. Briefly, the mice were shaved on the left flank and the skin was scratched with a needle. HSV-1-17 was then applied to the skin and the mice were observed daily for signs of infection and disease progression as described earlier. As seen in figure 5A, mice that received hsp70, gB498-505, and rgB faired most favorably, developing either no or only minimal signs of infection. Mice that received hsp70 and gB498-505 were resistant to death and disease progression (Figure 5B), but were not protected from initial infection. In stark contrast, control mice that received hsp70 and OVA257-264 developed severe HSV-1 lesions and encephalitis, with approximately 75% succumbing to the infection. Mice that received hsp70, OVA257-264, and rgB also were able to resist infection, comparable to groups that received gB498-505 along with hsp70 and rgB. In addition, rgB alone resulted in enhanced

immunity to zosteriform challenge, with mice following a similar course of disease to that of other partially protected groups. Therefore, it appears that $CD8^+$ T cell immunity is important for preventing the spread of the infection, but $CD4^+$ T cell responses and specific antibody may play an even greater role in controlling the initial infection [37]. Any effective neonatal vaccination strategy would most likely involve such a combinatorial approach.

Discussion

This study demonstrated that immunization of neonates with hsp70 and the immunodominant peptide MHC Class I-restricted epitope from gB along with recombinant gB in alum elicited detectable CTL responses during the neonatal period as well as strong CTL and Th1 memory recall responses during adult life. Most importantly, $CD4^+$ T cell memory responses in groups that had received hsp70 and gB protein showed a clear shift to Th1, demonstrating that neonatal tolerance was indeed averted. In contrast, either mixed or strong Th2 responses (IL-4 gB-specific production) were present in groups immunized as neonates with rgB in alum without hsp70. Although enhanced primary as well as memory CTL responses were noted (by intracellular staining, ELISPOT, and CTL analysis) following hsp70 immunization strategies, gB-specific antibody responses were not of the expected isotype. Strong gB-specific antibody responses were noted in sera of immunized mice at 6 weeks of age, but these responses were primarily IgG1 in makeup, not IgG2a, as would be expected to accompany a Th1 response. This observation may be attributed to the relative immaturity of the B cell at the time of priming, and similar outcomes have been observed in prior studies by others [40].

More importantly, we demonstrated that neonatal immunization with hsp70 based approaches can protect mice from subsequent challenge as adults with a lethal dose of HSV-1 (as demonstrated by the zosteriform challenge experiments). Protection is likely mediated by a combination of $CD4^+$ T cells, $CD8^+$ T cells, and antibody to gB. However, when mice were immunized with hsp70 and peptide, only partial protection was noted. Most of these mice showed signs of infection but managed to contain the spread of the virus and recover completely. These results are in accord with prior studies indicating that $CD4^+$ T cells are critical for effective $CD8^+$ T cell memory responses against HSV-1 infection [38].

It is striking that inclusion of $CD4^+$ T cell help is critical for both primary neonatal responses as well as secondary adult recall responses. It was previously demonstrated by Shen [29], Bevan [30], and Schoenberger [28]that $CD4^+$ T cell help is critical for memory development of $CD8^+$ T cell responses, but primary responses appeared to not be affected by the absence of help. It may be plausible that neonatal CTL responses are even more reliant upon strong $CD4^+$ T cell help (most likely a key source of IL-2 and CD40/CD154 interaction on DC) for initiation due to their lower precursor frequency than in adults.

Newborns are at high risk of exposure to a number of viral and bacterial pathogens, including HSV-1. Nevertheless, most immunizations are not performed until 2-3 months after birth in humans, since the neonatal immune system preferentially mounts Th2 responses following antigenic challenge. This defect in the neonate has been attributed to both inadequate presentation (both nature and quality) by neonatal APC, as well as insufficient numbers. We have chosen to use the age of 1 week after birth for

these studies, since this time has been suggested by others to most closely match the maturity level of a newborn human. Successes have been noted in immunization of neonatal mice with a number of agents, including plasmid DNA alone as well as CpG oligonucleotides in combination with plasmid DNA or proteins. Unfortunately, responses in neonates may be weaker than what is observed in adult mouse models for plasmid DNA immunizations [14]. In addition, Th responses in plasmid DNA immunizations may not be fully directed to Th1 recall responses without additional factors such as IL-12 [15]. Furthermore, CpG immunizations may be toxic to neonates, as enhanced morbidity and mortality has been noted in neonatal immunizations [41]. Other successes in the neonate include the use of recombinant HSV-1 DISC, which is capable of replication only once [9], as well as a low pfu of retrovirus (1 pfu) for infection [8]. Success has also been observed using mucosal immunization with attenuated recombinant adenoviral vectors. However, safety concerns and the possibility of a reversion to wild-type virus are always a concern when using live virus in a highly susceptible neonate. Other prior approaches include CFA as an adjuvant to prevent neonatal tolerance, but drawbacks of this approach exist as well, since CFA use can lead to oil induced arthritis [42]. In addition, CFA is not approved for use in humans. However, alum is approved for use in humans, and we have exploited its ability to create an antigen depot in these studies. Hsp70 also appears to be a safe adjuvant in mice, since excess amounts do not lead to TNF-α-mediated shock in adult mice [27]. Therefore, use of a protein based adjuvant in alum, such as hsp70, appears ideal for safe, effective stimulation of the neonatal immune system. It was interesting to note that all groups that received rgB demonstrated enhanced $CD8^+$ T cell responses. This observation could be attributed to possible adjuvant effects exerted by the

glycoprotein itself, which could conceivably activate a toll-like receptor (TLR). Viral glycoprotein activation of TLR2 has been observed by the Compton group [43].

Infection of the neonate with HSV-1 can even lead to life-threatening situations, most likely due to the inability of the neonate to mount robust $CD4^+$ and $CD8^+$ T cell responses in a timely manner to contain HSV-1 [19]. Neonatal exposure and infection are also quite common in HSV-1, allowing the virus to set up latency and establish a life-long infection. Therefore, early intervention is necessary to break the chain of events and prevent initial infection. Neonatal immunization approaches utilizing the unique adjuvant and cross-priming characteristics of hsp70 may prove therapeutically useful in allowing the neonate to mount a protective response at this vulnerable, early point in life. In addition, these early responses should not prove detrimental upon pathogen encounter, as one might have expected if Th2 recall responses had been observed.

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Appendix

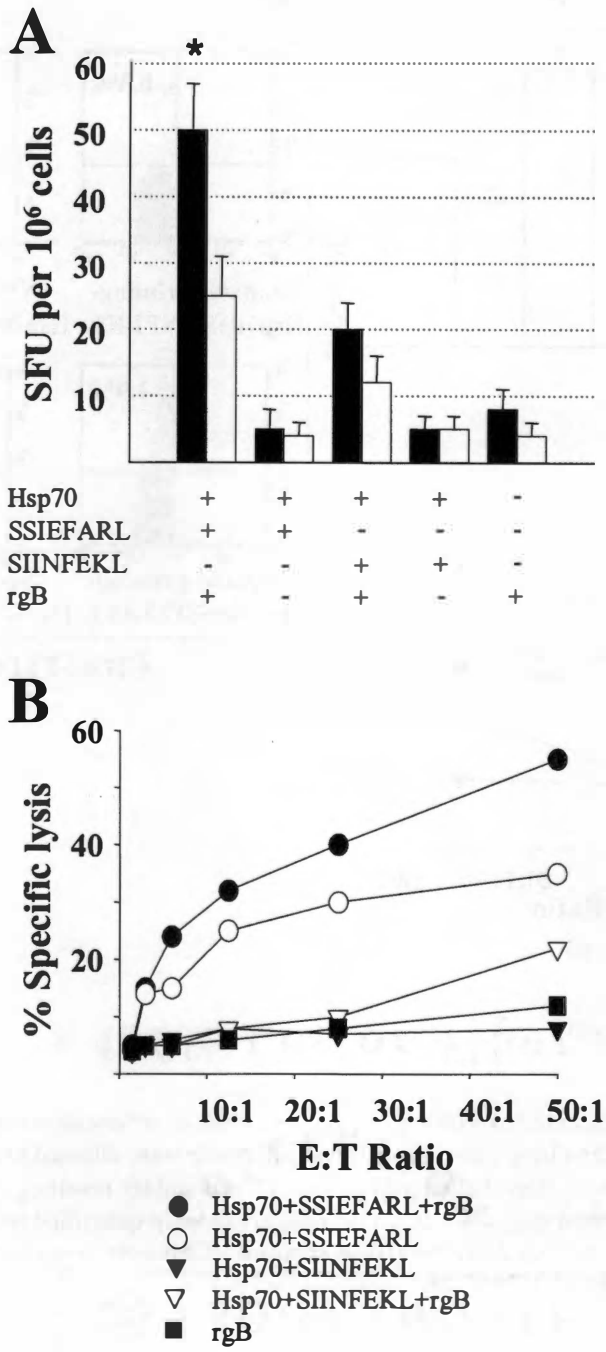

Figure 1. *Neonatal vaccination generates enhanced primary responses in the neonate.* 7 days post immunization the spleens were removed and ELISPOT for IFN-γ production against gB498-505 (filled bars) or rgB (open bars) was performed (A) or cells were stimulated in vitro for 5 days and a standard ^{51}Cr release assay was performed (B). *$p<0.05$

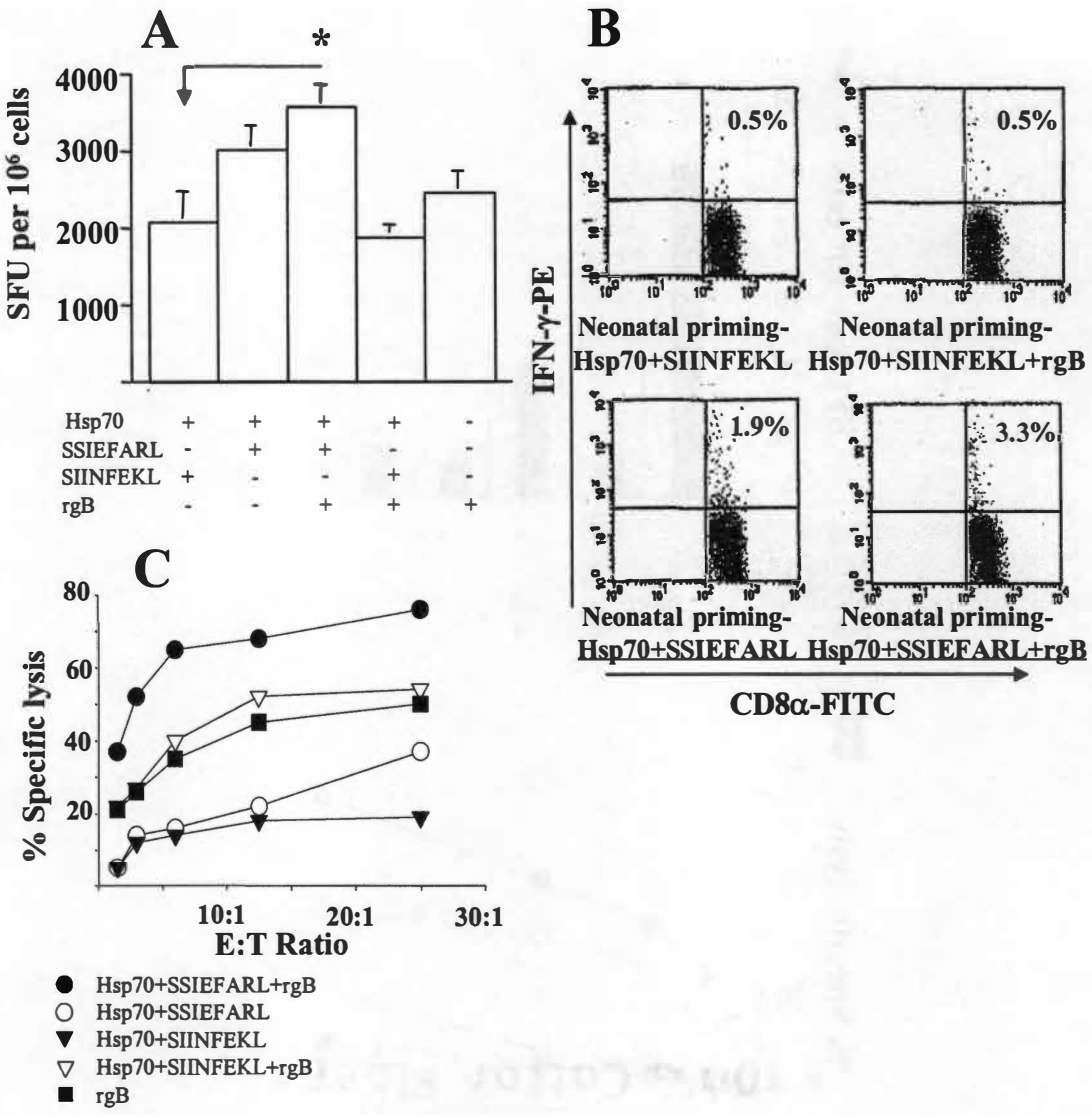

Figure 2. *Neonatal vaccination with hsp70 regimens leads to enhanced memory recall responses.* Mice that had been immunized 7 days after birth were allowed to mature to 7 weeks of age. They were then challenged i.p. with VV-gB and the resulting cytokine and CTL responses were then measured. IFN-γ secreting cells were quantified by ELISPOT (A) and intracellular staining for IFN-γ (B). A standard ^{51}Cr release assay was used to determine CTL activity (C). *$p<0.05$

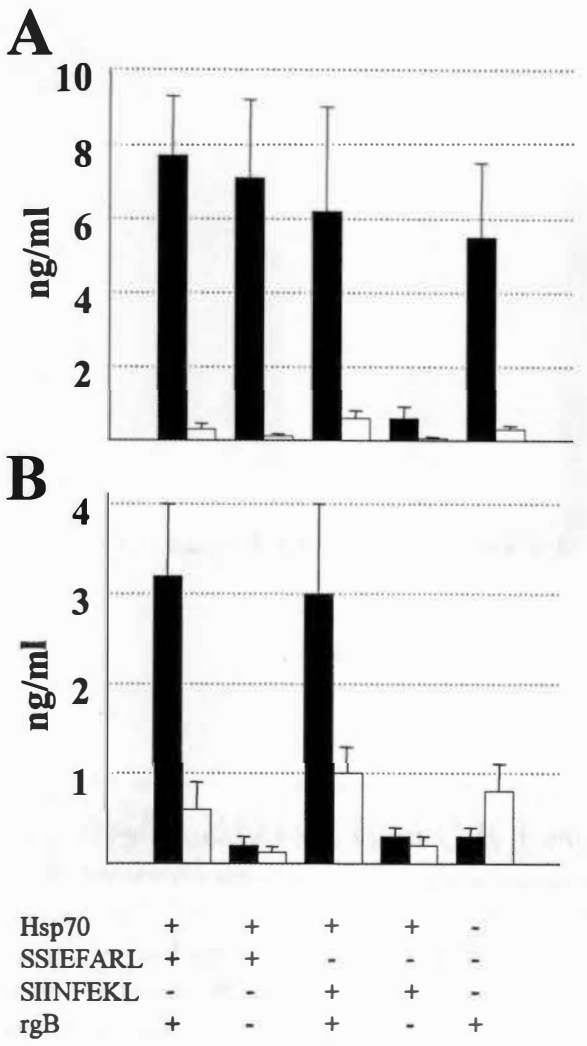

Figure 3. *Mice receiving hsp70 and antigen generate Th1 dominated recall responses*. Spleen cells from recall response mice were incubated with either 5 μg/ml gB498-505 (A) or 20 μg/ml rgB (B) for 48 h. The supernatant was then tested for either IFN-γ (filled bars) or IL-4 (open bars) by ELISA.

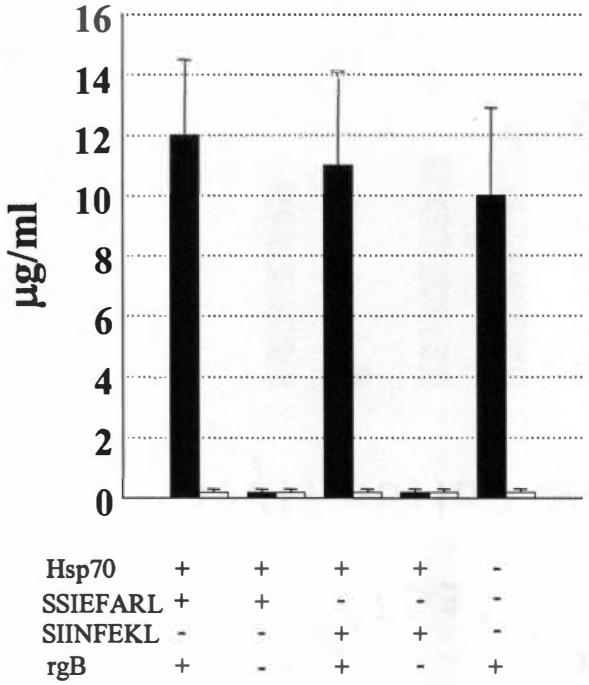

Figure 4. *Despite a predominant Th1 recall response, mice immunized as neonates mount an IgG1 gB-specific response following challenge with VV-gB during adulthood.* Responses from various serum dilutions taken 5 d p.i. with VV-gB were measured by ELISA using gB coated plates (1 µg/ml) followed by incubation with the appropriate HRP-goat anti-mouse detection antibody. IgG1-specific responses are depicted as filled bars while IgG2a-specific responses are designated as open bars.

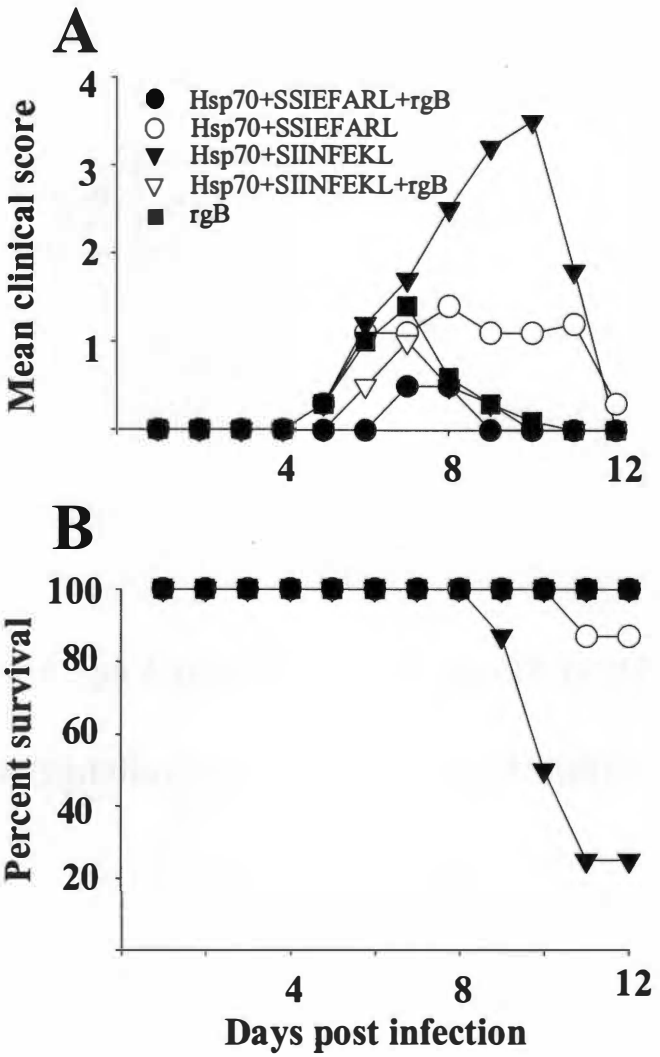

Figure 5. *Mice that received hsp70 and gB antigen display enhanced resistance to zosteriform challenge as well as reduced mortality.* A zosteriform challenge with HSV-1 strain 17 was carried out by shaving the left dorsal flank and sraping the skin with a 27G needle prior to exposure with HSV-1. Mice were graded daily for signs of disease (A). Scoring was as follows: 1) local inflammation, 2) defined local lesion, 3) spreading of local lesion up the flank, 4) paralysis of hind limbs due to encephalitis, and 5) death. Survival rate was also recorded (B). Each group consisted of 8 mice and the results are representative of 2 independent experiments.

Part III.

An Intranasal Heat Shock Protein Based Vaccination Strategy Confers Protection Against Mucosal Challenge with Herpes Simplex Virus

Abstract

Herpes simplex virus (HSV-1) represents a significant obstacle for vaccine designers, despite decades of investigation. The virus primarily infects the host at vulnerable mucosal surfaces that progresses to lesion development, latency in nervous tissue, and possible reactivation. Therefore, protection at the site of infection is crucial. Mucosal adjuvants are critical for the development of an effective vaccine approach, and heat-shock protein 70 (Hsp70) represents an attractive candidate for this purpose. This study demonstrates that Hsp70 coupled to gB498-505 from HSV-1 induced mucosal and systemic priming of CD8+ T cells capable of protecting C57BL/6 mice against a lethal vaginal challenge. Elevated gB-specific cytotoxicity was observed in the spleen of Hsp70+peptide immunized mice. In addition, both vaginal IFN-γ levels and viral clearance were enhanced in mice mucosally immunized with Hsp70 and gB peptide versus peptide only control mice or mice receiving Hsp70 and a control peptide. These studies demonstrate that Hsp70 can be used as an effective mucosal adjuvant capable of generating a protective cell-mediated immune response against HSV-1.

Introduction

Despite decades of research and development, effective vaccines against a number of viral pathogens, such as HIV and a number of human herpesviruses, are still lacking. It appears that T cell immunity, including both $CD4^+$ and $CD8^+$ T cells, are crucial for controlling and eliminating these chronic viral infections. Despite decades of attempts, no effective vaccine strategy has proved effective against herpes simplex virus

(HSV) in human trials. Therefore, additional, novel approaches are necessary and warrant investigation. A number of vaccine approaches, both systemic and mucosal, have been investigated against HSV [1]. Some examples include DNA vaccines along with various cytokine and chemokine adjuvants [2-5], CpG oligonucleotide motifs along with peptide [6,7], heat-shock protein/peptide complexes [8,9], recombinant vaccinia virus expressing HSV glycoproteins [10], and many others. Moreover, it appears that heat shock protein 70 (hsp70) coupled to viral peptides may prove useful as vaccine candidates for HSV. Hsp70 has been demonstrated to induce the maturation and migration of dendritic cells (DC) [11], leading to the upregulation of costimulatory molecules and the secretion of strong proinflammatory cytokines as well as Th1 associated chemokines [12-14]. Recent studies from our group demonstrated that when hsp70 was coupled to gB498-505, the dominant $CD8^+$ T cell epitope in C57BL/6 mice against HSV-1, it induced potent acute responses marked by enhanced levels of IFN-γ secreting cells and strong cytotoxic T cell (CTL) activity. Unfortunately, these responses were not durable, as mice challenged in the memory phase gradually lost resistance to viral challenge in comparison to groups that were immunized with UV-inactivated HSV-1 or a recombinant vaccinia virus encoding gB498-505 as a minigene [8]. This decrease was attributable to the lack of $CD4^+$ T cell help during the priming phase. Therefore, additional studies including cognate $CD4^+$ T cell help increased long-term effectiveness and memory responses [9].

HSV is primarily transmitted via mucosal surfaces, such as the oral and genital mucosae. Therefore, efficient immune responses are critical for protection at these vulnerable sites and development of vaccine approaches to enhance mucosal immunity against HSV is a major goal [15,16]. The respiratory tract is an attractive route of

administration to induce protective immune responses to mucosal pathogens, especial since immune cells primed at the primary mucosal site appear to migrate to distal mucosal sites, such as the vaginal mucosa. This process has been referred to as the common mucosal concept [17]. Several effective mucosal adjuvants have been tested in HSV vaccine trials, and one of the more potent of these in clinical trials in humans was 3-O-deacylated monophosphoryl lipid A given along with recombinant HSV-2 glycoprotein D in aluminum hydroxide [18]. Unfortunately, the vaccine was only effective in women seronegative for both HSV-1 and HSV-2. Other bacterial toxins, such as cholera toxin and *E. coli* enterotoxin, are strong mucosal adjuvants, but significant side effects prevent their practical use in humans [19]. Another possible mucosal adjuvant, CpG motifs, has also been investigated in mucosal HSV vaccine studies by the Rosenthal group [7]. Mucosal CpG administration along with recombinant protein from HSV was an effective approach, generating high levels of $CD4^+$ and $CD8^+$ T cell responses as well as HSV-specific antibody responses. Even CpG alone was surprisingly effective at preventing vaginal HSV infection for a short period, presumably because of enhanced stimulation of innate immunity. In this study we evaluate the potential use of hsp70 as a mucosal adjuvant. Mice were given hsp70 bound to gB 498-505 intranasally (i.n.) or intraperitoneally (i.p.) and the resulting systemic CTL response was measured. Resistance to vaginal challenge with HSV-1, vaginal cytokine production, and clearance of virus was also examined. Our results show systemic responses to gB498-505, increased vaginal IFN-γ levels, enhanced viral clearance rates, and resistance to vaginal challenge.

Materials and Methods

Mice

Four- to five-week-old C57BL/6 ($H\text{-}2^b$) mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). In conducting the research described in this work we adhered to the *Guide for the Care and Use of Laboratory Animals* as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences of the National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Peptides

The HSV gB (amino acids 498 to 505) peptide SSIEFARL and the chicken ovalbumin (aa 257 to 264) peptide SIINFEKL were synthesized and supplied by Research Genetics (Huntsville, AL)

Viruses

HSV-1 McKrae strain was grown on Vero cells (ATCC catalog no. CCL81), titrated, and stored in aliquots at -80°C until use. Viral titrations were performed on Vero cell monolayers and PFU/ml was calculated.

Recombinant hsp70 protein preparation

The murine *hsp70* gene was amplified using high-fidelity PCR from the *hsp70.1* gene from a plasmid kindly provided by S. Calderwood [20]. It was then inserted into the pET-22b$^+$ expression vector (Invitrogen, Carlsbad, CA) using an engineered 5' NdeI site and a 3' BamHI site. The insert was then sequenced for confirmation. Recombinant protein was then produced in transformed BL21 (DE3) *E. coli* (Invitrogen) and purified as previously described [21]. In brief, the bacteria were lysed using a french press following a 4 h IPTG induction and then dialyzed in binding buffer. The dialyzed lysate was then purified using ATP-agarose affinity chromatography, dialyzed in binding buffer, and then further purified by ion exchange chromatography to remove any contaminating DnaK (Q sepharose was used rather than POROS HQ). The final elute was then dialyzed in PBS. Western blot analysis demonstrated a single band at approximately 70 kD. Limulus amebocyte lysate testing (BioWhittaker, Walkersville, MD) revealed that the final protein product contained only trace levels of endotoxin (3 EU/ml).

Cell lines

Vero (African green monkey kidney cell line) was used for growing of viral stocks and MC38 was used as a target cell (C57BL/6 adenocarcinoma, *H-2^b*). All cell lines were cultured in Dulbecco's Modification of Eagle's medium (Mediatech, VA) supplemented with 10% heat-inactivated fetal bovine serum, 5 mg/L of gentamicin sulfate, and 2 mM L-glutamine. T cell stimulation assays were carried out in 25 mM Hepes buffered RPMI-

1640 media (Sigma, ST. Louis, MO) containing 10% heat-inactivated fetal bovine serum, 5 mg/L of gentamicin sulfate, 0.05 mM 2-ME, and 2 mM L-glutamine.

Hsp70 and peptide binding

SSIEFARL and SIINFEKL peptides were loaded onto hsp70 according to a previously described procedure [22]. Briefly, peptides were incubated with hsp70 in binding buffer (phosphate-buffered saline with 2 mM $MgCl_2$) at 37°C for 60 min (10 μg each in 50 μl total vol, 75:1 molar ratio). ADP was then added to 0.5 mM (Sigma) and the incubation was continued for another 60 min.

Immunizations

C57BL/6 mice were immunized either i.n. or i.p. with either 10 μg of SSIEFARL or SIINFEKL coupled to 10 μg of hsp70 in binding buffer for a total vol of 20 μl (i.n.) or 100 μl (i.p.). Some groups received only peptide. Immunizations were performed on d0, d7 and d21. Spleen responses were then assessed 5 d after the 3rd immunization.

CTL assay

The CTL assay was performed as described earlier [6]. Briefly, effector cells generated after a 5 d in vitro expansion (with SSIEFARL-loaded, irradiated splenocytes) were analyzed for their ability to kill major histocompatibility complex (MHC)-matched antigen-presenting targets. The cells were mixed with the target at various ratios and incubated for 4 h. The targets included ^{51}Cr-pulsed MHC-matched SSIEFARL-pulsed as well as control SIINFEKL-pulsed MC38 target cells. Percent specific lysis was then calculated according to the following formula:

100 x [(experimental release - spontaneous release)/ (maximum release - spontaneous release)].

ELISA

ELISA was performed as previously described to determine cytokine levels (IFN-γ) in vaginal washings [6]. Briefly, 96-well EIA/RIA plates were coated with capture antibody for IFN-γ (RA-6A2)(BD Pharmingen) ON @ 4°C (2 μg/ml) in 1 M Na_2HPO_4. The wells were then washed with PBS-Tween and vaginal washings taken every 24 h with PBS were incubated ON @ 4°C. For detection wells were washed with PBS-Tween and biotin anti-mouse IFN-γ (XMG1.2) (BD Pharmingen) (1 μg/ml in PBS 3% BSA) was then added to each well and incubated for 1h @ RT. Wells were then washed with PBS-Tween and streptavidin-conjugated peroxidase (Jackson Laboratories, ME) in PBS 3%

BSA was added to each well (1 μg/ml) for 30 min @ RT. Plates were then washed and developed using ABTS (Sigma). ELISA readings were taken on an automated ELISA reader (SpectraMAX 340, Molecular Devices, Sunnyvale, CA).

Virus challenge

Since mice are irregularly susceptible to genital infection with HSV-1 unless they are synchronized into diestrous, mice were injected subcutaneously with 2 mg of Depo-Provera (medroxyprogesterone) (Pharmacia and Upjohn, Kalamazoo, MI) to synchronize the ovarian cycle [23]. Five days later each mouse was anesthetized with avertin and infected intravaginally with 10^6 PFU of HSV-1 McKrae strain. Every 24 h a genital lavage specimen (washing with 200 μl PBS) was collected for viral titration and cytokine analysis. Mice were monitored daily for clinical disease and pathology and graded according to the following: 0, no apparent infection; 1, mild inflammation of the external genitalia; 2, redness and swelling of external genitals; 3, severe redness, inflammation, and genital ulceration with loss of hair; 4, hind limb paralysis; 5, death.

Statistical analysis

The data were analyzed by using a 2-tailed student's *t* test, and p values less than .05 were deemed significant.

Results

Mucosal immunization with hsp70 and gB peptide results in systemic gB-specific cytotoxic T cell responses

A standard cytotoxic T cell assay was used to examine the level of systemic CTL activity that was generated in each experimental group. Mice, as previously described, were immunized a total of 3 times i.n or i.p. with hsp70 and gB peptide. Five days after the final boost the spleen was removed and restimulated for 5 days in the presence of irradiated syngeneic splenocytes loaded with gB peptide. A standard Cr^{51} release assay was then performed to assess cytotoxic function. As seen in figure 1, both groups that received hsp70 coupled to gB peptide mounted robust CTL responses in comparison to control groups immunized with hsp70 and control peptide or gB peptide alone. At the highest effector to target ratio (50:1) mice i.n. immunized with hsp70 and gB peptide yielded a specific lysis of over 60%, while control mice given gB peptide alone showed less than 20% lysis. It is interesting to note that mice receiving i.n. hsp70 and SSIEFARL mounted slightly higher CTL activity in comparison to i.p. administered mice (60% versus 34% specific lysis). Mice given hsp70 and ova peptide yielded minimal killing (less than 10%), regardless of the route of administration.

Mucosal immunization with hsp70 and gB peptide grants protection against a lethal vaginal challenge with HSV-1

In an effort to test if mucosal immunization with hsp70 and gB peptide was effective at controlling or preventing a vaginal HSV-1 infection i.n or i.p. immunized mice were

subjected to a lethal challenge (10^6 pfu) with HSV-1 McKrae, a highly virulent strain that often results in paralysis and death in immunized mice. Mice were immunized as previously described and then synchronized as described in the materials and methods prior to challenge. According to previous studies synchronization of the estrous cycle is an important factor for susceptibility to vaginal infection with HSV-1 [23]. Mice were vaginally challenged and scored for disease as described in materials and methods. As evident in figure 2, only mice that were immunized i.n with hsp70 and gB peptide were virtually completely protected from significant disease and death. Mice that received hsp70 and SSIEFARL i.p. demonstrated a reduced disease course and moderate resistance to encephalitis and death, as 66% of the mice fully recovered. Mice that received hsp70 and control ova peptide or mice that received gB peptide alone developed severe disease, and most, if not all, succumbed to infection in both control groups. Surprisingly, i.n. hsp70 and gB peptide immunized mice only developed extremely mild disease or none at all, and all mice were protected from death.

Mice immunized mucosally with hsp70 and gB peptide secrete elevated levels of IFN-γ in vaginal washings during challenge with HSV-1

Throughout the challenge with HSV-1 McKrae daily vaginal washings were collected to examine IFN-γ levels throughout the course of infection. Figure 3 shows that mice that had received hsp70 and gB peptide i.n. demonstrated higher initial levels (2-fold enhancement) of IFN-γ early on during infection and also mounted a delayed production of IFN-γ (400 +/- 153 pg/ml), observed on day 4 p.i. which was completely absent in all

other groups. This delayed production of IFN-γ is most likely attributable to infiltrating memory $CD8^+$ T cells. The early enhanced IFN-γ response in mice i.n. immunized with hsp70 and gB498-505 is most likely due to a combination of tissue residing effector memory $CD8^+$ T cells or an elevated NK cell response in relation to the other groups. However, high NK cell responses were observed in all groups.

Mucosal immunization with hsp70 and gB peptide results in an enhanced viral clearance rate

We next set out to determine if such enhanced $CD8^+$ T cell responses were capable of affecting the rate of viral clearance following a high dose mucosal vaginal challenge with HSV-1 McKrae. Mice were synchronized with Depo-Provera as before and challenged with HSV-1 on day 4 after the final booster. Vaginal washings were then taken on each day to determine the level of virus present. As seen in table 1, Mice that were previously immunized i.n. with hsp70 and gB peptide were able to clear virus from the vagina at a slightly faster rate than in comparison to control groups. In addition, groups receiving hsp70 and gB peptide, i.n and i.p., were able to completely clear virus one day faster than control groups (4 days versus 5 days for control groups). This suggests that viral clearance rate is enhanced moderately following either mucosal immunization or systemic immunization with hsp70 and gB peptide in comparison to hsp70 and control peptide or gB peptide alone.

Discussion

The present study assesses the efficacy of mucosal immunization with hsp70 in conjunction with the immunodominant CD8 epitope found in C57BL/6 mice from HSV-1, gB 498-505. In addition, the mucosal route of administration was compared to another systemic route, i.p. administration. These two vaccination approaches were then compared in their ability to generate systemic CTL activity, increase resistance to vaginal challenge, enhance vaginal cytokine levels, and enhance viral clearance rate. Results demonstrate that hsp70 coupled to gB498-505, regardless as to the route of administration, primes a systemic response in the spleen as measured by a standard Cr^{51} release assay. In addition, it appears that i.n. administration of hsp70 and gB498-505 leads to enhanced IFN-γ levels in the vaginal lumen, faster clearance of virus, and elevated resistance to vaginal challenge. Enhanced levels of vaginal IFN-γ may be a critical factor for the observed resistance to disease, and this has been carefully documented before [24]. It is also interesting to speculate upon the source of the enhanced early levels of IFN-g in vaginal washings (24 h). The source is most likely NK cells, which have shown to play a critical role in the early control of HSV [25], or effector memory CD8 T cells. More interesting, it appears that mucosal administration of hsp70 and gB498-505 is more effective than i.p. administration in reference to the ability of each regimen to protect against vaginal challenge.

Several reports have demonstrated the efficacy of hsp70 and peptide to act as an effective vaccine against viral infection, most notably in the LCMV system by the Welsh group and studies in our own lab [8,22]. However, all hsp70 strategies to date have

utilized either i.p., intradermal (i.d.), or s.c. immunization routes. Our own group has demonstrated that i.p. immunization with hsp70 and gB498-505 generates potent acute responses, but these responses decay over time in comparison to groups immunized with a recombinant vaccinia virus expressing gB498-505 [8]. This difference was most likely attributable to a lack of $CD4^+$ T cell help during priming in the hsp70 groups, and additional work showed that the addition of cognate $CD4^+$ T cell help was sufficient to greatly improve long-term responses [9]. In addition, studies from our own lab using CpG and gB498-505 also demonstrated a lack of long-term durability, as mice challenged in the memory phase showed reduced resistance to viral challenge [6]. It is likely that long-term protection would also diminish in mice mucosally immunized with hsp70 and peptide, much like that observed in earlier studies. Clearly, additional work is needed including possible CD4 epitopes, such as including recombinant full-length gB, to possibly enhance the acute as well as long-term memory response. Other possibilities for improvement include the use of hsp70 and peptide as either a priming or boosting agent along with recombinant vaccinia virus expressing gB or plasmid DNA encoding gB. Prime-boost mucosal vaccination against HSV-1, as previously demonstrated, was more effective when the recombinant vaccinia virus rather than plasmid DNA when used during the priming step rather than the boost [26]. Perhaps hsp70 and peptide approaches would also be more effective as a booster immunogen, especially since $CD4^+$ T cell help may not be as essential during the booster stage. This concept is currently under investigation. In addition, further work is warranted to determine the effects upon maturation and migration that hsp70 exposure may exert upon respiratory DC, which are

typically resistant to maturation signals in comparison to other tissue compartments such as the spleen.

Overall, this study is the first to investigate the possible use of hsp70 as a mucosal adjuvant capable of priming protective $CD8^+$ T cell responses. In humans it has been demonstrated that $CD8^+$ T cell responses are closely linked to HSV control and clearance. Therefore, approaches stimulating immunity may prove beneficial in a clinical setting, especially since many HSV peptide epitopes for several common HLA Class I haplotypes have been discovered in recent years [1]. We demonstrate that hsp70 is indeed a safe, effective mucosal adjuvant capable of inducing elevated IFN–γ levels in the vagina in response to infection, enhancing viral clearance, as well as elevating systemic CTL activity and overall protection from disease. Additional work fully characterizing the nature and level of the $CD8^+$ T cell response within mucosal tissues should add further support for this novel, promising approach.

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Appendix

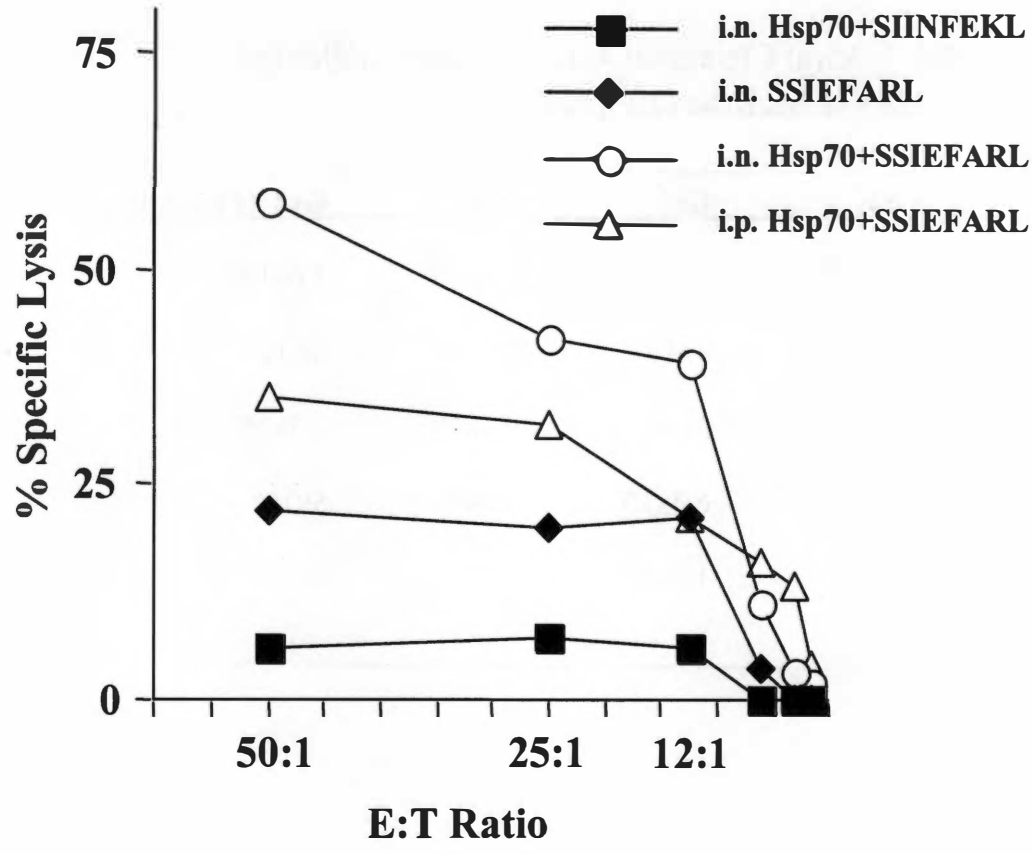

Figure 1. *Spleen CTL activity following systemic or mucosal Hsp70+SSIEFARL immunization 7d after the final boost using a standard 4 h ^{51}Cr release assay reveals enhanced levels of cytotoxicity in hsp70 mucosally immunized mice.* Various E:T ratios were performed using MC38 target cells loaded with gB498-505.

Table 1. Viral Clearance After Vaginal Challenge with HSV-1 McKrae (10^6 pfu)

Day	Gr1	Gr2	Gr3	Gr4 (PFU/ml)
1	$4.3x10^5$	$1x10^5$	$6.3x10^4$	$2.6x10^5$
2	$7x10^4$	$4x10^4$	$2.1x10^4$	4X10
3	$1.9x10^3$	$3x10^3$	$2.5x10^3$	$1.3x10^3$
4	$1x10^3$	$6.3x10^2$	$2x10^2$	$5x10^2$
5	$3x10^3$	$1.5x10^3$	0	0

Gr1-i.n. Hsp70+SIINFEKL
Gr2-i.n. SSIEFARL
Gr3-i.n. Hsp70+SSIEFARL
Gr4-i.p. Hsp70+SSIEFARL

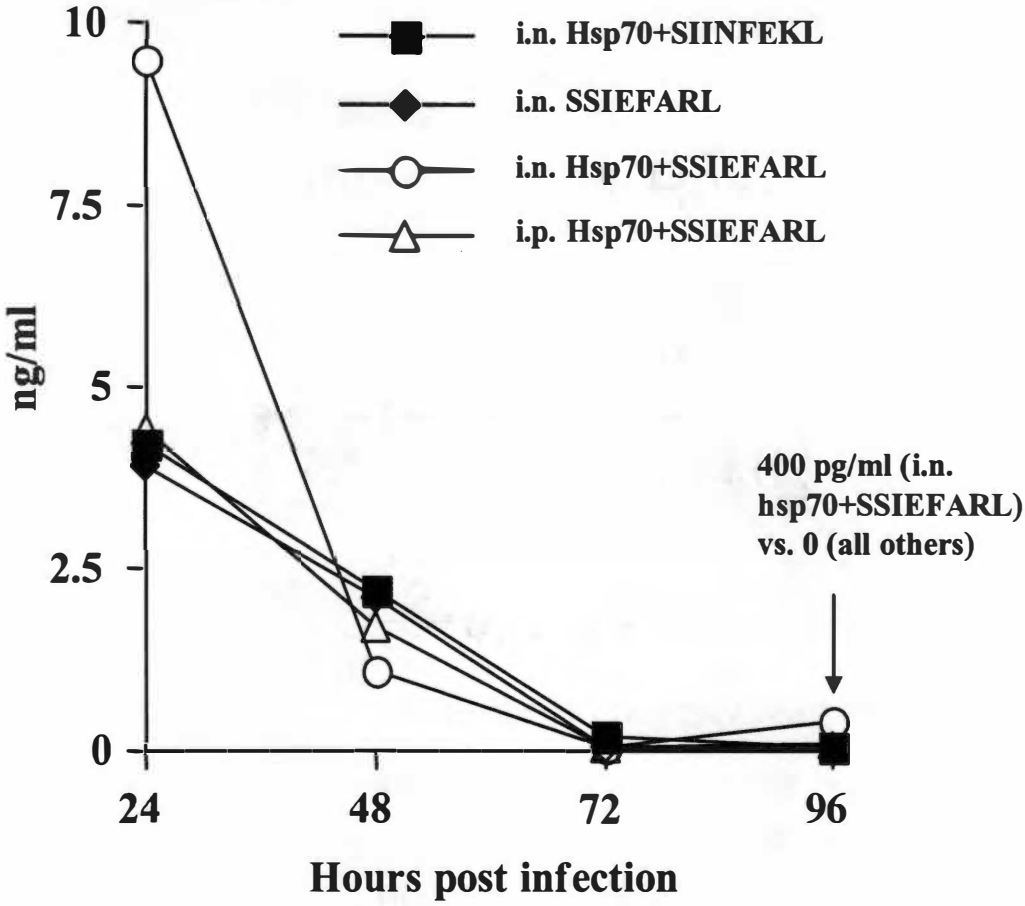

Figure 2. *Enhanced levels of IFN-γ are secreted in response to HSV-1 McKrae vaginal infection from mice mucosally immunized with hsp70 and SSIEFARL.* Data were obtained from vaginal washings performed every 24 h with PBS followed by ELISA for detection of IFN-γ.

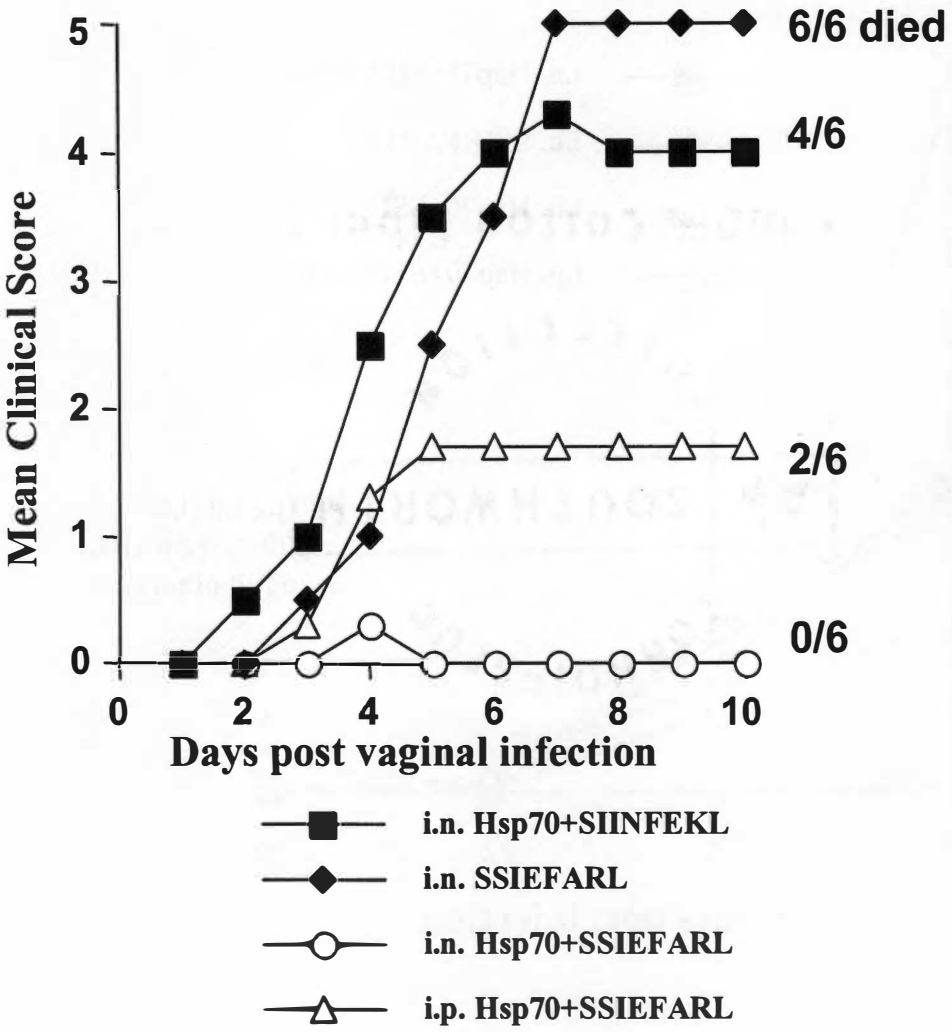

Figure 3. *Mice that received mucosal administration of hsp70 and SSIEFARL were resistant to vaginal challenge with HSV-1 during the acute phase response.* Mice were vaginally challenged with HSV-1 McKrae strain (10^6 pfu) on d5 following the final treatment. Grading was performed daily as follows: 1) mild vaginal inflammation, 2) moderate inflammation and swelling, 3) severe inflammation, ulceration, and hair loss, 4) paralysis, and 5) death.

Part IV.

Binding of an Anti-DNA Antibody to Plasmid DNA Enhances Cytotoxic T Cell Immune Responses

Abstract

Even though it has been over a decade since genetic vaccines were first introduced they have failed to generate effective responses in primates, despite strong observed responses in rodents. Therefore, a number of approaches have been investigated to improve DNA vaccine efficacy. In this study we describe a novel method for improving plasmid DNA delivery to antigen presenting cells by first binding an anti-DNA antibody to the plasmid DNA. Antibody-bound plasmid DNA is most likely taken up more efficiently via Fc receptors, thereby leading to higher levels of transcription and enhanced presentation to T cells. This antibody-bound plasmid DNA vaccine regimen, in comparison to plasmid DNA alone, also leads to an enhancement of the immune response to the encoded antigen as measured by IFN–γ secreting cells and cytotoxic T cell activity.

Introduction

Following the initial discovery that protective immunity and cytotoxic T cell responses (CTL) could be generated in vivo by plasmid DNA injection [1], many groups have used this approach in a multitude of vaccine candidates [2-7]. Despite success in rodents, genetic vaccination in primates has never reached the same level of efficacy, primarily due to low immunogenicity. It has been well documented that DNA given intramuscularly (i.m.) or intradermally is primarily taken up by muscle cells or keritinocytes, respectively. However, these cell types are unable to prime immune responses. It is currently assumed that these cells take up the DNA, express the encoded

protein, and then transfer this protein to professional antigen presenting cells (APC), such as dendritic cells [5,8].

A number of strategies exist that improve the potency of DNA vaccines. One way to enhance DNA vaccine potency involves modification of the vector to enhance antigen presentation (which may involve intracellular targeting or targeting to other cell types) [3,9,10]. Another general strategy deals with improving delivery of plasmid DNA, thereby enhancing transfection efficiency. Popular variations of this strategy include the use of recombinant bacteria such as salmonella [11], the use of bacterial ghosts [12], M cell targeting of plasmid DNA via attachment to a reoviral protein (thereby enhancing mucosal DNA delivery) [13], cationic microparticles [14], and in vivo electroporation [15]. In essence, each of these strategies attempts to increase the relative number of cells that take up the plasmid DNA. A third approach for improving DNA vaccine potency involves the inclusion of genetic adjuvants (such as cytokines, chemokines, or costimulatory molecules), either as a gene or a coadministered agent [16-19]. In this study we investigate the potential of using anti-DNA antibody to target plasmid DNA to APC, such as dendritic cells and induce cell-mediated immunity. Since DC express receptors for the Fc portion of IgG (Fcγrs), which mediate internalization of antigen-IgG complexes and promote efficient presentation of these internalized antigens, we investigated if this process might also be able to enhance delivery and uptake of plasmid DNA. In addition, Fc receptor binding has been shown to lead to the maturation of DC, a process that might further enhance the immune response to the encoded antigen [20].

Materials and Methods

Mice

Four- to five-week-old C57BL/6 (*H-2*^{b}) mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). In conducting the research described in this work we adhered to the *Guide for the Care and Use of Laboratory Animals* as proposed by the committee on care of Laboratory Animal Resources Commission on Life Sciences of the National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Peptides

The HSV gB (amino acids 498 to 505) peptide SSIEFARL and the chicken ovalbumin (aa 257 to 264) peptide SIINFEKL were synthesized and supplied by Research Genetics (Huntsville, AL)

Cell lines

Vero (African green monkey kidney cell line) was used for growing of viral stocks and MC38 was used as a target cell (C57BL/6 adenocarcinoma, *H-2*^{b}). All cell lines were cultured in Dulbecco's Modification of Eagle's medium (Mediatech, VA) supplemented with 10% heat-inactivated fetal bovine serum, 5 mg/L of gentamicin sulfate, and 2 mM L-

glutamine. T cell stimulation assays were carried out in 25 mM Hepes buffered RPMI-1640 media (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum, 5 mg/L of gentamicin sulfate, 0.05 mM 2-ME, and 2 mM L-glutamine.

MRSS-1 production and purification

MRSS-1 is a B cell hybridoma which makes a mouse IgG3 isotype anti-DNA antibody (ATCC #HB-69, Manassas, VA) [21]. The hybridoma was grown in roller bottles in DMEM 5% FCS (Mediatech, Herndon, VA and Hyclone, Logan, UT, respectively). The cells were filtered out using Whatman paper (Middlesex, UK) and the supernatant was frozen at -20°C until a later date. The supernatant was then thawed and mixed 1:1 with a saturated ammonium sulfate solution and allowed to sit at 4°C overnight for precipitation of immunoglobulins and other high-molecular weight proteins. The precipitated proteins were passed over a protein L column (Pierce, Rockford IL) to isolate mouse Ig by affinity chromatography. The concentration of the antibody was determined by UV spectroscopy.

Plasmid DNA preparation

The plasmid pcDNA-gB was created as described by Manickan et al [22]. It was propagated in *E. coli* DH5-α cells and grown in LB broth (both Invitrogen, San Diego, CA) and purified with an Endofree Plasmid Mega kit (Qiagen, Valencia, CA).

DNA/Antibody binding assay

pcDNA-gB was bound to a standard ELISA plate (Corning, Acton, MA) at [DNA]=5 μg/ml, 100ul/well using ReactiBind (Pierce, Rockford, IL) to bind the DNA to the plate. MRSS-1 was then added at various concentrations. Negative control wells contained non-specific mouse IgG (Southern Biotech Associates, Birmingham, AL). All wells were done in duplicate and the ELISA was performed as previously described [22].

T cell activation assay

An in vitro T cell activation assay was performed by first allowing splenic adherent cells (splenocytes adhered to plastic tissue culture plates for 2 h @ 37°C and then washed, scraped, and counted), consisting mainly of macrophage and DC, to take up plasmid DNA (pcDNA-gB), express protein, and present processed peptides to T cells. Antigen sources were as follows: 0.1 μg/ml of MRSS-1 anti-DNA antibody was incubated with 0.1 μg/ml of pcDNA-gB for 1 h @ 37°C (total vol 100 μl). This complex was then added to 500,000 (total vol 50 μl) splenic adherent cells in a 96 well U-bottom plate and allowed to incubate for 24 h 37°C, 5% CO_2 . Plasmid DNA without antibody as well as a group with an irrelavent control antibody were also included. For a positive control adherent splenocytes were either transfected with the same amount of pcDNA-gB as above using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen), or gB498-505 peptide (1 μg/ml) was used. Following the 24 h incubation 50, 000 (total vol 50 μl) T cell hybridoma specific for gB498-505, clone 2E2 [23]

(provided by F. Carbone) were added and the incubation was continued for another 24 h. Supernatant was then collected and tested for IL-2 production by ELISA.

ELISPOT

ELISPOT assay for IFN-γ secreting cells was performed as previously described, except for slight modifications [24]. Briefly, 96-well filter plates (Millipore HA) were coated with capture antibody for IFN-γ (RA-6A2) (BD Pharmingen) ON @ 4°C (BD Pharmingen) (2 μg/ml) in PBS. Plates were then washed with sterile PBS and blocked with culture media containing 10% FCS. Splenocytes from a single cell suspension were incubated at various effector:stimulator ratios. 10^5 irradiated, SIINFEKL or SSIEFARL-pulsed irradiated (3000 rads), syngeneic splenocytes were used as stimulators. Serial 2-fold dilutions of effectors were then incubated with stimulators starting with 5×10^5 and ending with 6.25×10^4. 20 U/well of IL-2 was also added to the culture. Cells were then incubated for 48 h at 37°C, 5% CO_2. Wells were then washed with PBS, followed by PBS-Tween. Biotin anti-mouse IFN-γ (XMG1.2) (BD Pharmingen) (1 μg/ml in PBS 3% BSA) was then added to each well and incubated ON @ 4°C. Wells were then washed with PBS-Tween and streptavidin-conjugated peroxidase (Jackson Laboratories, Bar Harbor, ME) in PBS 3% BSA was added to each well (1 μg/ml) for 30 min @ 37°C. Wells were then washed with PBS-Tween and developed using 3-amino-9-ethylcarbazole (AEC) (Sigma) in 0.1 M acetate buffer pH 5.0, containing 0.05% H_2O_2. Reactions were

allowed to proceed for 10 min and were ended by extensive washing with dH_2O. Plates were allowed to dry and then counted on a stereo microscope.

CTL assay

The CTL assay was performed as described earlier [24]. Briefly, effector cells generated after a 5 d in vitro expansion (with SSIEFARL-loaded, irradiated splenocytes) were analyzed for their ability to kill major histocompatibility complex (MHC)-matched antigen-presenting targets. The cells were mixed with the target at various ratios and incubated for 4 h. The targets included ^{51}Cr-pulsed MHC-matched SSIEFARL-pulsed as well as control SIINFEKL-pulsed MC38 target cells. Percent specific lysis was then calculated according to the following formula:

100 x [(experimental release - spontaneous release)/ (maximum release - spontaneous release)].

ELISA

ELISA was performed as previously described [24]. Briefly, 96-well EIA/RIA plates were coated with capture antibody for IL-2 (BD Pharmingen) ON @ 4°C (2 μg/ml) in 1 M Na_2HPO_4. The wells were then washed with PBS-Tween and supernatant taken after 24 h was added and the plates were incubated ON @ 4°C. For detection wells were

washed with PBS-Tween and biotin anti-mouse IL-2 (BD Pharmingen) (1 μg/ml in PBS 3% BSA) was then added to each well and incubated for 1h @ RT. Wells were then washed with PBS-Tween and streptavidin-conjugated peroxidase (Jackson Laboratories, ME) in PBS 3% BSA was added to each well (1 μg/ml) for 30 min @ RT. Plates were then washed and developed using ABTS (Sigma). ELISA readings were taken on an automated ELISA reader (SpectraMAX 340, Molecular Devices, Sunnyvale, CA).

Immunizations

90μg/mouse pcDNA-gB was complexed to 10μg/mouse MRSS-1 for 1 h @ 37°C. This preparation was then split in half and administered to both of the tibialis anterior muscles of the mouse on day 0. Negative controls included pcDNA-gB alone and pcDNA-gB + control mouse IgG (Southern Biotech Associates, Birmingham, AL). Both underwent the same incubation as the experimental preparation. Tibialis anterior administration of 10^6 pfu recombinant vaccinia virus expressing gB (VV-gB) served as a positive control.

Statistical analysis

The data were analyzed by using a 2-tailed student's *t* test, and p values less than .05 were deemed significant.

Results

The anti-DNA antibody MRSS-1 specifically binds to plasmid DNA

Initial experiments were first undertaken to determine if the MRSS-1 antibody could indeed bind to plasmid DNA encoding gB from HSV-1. As can be seen in figure 2, MRSS-1 antibody was able to bind pcDNA-gB, which was used for coating of the plate, in a dose dependent manner. Binding reached a saturation point at approximately 1 μg/ml. Control mouse IgG showed no significant binding to pcDNA-gB.

MRSS-1 antibody binding to plasmid DNA enhances antigen presentation to T cells

We next wanted to test if antibody-bound plasmid DNA could be more efficiently internalized into professional antigen presenting cells (APC), such as dendritic cells (DC), and the encoded gB be transcribed, translated, and presented to $CD8^+$ T cells. To this end we employed a T cell activation assay using a T cell hybridoma generated from a transgenic mouse in which 98% of the $CD8^+$ T cells recognize gB498-505, the dominant Class I-restricted epitope in C57BL/6 mice [25]. Adherent splenoytes were first incubated for 24 h with MRSS-1 antibody bound to pcDNA-gB, pcDNA-gB only, control mouse IgG and pcDNA-gB, MRSS-1 alone, lipofectamine and pcDNA-gB, or gB498-505 peptide. After allowing 24 h for expression and presentation, the gB-specific T cell hybridoma (2E2) was added to each well and after 24 h supernatant from each well was analyzed for production of IL-2. As depicted in table I, MRSS-1 bound pcDNA-gB lead to a 3 fold increase over pcDNA-gB alone in the amount of IL-2 (48 versus 16 pg/ml). However, lipofectamine and pcDNA-gB, which is far more efficient, generated strong

production of IL-2, with levels exceeding 1000 pg/ml. Only background levels of IL-2 (15-20 pg/ml) were detected with control mouse IgG + pcDNA-gB or MRSS-1 antibody alone. Despite a relatively low level of IL-2 production in comparison to lipofectamine or peptide, these data are promising and do suggest that MRSS-1 antibody might be facilitating the entry of pcDNA-gB into APC via Fc receptor-mediated uptake.

The anti-DNA antibody MRSS-1, when co-delivered with plasmid DNA, increases the number of IFN-γ responding $CD8^+$ T cells

We next wanted to assess the ability of anti-DNA antibody to enhance genetic vaccination. Plasmid DNA encoding gB was either injected alone or in combination with MRSS-1 anti-DNA antibody. After 8 days the spleen was removed and an ELISPOT assay was performed as described in materials and methods. As shown in figure 3, inclusion of MRSS-1 lead to a significant (2-3 fold) enhancement of IFN-γ secreting gB-specific T cells in comparison to mice immunized with pcDNA-gB. However, when compared to levels generated with a recombinant vaccinia virus encoding gB, levels were only modest in comparison. Control groups immunized with mouse IgG and pcDNA-gB did not demonstrate any significant increase in response over pcDNA-gB alone. In addition, MRSS-1 antibody administered without pcDNA-gB did not elicit a gB-specific response.

Addition of anti-DNA antibody to plasmid DNA vaccination increases cytotoxic T lymphocyte activity

We next tested the cytotoxic capability of CD8+ T cells that were generated from immunization with either pcDNA-gB, MRSS-1 anti-DNA antibody along with pcDNA-gB, or a control mouse IgG and pcDNA-gB. Vaccinia virus expressing gB was used as a positive control. Immunization with MRSS-1 only does not generate a significant CTL response (data not shown). As depicted in figure 4, addition of the anti-DNA antibody significantly increases (2 fold) CTL activity in comparison to immunization with plasmid DNA alone. At an effector:target ratio of 25:1, mice that were immunized with MRSS-1 and pcDNA-gB showed 30% killing of target cells, while pcDNA-gB immunized mice only had 15% specific lysis. Mice immunized with control mouse IgG and pcDNA-gB mounted a similar level of killing as pcDNA-gB only mice, suggesting that IgG is not exerting a non-specific effect upon immune response induction. Mice immunized with VV-gB had a much higher level of killing, with over 50% specific lysis at the same effector:target ratio.

Discussion

Genetic vaccination, despite over a decade of study and steady improvements, still lacks the potency and duration of live viral infection. Various strategies have been adopted to improve the efficacy of plasmid DNA vaccination. Among these include vector modification to increase expression or the inclusion of additional CpG stimulatory motifs. Other approaches focus on improving the immune response by adding adjuvants, either as a gene or protein, such as inflammatory cytokines and chemokines [5,19]. Still

other strategies include efforts to improve delivery of the plasmid DNA to cells of interest. Most reports regarding this approach do not specifically target a specific cell population, but instead increase the overall transfection efficiency (most commonly of myocytes). Examples include in vivo electroporation and cationic microparticle delivery of the DNA [14,15]. A recent study by the Pascual group was one of the first to attempt to target plasmid DNA to a particular cell type [13]. They used a protein from from reovirus, σ1 ,that attaches to M cells. Attachment of this reoviral protein to plasmid DNA encoding HIV gp160 greatly enhanced both humoral and cell-mediated mucosal immune responses. Therefore, specific targeting of plasmid DNA to a critical cell type, such as DC, may prove advantageous for the generation of a protective response.

In order to target pcDNA-gB to APC we used an anti-DNA antibody, MRSS-1, which is a non sequence specific antibody that binds structural regions of the phosphate backbone of DNA. MRSS-1 binds plasmid DNA and most likely improves the ability of plasmid DNA to be uptaken by APC, thereby increasing the level of gene transcription, translation and ultimately presentation of the foreign protein. This should then permit T cell activation, a process not observed in vitro with naked plasmid DNA. In addition, plasmid DNA immunizations often require several boosters to obtain measurable responses. Here we show that only one immunization is necessary to generate $CD8^+$ T cell responses. This strategy presumably operates via Fc-mediated internalization of the DNA/antibody complex, thereby increasing the transfection rate of APC by specific targeting. Our results suggest that this approach also increases cell mediated immunity, as measured by the level of HSV-specific IFN-γ secreting cells and specific cytotoxic activity. Since Fc-receptor internalization results in the targeting of material to

endosomes (and ultimately the Class II-restricted pathway), it is surprising that any material escapes to the cytosol. However, reports do exist demonstrating that material internalized via Fc receptors can reach the cytosol and lead to cross-priming, the activation of $CD8^+$ T cells with exogenously obtained antigen [26,27]. If proteins can access this minor pathway perhaps plasmid DNA can as well. At the present it is not known which Fc receptor is responsible for the observed enhancement. Additional work will focus on determining the role of individual Fc receptors in this process by antibody blocking experiments, both in vitro and in vivo. In addition, this approach would ideally use a sequence specific anti-DNA antibody, which has been described [28], to avoid possible autoimmune reactions. Other critical factors to consider include the possible effects of antibody dependent cell-mediated cytotoxicity (ADCC), a process which may occur in vivo since DC may keep anti-DNA antibody (IgG3)/DNA complexes on their surface for a short period of time bound to CD16 (FcγRIII), allowing for the possibility of NK cell-mediated lysis. Delivery of a maturation stimulus through binding of Fc receptors on the DC may also enhance immune responses, much like a general adjuvant [20]. This additional stimulus would only add to the pre-existing TLR9 stimulation from the CpG motifs of the plasmid DNA [29]. Work is currently underway to address the contribution and effect of Fc-mediated maturation on DC. In addition, we are investigating the relative impact of anti-DNA antibody/plasmid DNA vaccination upon gB-specific $CD4^+$ T cell and antibody responses. However, the ultimate test of any vaccine candidate is challenge with the infectious agent of interest. Studies assessing the effect of this novel approach against a cutaneous HSV-1 infection are underway using a

zosteriform challenge model [22]. These additional experiments should reveal the overall effectiveness of this novel approach.

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Appendix

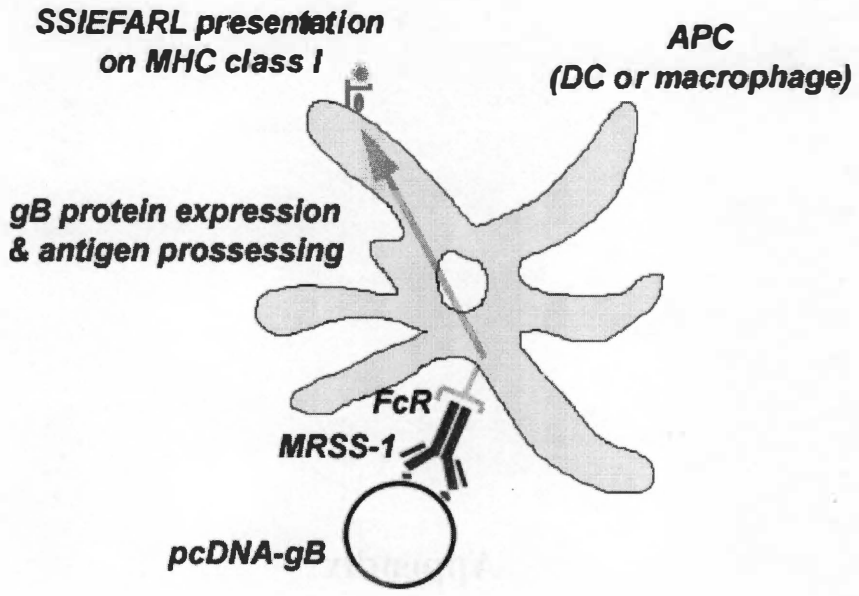

Figure 1. *Schematic representation of plasmid DNA/anti-DNA antibody vaccination approach*

Table 1. MRSS-1 antibody bound to pcDNA-gB activates a T cell hybridoma specific for gB498-505

Adherent Splenocytes loaded with:	IL-2 (pg/ml)
pcDNA-gB (0.5 mg/ml)	16
pcDNA-gB (0.5 mg/ml) +MRSS-1 mAb (0.5 mg/ml)	48
pcDNA-gB (0.5 mg/ml)+ mouse IgG (0.5 mg/ml)	15
MRSS-1 mAb alone	17
Lipofectamine + pcDNA-gB	1005
gB498-505 (1 mg/ml)	1553

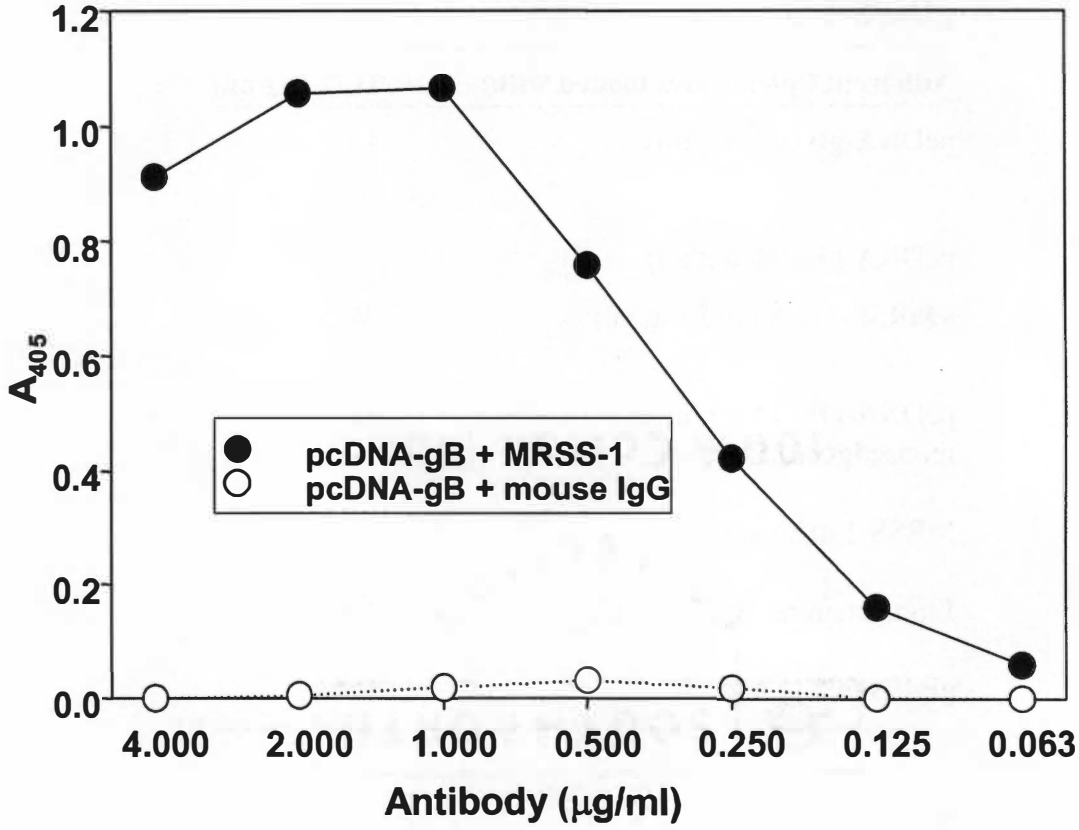

Figure 2. *MRSS-1 antibody binds plasmid DNA (pcDNA-gB) in a dose dependent manner*

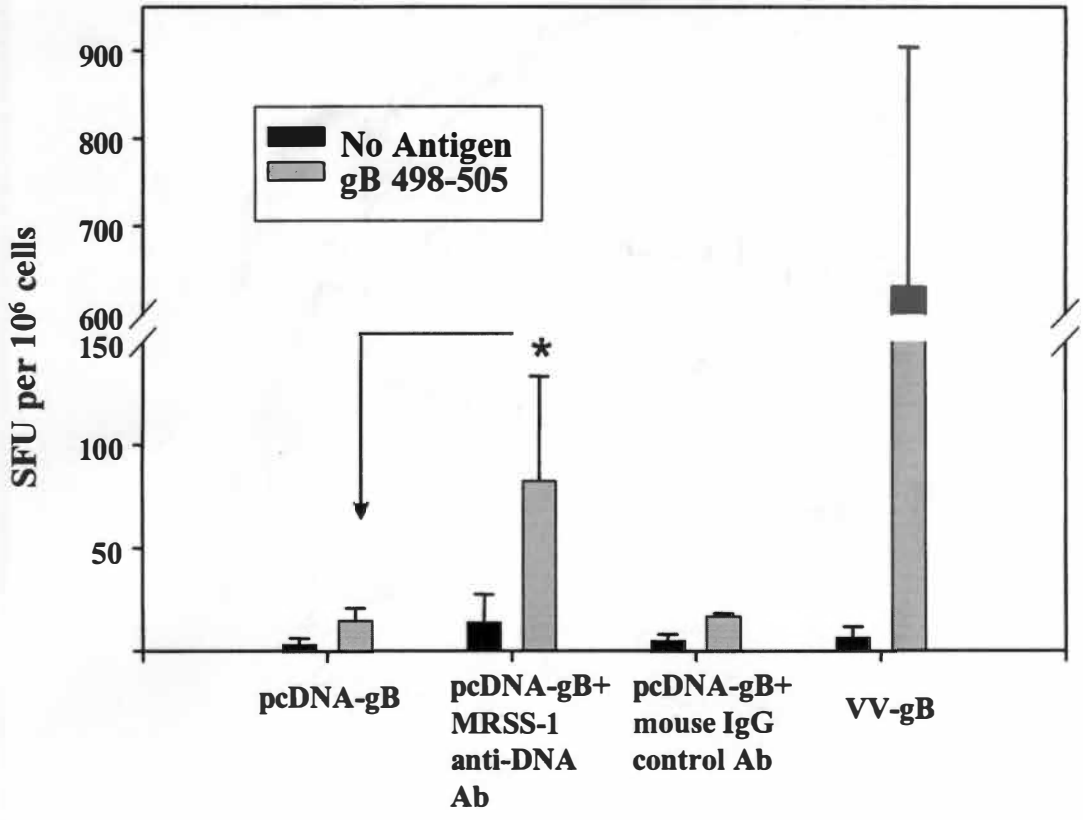

Figure 3. *MRSS-1 bound pcDNA-gB increases the number of IFN-γ secreting cells during the acute phase response as measured by ELISPOT*

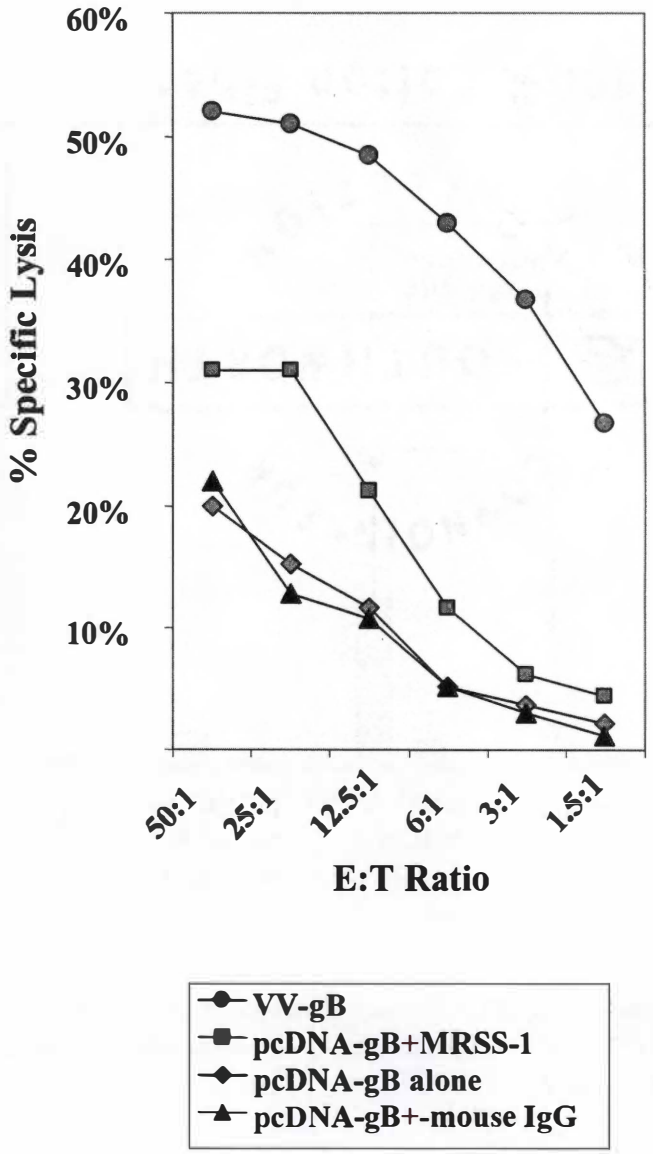

Figure 4. *CTL Activity is enhanced in the spleen of mice immunized with pcDNA-gB and MRSS-1 anti-DNA antibody*

Vita

Christopher Dean Pack was born on January 28, 1973, in Knoxville, Tennessee. He grew up in Knoxville and attended Halls High School, graduating Salutatorian of his class in 1991. He then attended The University of Tennessee, Knoxville for his undergraduate education on an Alumni Merit scholarship. He majored in microbiology and developed a keen interest in immunology towards the end of his undergraduate tenure in the lab of Dr. Habib Zaghouani. He graduated Cum Laude from Tennessee in 1996 and was also inducted into Phi Beta Kappa in recognition of his achievements. For his undergraduate research he received the Lisa Kahn Memorial Award. At that time he continued work in the lab of Dr. Zaghouani, who further cultivated his interest in a scientific career. Chris completed a Master's degree in microbiology at Tennessee with honors in August of 2000. His research focused on mechanisms of immune tolerance, specifically in the neonate. After graduation he continued his education in the lab of Dr. Barry T. Rouse, also at the University of Tennessee. In Dr. Rouse's lab his work concentrated on vaccine design for herpes simplex virus. He was awarded the Alexander Hollaender Fellowship in 2003. He graduated with a Doctorate of Philosophy in Comparative and Experimental Medicine in December of 2004 and plans on pursuing further post doctoral training in immunology.