Published in final edited form as:

Expert Rev Vaccines. 2009 August ; 8(8): 1023–1035. doi:10.1586/erv.09.60.

New concepts in herpes simplex virus vaccine development: notes from the battlefield

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Abstract

The recent discovery that T cells recognize different sets of herpes simplex virus type 1 and type 2 epitopes from seropositive symptomatic and asymptomatic individuals might lead to a fundamental immunologic advance in vaccine development against herpes infection and diseases. The newly introduced needle-free mucosal (i.e., topical ocular and intravaginal) lipopeptide vaccines provide a novel strategy that might target ocular and genital herpes and possibly provide 'heterologous protection' from HIV-1. Indeed, mucosal self-adjuvanting lipopeptide vaccines are easy to manufacture, simple to characterize, extremely pure, cost-effective, highly immunogenic and safe. In this review, we bring together recent published and unpublished data that illuminates the status of epitope-based herpes vaccine development and present an overview of our recent approach to an 'asymptomatic epitope'-based lipopeptide vaccine.

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Keywords

animal model; asymptomatic; clinical trial; epitope; genital herpes; herpes simplex virus; immunoprophylactic; immunotherapeutic; lipopeptide; ocular herpes; symptomatic; vaccine

Herpes simplex virus (HSV) types 1 and 2 (HSV-1 and HSV-2) are two pathogenic agents that typically cause lifelong recurrent immunopathologic diseases in man, ranging from fatal disseminated disease in newborns, to skin lesion (cold sores), genital ulcerations, blinding eye lesions and fatal encephalitis in adults [1–6]. While many people have frequent recurrences of herpes disease (i.e., ‘symptomatic’ or high recurrent disease patients, with five or more episodes of recurrent disease per year), others have no history of recurrent disease (i.e., ‘asymptomatic’ patients). The cellular and molecular immune mechanisms underlying herpes disease recurrences are unknown, yet essential to understand in order to develop efficient immunoprophylactic and immunotherapeutic vaccine strategies.

We recently made the unique observation that a set of ‘promiscuous’ human HSV-1 and HSV-2 epitopes was strongly recognized by T-effector cells (T_{eff}) cells from asymptomatic patients but not by T cells from symptomatic patients [7,8]. By contrast, a different nonoverlapping set of epitopes was strongly recognized by T cells from symptomatic patients, but not by T cells from asymptomatic patients. This finding suggests that a vaccine or immunotherapy that contains asymptomatic epitopes may promote the expansion of asymptomatic T cells, while the inclusion of symptomatic epitopes might promote immunopathological responses. The use of epitope-based vaccines (rather than whole virus or whole proteins) would allow the inclusion of asymptomatic epitopes and the exclusion of symptomatic epitopes, resulting in a new approach for the treatment of recurrent herpes diseases.

During primary infection the virus:

- Infects the host at mucocutaneous surfaces including the cornea, mouth, genital tract and skin
- Invades the local sensory nerves by propagating via neurons
- Establishes lifelong latency in the neuron bodies of sensory ganglia (sacral ganglion [SG] for genital herpes and trigeminal ganglia for ocular and oral herpes)

During latency, the viral genome is mostly transcriptionally inactive. Factors such as hormonal changes, stress and UV exposure trigger the virus to reactivate, travel back to and reinfect the primary site of infection (i.e., the eye, mouth or the genital tract). A good example of recurrent herpes disease is herpes stromal keratitis (HSK), an inflammatory disease of the cornea, which may result in corneal opacity and blindness, following reactivation of HSV-1 from latently infected trigeminal ganglia (TG). Despite multiple approaches to therapy and prevention, HSV-1 and HSV-2 remain among the most common infectious agents of humans [7,9,10]. Existing treatments include topical and/or oral administration of antiviral drugs [8]. There are currently no US FDA-approved vaccines available for HSV. Although the standard antiviral drug regimens (e.g., acyclovir) reduce/suppress recurrent symptomatic disease, asymptomatic shedding and transmission, they do not clear the infection or stop recurrent disease. Antiviral drug therapy shortens the duration of lesions and reduces their recurrence. However, development of drug-resistant virus strains is possible, especially in immunocompromised individuals, such as AIDS patients. Symptomatic herpes infections can be clinically recognized and reduced by antiviral therapy. However, many HSV infections are clinically unrecognized (asymptomatic) and are therefore not treated by available therapies. The ideal vaccine would not only prevent the acute disease produced by initial infection, but also prevent latency or reduce reactivations, which are the source for recurrent infections.

Given the worldwide magnitude of the number of HSV-infected individuals, effective vaccines offer the best hope and would be the most powerful tool for controlling the spread of HSV-induced diseases. Since genital herpes increases HIV-1 transmission by up to sixfold, an efficient vaccine strategy that controls HSV-2 would probably help decrease the spread of HIV [11,12]. Thus, development of a 'heterologous' needle-free mucosal (i.e., intravaginal) herpes vaccine deserves urgent attention and could become a significant approach towards preventing the spread of HIV-1 [13]. However, subunit formulations delivered into the genital tract (GT) are poorly immunogenic compared with other mucosal routes (e.g., the intranasal route) [14–16]. Thus, the progress towards an intravaginal vaccine still faces significant challenges, including:

- The identification of critical human 'protective' CD4⁺ and CD8⁺ T-cell epitopes (i.e., epitopes mostly recognized by T cells from asymptomatic patients)
- The improvement of protective 'naturally processed' T-cell epitopes
- The development of an efficient and safe intravaginal immunization strategy

We made several unique observations demonstrating that intravaginal or ocular delivery of needle-free lipopeptides containing herpes simplex epitopes in 'string-of-pearls' CD4⁺ and CD8⁺ T-cell pairs can induce strong local and systemic immunity with protective efficacy against herpes [6,13,17–20].

In general, development of an effective vaccine against HSV is complicated by some of the unique characteristics of herpes viruses [15,21–23]. These include:

- The complexity of the virus replication cycle (i.e., primary, latent and recurrent phases of infection) [24]
- The relatively poor understanding of the major effectors of herpes immunity that control each of the three phases of infection [25]
- The sophisticated immunoevasion strategies that HSV-1 and HSV-2 have evolved to dampen the immune response (e.g., the downregulation of antigen-presenting machinery by the *ICP47* gene)
- The identification of protective antigens and epitopes from over 84 protein candidates encoded by the large and complex herpes genome
- The primary infection does not induce natural immunity that efficiently prevents spontaneous reactivation and recurrent disease

Ocular herpes: a leading cause of corneal blindness due to an infectious agent

Often, the initial HSV-1 infection of the eye does not produce any significant clinical symptoms and can go unnoticed. Latency is established in sensory neurons and sporadic reactivations occur throughout life, even in those who never develop any clinical disease. In the USA, approximately 500,000 people have a history of recurrent ocular herpes. The number of asymptomatic individuals, who sporadically shed infectious reactivated virus in their tears that can be transmitted to others, is much greater [26–28]. Current estimates of latent HSV-1 range between 80 and 90% of adults in the USA, with one study suggesting that as many as 98% of adults sporadically shed the virus [26–28].

Recurrent ocular disease can include corneal epithelial and stromal tissue damage and development of chronic vision-impairing lesions known as HSK. In a murine model, HSK is a T-cell-mediated immunopathological lesion. Lesions do not occur in athymic mice unless they are reconstituted with T cells [13,29–31]. Similarly, HSK is not common in

immunodeficient humans [32]. These observations prompt many researchers to believe that HSK is the consequence of T-cell-mediated immunopathogenesis. In murine models of ocular herpes, a large number of inflammatory cells infiltrate into the cornea following acute infection, including polymorphonuclear neutrophils (PMNs), macrophages, Langerhans cells, natural killer (NK) cells, plasma cells and T cells. CD4⁺ helper T cells of Th1 type are the most profoundly activated lymphocytes involved in the pathogenesis of HSK [33]. It should be noted that spontaneous reactivation of HSV-1 does not occur in mice (or does so at extremely low levels) and mice therefore do not develop spontaneous recurrent disease. Thus, mouse studies of HSK are usually limited to the results of the acute infection, which is likely to be significantly different from recurrent HSK in humans. HLA-transgenic (Tg) mice that mount human-like T-cell responses exist, but since they also lack spontaneous reactivation, studies are still limited to primary infection.

Rabbits are also widely used as a model for ocular herpes. They have the advantage of supporting HSV-1 spontaneous reactivation, and one model develops a high level of recurrent HSK [34–36]. Unfortunately, similar to wild-type mice, rabbits do not respond specifically to human HLA-restricted T-cell epitopes. Recently, a Tg rabbit line expressing human HLA class I was developed and is now being used in our laboratories [37].

Genital herpes: a cofactor in HIV transmission & acquisition

Genital herpes is mainly caused by HSV-2, although the incidence of genital herpes caused by HSV-1 appears to be increasing [38,39]. Genital herpes is one of the most prevalent sexually transmitted infections (STIs). Primary genital infection with HSV is followed by viral latency in the sensory SG. Spontaneous reactivation of the virus in SG, and its return to the genitals, results in shedding of infectious virus. Most spontaneous reactivations are asymptomatic, that is, they cause no significant genital disease, while some spontaneous reactivations can lead to severe recurrent herpetic disease.

HSV-2 may be an important cofactor in the spread of HIV since, based on epidemiological evidence, individuals with a history of recurrent genital herpes lesions are up to six-times more likely to contract HIV [40,41]. Mathematical modeling predicts that, in populations where HSV-2 prevalence is 60% or more, almost half of sexually acquired HIV can be attributed to genital herpes [40,42–45]. Recent studies concluded that genital herpes has played a more important role than any other STI in driving HIV prevalence in Africa [40,45–47]. The role of genital herpes in increasing acquisition of HIV is most likely due to herpes genital lesions providing an efficient portal for entry of HIV. If this is correct, then both a prophylactic herpes vaccine that decreases the incidence of acquiring genital herpes and a therapeutic herpes vaccine that decreases the incidence of recurrent genital herpes lesions would probably help reduce the spread of HIV [35].

Vaccination approaches against herpes infection & disease

Over the last two decades, numerous efforts have been made to develop a vaccine against ocular and genital herpes infection and disease. Despite promising results of several candidate vaccines in animal studies and early phases of human trials, most large clinical HSV-2 vaccine trials have been disappointing. These vaccine strategies include:

- Genetically attenuated live virus [15,48]
- Whole inactivated virion preparations [49]
- Recombinant subunit (glycoprotein) vaccines [50,51]
- Replicating nonpathogenic vectors that express one or more HSV antigens [52–54]

- DNA plasmids expressing one or more HSV proteins (genetic immunization) [31, 55–58]

Neutralizing antibody does not appear to produce efficient protection against herpes infection in humans [59,60]. In addition, spontaneous reactivation and shedding of infectious virus occurs at high levels in individuals latently infected with HSV [46,61], indicating that the natural immunity acquired following primary infection and subsequent reactivations is not sufficient to prevent subsequent spontaneous reactivations or shedding of infectious virus capable of spreading the infection. It also suggests that, in some individuals, natural immunity can completely control (prevent) recurrent disease (i.e., asymptomatic individuals), while in other individuals natural immunity is insufficient to prevent recurrent disease (symptomatic individuals), even though there is no apparent difference in virus shedding between these groups. This is consistent with our findings that although symptomatic and asymptomatic individuals recognize many of the same HSV epitopes, they appear to recognize different subsets of epitopes (see later).

The current paradigm in herpes vaccine development is that a highly efficacious vaccine will need to induce a more vigorous and/or different T-cell response than the suboptimal immunity induced by natural infection [23,48,62]. Decreasing recurrent infections more efficiently than natural suboptimal immunity is likely to require stronger T-cell immunity of both a higher magnitude and wider breadth, or a selective induction of T-cell responses to a specific subset of viral epitopes.

Glycoproteins & tegument protein-based vaccine

Glycoproteins

HSV-1 specifies at least 11 glycoproteins that are expressed in infected cells. Most of the research on subunit vaccines has used HSV envelope glycoproteins, specifically glycoprotein (g)B and/or gD, as immunogens since these are the dominant targets for neutralizing antibody production in HSV-infected people. gB and gD are attractive choices for subunit vaccines because they are the targets for humoral (neutralizing and antibody-dependent cellular cytotoxicity) and cell-mediated immunity (class I and class II restricted). In addition, gB and gD have high sequence similarity in HSV-1 and HSV-2 and may, therefore, provide protection against both HSV-1 and HSV-2 infections.

Clinical trials of a gB and gD subunit vaccine using MF59 as adjuvant did not show any protection despite induction of high neutralizing serum antibody titers [16,51,60]. More recently, intra-muscular vaccination with a recombinant HSV-2 gD vaccine, using 3'-O-deacylated-monophosphoryl lipid A as an adjuvant, protected approximately 70% of women who were HSV-1 and HSV-2 seronegative; however, there was no protection among men or among HSV-1-seropositive women [51]. These results raised important questions regarding the role of gender-related factors on vaccine efficacy. In this clinical trial, despite the vaccine inducing high neutralizing antibody titers that exceeded those of natural immunity, recurrent disease was not reduced, suggesting that induction of a vigorous cellular immunity is critical for therapeutic protection.

Tegument proteins

Tegument proteins are sandwiched between the envelope and capsid protein core of HSV and have been reported to be major targets for T-cell responses. A recent study used an enzyme-linked immunospot assay that utilized pools of overlapping synthetic peptides presented to purified CD8⁺ T cells through autologous dendritic cells. The response to individual open reading frames ranged from 5 to a maximum of 70%, with the greatest responses directed against the tegument proteins *UL39*, *UL25*, *UL27*, *ICP0*, *UL46* and *UL47* (in descending order).

These six tegument proteins are therefore considered to be good candidates for T-cell based vaccines [63]. However, whether the T-cell responses of asymptomatic versus symptomatic individuals to these tegument proteins were similar or different remains to be determined.

Identification of symptomatic & asymptomatic T-cell epitopes: a novel concept in herpes vaccine development

Substantial research has recently been directed towards the development of a new generation of vaccines that are based on the identification and inclusion of immunogenic ‘epitopes’ in the subunit vaccines. However, there is evidence that under certain conditions some immunogenic epitopes can induce pathological events and do more harm than good. We therefore recently suggested that if such pathogenic or symptomatic epitopes can be identified, they should be excluded from herpes vaccines as their inclusion might decrease the vaccine’s immunotherapeutic potential by inducing harmful immune responses. As mentioned in the introduction, we recently found that there is a set of ‘promiscuous’ human HSV-1 and HSV-2 epitopes that are strongly recognized by T_{eff} cells from asymptomatic patients but not by T cells from symptomatic patients and vice versa [9,10]. This suggests that a vaccine containing only asymptomatic epitopes while excluding symptomatic epitopes might promote immunotherapeutic responses while avoiding induction of immunopathological responses. Therefore, a good starting point for the development of an efficient immunotherapeutic vaccine against herpes diseases would be to identify as many symptomatic and asymptomatic HSV T-cell epitopes as possible from glycoprotein and tegument target antigens.

Results from a number of studies indicate that gB and gD, the two major HSV-1/2 antigens, produce some protective immunity against herpes disease in both animal models and humans, and are recognized by $CD4^+$ and $CD8^+$ T cells from both symptomatic and asymptomatic HSV-seropositive humans. In the early 1990s, Zarling and coworkers generated gB- and gD-specific $CD4^+$ T clones from severely infected HSV-2-seropositive symptomatic patients who had recurrent HSV-2 genital infections approximately every 8 weeks with recurrent oral lesions [7]. Koelle and coworkers were able to recover both $CD4^+$ and $CD8^+$ HSV-specific T-cell clones from the HSV-2 lesions of five patients [53]. Burke and coworkers also isolated HSV-specific $CD8^+$ T-cell clones from a patient with recurrent genital herpes that lysed vaccinia virus/gD2-infected target cells [48]. Cunningham and coworkers reported that gB and gD are the major target glycoproteins for both $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes (CTLs) using human epidermal keratinocytes [22]. Although the aforementioned studies suggest that gB and gD are the targets of both symptomatic and asymptomatic $CD4^+$ and $CD8^+$ T cells, no specific symptomatic or asymptomatic gB or gD epitopes were defined. If people with a history of severe recurrent disease (i.e., symptomatic individuals) tend to develop T cells that recognize a subset of epitopes (i.e., symptomatic epitopes) that differs from those recognized by T cells from asymptomatic individuals (i.e., asymptomatic epitopes), it would be logical to exclude such symptomatic epitopes from vaccines on the grounds that they may enhance rather diminish the recurrent disease. In 2008, we made the unique observations that a set of promiscuous human HSV-1 and HSV-2 gB epitopes was strongly recognized by T_{eff} cells from asymptomatic patients but not by T-cells from symptomatic patients [64]. By contrast, a different nonoverlapping set of gB epitopes was strongly recognized by T cells from symptomatic patients, but not by T cells from asymptomatic patients. The strategy and criteria used for the selection of symptomatic and asymptomatic epitopes from tegument protein UL25, is summarized in Figure 1. We should emphasize that although qualitative criteria are used in the segregation of symptomatic and asymptomatic epitopes from HSV-1 antigens, we do not exclude the possibility that qualitative differences such as different cytokine profiles and/or different cytolytic capabilities may be associated with recognition of particular epitopes and, if so, these findings should emerge from our future studies. More importantly, we also observed that immunization of susceptible double-Tg mice expressing both type 1 and type 2

human leukocyte antigens (i.e., HLA-DR and HLA-A2.1) with asymptomatic T-cell epitopes, but not with symptomatic T-cell epitopes, reduced the severity of herpetic lesions after ocular or intravaginal challenge with virulent strains of HSV-1 and HSV-2.

Concept of multivalent herpes vaccine

A question of practical importance is the translation of the current findings using a HLA-DRB1*0101-HLA-A2.1 double-Tg mouse strain in the development of self-adjuvanting clinical Th-CTL lipopeptide vaccines for a genetically heterogeneous human population. Although the high degree of HLA polymorphism is often pointed to as a major hindrance to the use of epitope-based vaccines, this constraint can be addressed through the inclusion of multiple supertype-restricted epitopes recognized in the context of diverse related HLA alleles, and by designing Th-CTL lipopeptide vaccines with higher epitope densities. A Th-CTL lipopeptide-based herpes vaccine could include multiple CD8⁺ T-cell epitopes present in diverse herpesvirus protein antigens that are chosen to represent at least the HLA-A2, -A3 and -B7 supertypes, known to provide recognition in up to 95% of the global population, regardless of race and ethnicity. Hence, with the particular properties required of human vaccination in mind, we have conceived studies to identify HLA class I-degenerate T-cell epitopes in HSV protein antigens targeted by CD8⁺ T cells from seropositive humans and HLA-Tg mice [64]. These epitopes, along with similarly identified HLA-DR supertype-restricted HSV CD4⁺ T epitopes, would provide the database needed to develop multi-epitope Th-CTL lipopeptide vaccines that are broadly recognized in the majority of outbred racial and ethnic populations. Such multivalent Th-CTL lipopeptide vaccines might contain asymptomatic CD4⁺ and CD8⁺ T-cell epitopes selected from envelope and tegument proteins.

Memory T cells & the pathogenesis of herpes disease

The intermittent reactivation of HSV-2 viral infection provides a unique situation to examine how repeated antigenic challenges shape memory T-cell repertoires [7,65]. Posavad *et al.* found that peripheral blood mononuclear cells from HSV-2-infected individuals showed the persistence of CD8 CTL for up to 7 years [66]. The generation and maintenance of CD8⁺ memory T cells have been studied in both murine models and humans, but only limited work has been performed for CD4⁺ memory T cells. There is growing evidence that memory CD8⁺ T cells may reside in nonlymphoid tissues following viral infections. In one study, CD8⁺ T cells were shown to reside in multiple tissues that did not appear to harbor viral genome or proteins, leading to the suggestion that memory CD8⁺ T-cell retention might not be an antigen-driven process [67–69]. Preferential localization of effector memory cells is in nonlymphoid tissue. There appears to be a dynamic balance between HSV-1 latency and reactivation involving a tripartite interaction between the virus, the host neuron and the local immune components. At any given time, some neurons may escape these control mechanisms and viral reactivation may occur [24]. Frequent reactivation of HSV-1 in human TG may reflect a less efficient CD8⁺ T-cell response. It is still unclear as to which CD4⁺ and CD8⁺ T-cell subset is most beneficial for conferring long-term protective versus immunopathological memory. We hypothesize that the clinical spectrum of herpes, ranging from asymptomatic to frequently distressing outbreaks, may be reflected in memory CD4⁺ and/or CD8⁺ T-cell recognition of different sets of epitopes from one or several HSV protein antigens. Thus, the recognition of a set of viral epitopes by ‘pathogenic’ memory T cells might be associated with severe immunopathologic diseases, while recognition of a different nonoverlapping set of viral epitopes by protective memory T cells designated asymptomatic might, in turn, lead to immunoprotection.

Involvement of T-cell-dependent heterologous immunopathology in herpes disease

It must be remembered that humans are not immunologically naive and they often have a higher number of memory T-cell populations that can cross-react with, and may disproportionately contribute, to other infectious pathogens. These cross-reactive T cells can become activated and modulate the immune response and outcome of subsequent heterologous infections, a phenomenon termed T-cell-dependent heterologous immunity and immunopathology. It is now clear that memory T cells laid down as a consequence of one infection can influence protective immunity to an unrelated virus (reviewed in [70]). CD4⁺ and CD8⁺ T-cell cross-reactivity between viruses can also produce damaging immunopathological responses, a phenomenon known as heterologous immunopathology. Thus, memory T cells from a virus infection outside the herpes family can influence protective immunity and immunopathology against herpes. The unique and private epitope-specific repertoire of each individual, known as 'private specificity', can also influence the pattern of heterologous immunity. Therefore, some of the symptomatic and asymptomatic HSV-specific CD4⁺ and CD8⁺ T cells identified so far might cross-react with self or other pathogen-derived epitopes [71–73]. Such scenarios have been recently reported in murine heterologous infection and may be true in humans. This has been shown for T-cell responses to cytomegalovirus and other pathogens [70–72].

Cellular immune mechanisms underlying symptomatic versus asymptomatic herpes disease

Considering the wealth of information addressing the role of T cells in animal models, it is surprising how few reports exist exploring the immunologic basis of symptomatic and asymptomatic HSV infection in humans. Identification of T-cell-mediated immune mechanism (s) by which asymptomatic patients control herpes disease and symptomatic patients do not is critical for the logical development of herpes vaccines. Among the multitude of complex mechanisms that might be in play are:

- Differences in precursor frequency, proliferative capacity and functional property of symptomatic versus asymptomatic epitope-specific T cells. Indeed, the T-cell repertoire of individuals with the same MHC restriction elements can vary significantly because of 'heterologous immunity' and 'private specificity';
- The differential level of infiltration/homing into sites of infection – specifically corneas, genital areas and/or sensory ganglia – of T cells specific to symptomatic versus asymptomatic epitopes, that would affect viral production and disease [65];
- Asymptomatic epitopes might trigger proliferation of 'protective' T cells within the sites of infection, while symptomatic epitopes might trigger pathogenic T cells. Indeed, there is evidence that under certain conditions some immunogenic epitopes can do more harm than good and might therefore be considered pathogenic or symptomatic;
- The symptomatic epitopes may direct T-cell responses away from those that are best suited to clear the viral infection with minimal pathogenic reaction;
- An immunopathogenic T-cell response might occur through stimulating low-affinity oligoclonal responses that inhibit broad-based T-cell responses to other well-presented high-affinity epitopes, thus deviating protective responses to damaging responses;
- Differences in T_{eff} cells lingering after recent shedding and/or disease compared with memory T cells maintained in the absence of antigenic exposure;

- T-cell cross-reactivity with epitopes from other viruses [71–73], which can also play a role in protective heterologous immunity versus damaging heterologous immunopathology [70].

Regardless of the mechanism(s), if symptomatic individuals tend to generate T cells that recognize a discrete set of symptomatic epitopes that differs from the set of asymptomatic epitopes, it would be logical to exclude such symptomatic epitopes from future herpes vaccines on the grounds that they may enhance rather than diminish recurrent herpes diseases.

Vaccines bearing asymptomatic peptide epitopes to stimulate ocular, oro-facial & genital mucosal immunity: an attempt for a change in path

Development of a herpes subunit vaccine has been motivated by the previous achievements obtained from other pathogens. Major hurdles include identification of antigens that focus the exquisite specificity of the immune system on HSV-1- and HSV-2-infected cells without harming uninfected cells. So far, the early clinical trials for gB- and gD-based subunit vaccines have induced merely low-to-moderate protection against herpes disease, which indicates the need for an improved delivery system as well as the identification of novel target antigens. However, this task is far from complete because of the large and complex herpes genome that encodes at least 80 polypeptides, each of which could be a potential target to a protective immune effector.

The mucocutaneous surfaces constitute an impressive first-line defense system that is frequently exposed to an array of exogenous antigens and infectious pathogens, including HSV-1 and HSV-2. The majority of traditional herpes vaccines are injected parenterally, which induces strong systemic immune responses. However, this approach rarely generates the significant mucosal T-cell immunity at the eye or at the GT or at sites of local draining lymph nodes, which is probably needed to limit severity of the disease [74–79]. We have recently found that:

- Topical ocular and intravaginal vaccination with herpes lipopeptides (i.e., peptides covalently linked to a fatty acid moiety, as a Toll-like receptor-2 agonist) induced higher local ocular and GT protective immune responses than parenteral immunization [13,36];
- Protective immunity against genital herpes infection and disease can be induced by highly immunogenic self-adjuvanting gD lipopeptide vaccines [18] [Chentoufi AA, Dasgupta G, Choudhury Z *et al.*, Submitted manuscript];
- Th-CTL chimeric epitopes extended by one palmitic acid moiety can induce HSV-1-specific effector CD8⁺ TC1 responses and protect mice against ocular infection [20];
- Lipopeptide antigens are taken up by mucosal dendritic cells (DCs)/Langerhans cells and can activate immature DCs to become mature DCs, the only form of DC capable of priming T cells at the mucosal level [13,19,13,35,36,80–83].

Since lipopeptides bearing murine CD4⁺ and CD8⁺ T-cell epitopes are able to cross genital mucosal surfaces [5,18,20], we hypothesize that herpes lipopeptides bearing multiple asymptomatic human CD4⁺ and CD8⁺ T-cell epitopes can target critical protective epitopes across mucosal genital membranes allowing epitope delivery to induce both local and systemic immune responses.

Development of a multivalent needle-free lipopeptide vaccine: a new cause for optimism

The multi-epitope approach should induce herpes immunity based on the induction of T cells specific to several asymptomatic epitopes from various proteins and glycoproteins. This relies on the identification and selection of protective asymptomatic CD8⁺ and CD4⁺ T-cell epitopes, which have recently been found in our laboratory. This will help to assess the type specificity of clonal T-cell responses induced by natural infections in both adults and neonates and provide insight into the nature of protective immunity against both HSV-1 and HSV-2.

Unmodified synthetic peptides usually fail to prime T-cell responses *in vivo* unless they are delivered with a potent immunological adjuvant. Peptide-based T-cell epitope vaccines have been emulsified with a variety of adjuvants, including Freund's adjuvant, MF59 and QS-21 [81–85]. Most of the adjuvants tested in small laboratory animals have limitations in clinical trials due to toxicity. Currently, alum (aluminum-based mineral salts) is the only adjuvant that has acceptable toxicity for widespread use in humans. However, peptides adsorbed to alum are effective only in inducing Th2-associated antibody responses. Alum is usually ineffective in inducing Th1 and CTL responses and both Th1 and CTL responses are crucial in protecting against genital herpes. As an alternative, lipopeptide vaccines have recently gained considerable interest and represent a promising novel approach [81–85]. As discussed above, past clinical trials of traditional subunit vaccines (such as recombinant glycoproteins gB–gD, or gD alone) applied parenterally with powerful adjuvants have shown poor efficacy [5,18–20,35,36]. A first wave of preclinical and clinical vaccine trials for many infectious diseases show that lipopeptides are efficient, safe and can be scaled-up to good manufacturing practice levels by modern methods of peptide synthesis [19,81,86]. In a recent Phase I/II clinical trial, a HIV lipopeptide vaccine produced strong and long-lasting CD4⁺ and CD8⁺ T-cell responses [87–90]. Both CD8⁺ CTL and CD4⁺ helper T lymphocyte cellular immune responses appear to be the dominant arms of defense in anti-herpes immunity. Since they are likely to be characteristic of a successful vaccine, it is crucial to determine the critical asymptomatic CD4⁺ and CD8⁺ T-cell epitopes within the HSV-1/2 glycoprotein and tegument proteins. Self-adjuvanting CD4–CD8 lipopeptides using these critical asymptomatic epitopes can then be constructed. We recently demonstrated that topical ocular and intravaginal delivery of a prototype lipopeptide vaccine can stimulate mucosal immune responses (Figure 2) [19].

These results constitute a proof-of-principal that will extend the application of immunogenic HSV-1/2 lipopeptide vaccines against ocular and genital herpes infection and disease.

The cellular and molecular mechanisms behind the immunogenicity of lipopeptides are the subject of considerable debate [5,83]. Our own view is that the lipid moiety likely exerts its adjuvant effect by interacting with Toll-like receptor-2 receptors, which trigger the MyD88-mediated endogenous signal transduction pathway, and by stimulating a variety of immune cell types *in vivo*, including DCs [5,84]. We have previously demonstrated that herpes lipopeptides are taken up more efficiently by DCs than monocytes/macrophages [83]. We also demonstrated recently that the HSV-1 gD1–29 lipopeptide epitope extended by a Ne-Palmitoyl lysine moiety can interact with and drive maturation of DCs [5].

Experimental animal models for the study of HSV latency, reactivation & immunity

Owing to the obvious ethical and practical considerations in studying human immune responses, a challenge for herpes immunologists is: what animal model is the most appropriate for studying how immunization with human HLA-restricted T-cell epitopes can decrease

recurrent herpes disease resulting from spontaneous viral reactivation? Ideally, the animal model should be able to produce an immune response to human HSV epitopes (such as HLA-A*0201-restricted epitopes), while mimicking all aspects of viral pathogenesis, neuropathology, neuroinvasiveness and latency. Unfortunately, such a natural animal model does not exist. Rabbits infected ocularly with HSV-1 develop ocular disease and latent infections in their TG with sporadic spontaneous reactivation detectable by infectious virus in tears (shedding) and rare incidences of recurrent corneal disease [91]. Guinea pigs infected genitally with HSV-2 develop genital disease, the virus establishes latency in SG, and sporadic spontaneous reactivation occurs resulting in infectious virus being detected in the GT along with high levels of recurrent genital disease [92,93]. However, neither of these animal models respond to HLA-restricted human epitopes. To our knowledge, no one has constructed any Tg guinea pigs, and Tg rabbits are rare.

For most immunologists, the mouse is a preferred model owing to the availability of unlimited inbred and Tg strains, specific immune molecule knockout strains and the well-characterized immunological probes available to study the mouse immune response to specific therapies. In addition, HLA-Tg mice are available. Although the mouse model has provided much important information regarding ocular herpes infection and immunity, and despite the tremendous advances in understanding the mouse immune system, the mouse model lacks significant HSV spontaneous reactivation.

Recently, a HLA-Tg rabbit line was developed [37]. Similar to their wild-type parents, ocular HSV-1 infection of these rabbits results in acute corneal disease and ganglionic latency, followed by sporadic spontaneous reactivation detectable in tears. Thus, we now have a humanized *HLA-A*0201*-Tg rabbit model of ocular HSV-1 that has spontaneous reactivation, produces recurrent HSK similar to the clinical disease and expresses human, rather than rabbit, HLA class I molecules. These susceptible humanized HLA Tg rabbits therefore recognize and mount immune responses to HSV-1 human CD8⁺ T-cell epitopes [Chentoufi AA, Dasgupta G, Choudhury Z *et al.*, Submitted manuscript]. To our knowledge, this HLA Tg rabbit model is the only animal model with spontaneous HSV-1 reactivation that can develop humanized CD8⁺ T-cell responses to human HSV epitopes. Thus, we now have a HLA animal model with HSV-1 spontaneous reactivation that will allow us for the first time to investigate the role of human-specific CD8⁺ T cells in reducing HSV-1 spontaneous reactivation. In addition, we now have a HSV mutant (CJLAT) that produces high levels of recurrent HSK in rabbits (up to 70% of eyes, compared with ~1–2% of wild-type HSV-1-infected eyes) [94,95]. This will now allow us to assess the role of human-specific CD8 T cells in the pathogenesis of recurrent HSK.

Expert commentary

The recent findings that different sets of HSV epitopes are recognized by T cells from symptomatic versus asymptomatic individuals might lead to a fundamental immunologic advance in vaccine development against herpes infection and/or diseases. An efficient immunotherapeutic herpes vaccine would include only the protective (asymptomatic) T-cell epitopes and exclude the pathogenic (symptomatic) epitopes. The lack of an appropriate animal model with a humanized immune response (HLA-Tg) and spontaneous HSV reactivation from latency has stalled the preclinical development of an immunotherapeutic vaccine against the virus. To our knowledge, the recently developed HLA Tg rabbit model is the only animal model with spontaneous HSV-1 or HSV-2 reactivation that can respond to humanized T-cell epitopes, and thus can be used for human vaccine development. Finally, newly introduced needle-free mucosal (i.e., topical ocular and intravaginal) lipo peptide vaccines provide an unprecedented strategy against ocular and genital herpes.

Five-year view

In recent years, there have been remarkable advances in the development of new technologies of immunological monitoring, genomics, proteomics and epitope mapping, resulting in a better understanding of the immune system and its working mechanisms. It is necessary to understand why past clinical HSV vaccine trials, such as recombinant gB and gD, did not meet expectations before we can rationally develop the next generation of vaccines. Future HSV vaccine development will depend on our capacity to validate and compare new vaccine strategies in appropriate preclinical animal models. The ongoing identification of herpes symptomatic and asymptomatic epitopes has increased the possibility that a vaccine could be developed to protect against herpes infection and reactivation, and/or recurrent disease in the near future.

We believe that in the next 5 years research should focus on:

- Identifying more asymptomatic versus symptomatic herpes epitopes;
- Qualitatively and quantitatively analyzing T cells in symptomatic versus asymptomatic patients that could break new ground in our understanding of the immune mechanisms underlying herpes pathogenesis in humans;
- Incorporating promiscuous asymptomatic epitopes into vaccines;
- Using mucosal vaccine strategies, such as lipopeptides, to immunize against herpes;
- Using ‘humanized’ susceptible HLA-Tg mice and rabbits to assess the immunogenicity and protective efficacy of herpes epitopes against primary and recurrent infection.

Owing to the high cost and incomplete protection with anti-viral drugs, along with the possibility of generating drug-resistant HSV strains, a cost-effective immunotherapy that can prevent HSV reactivation from latency would be highly useful against herpes [19,96,97]. Such immunotherapies should induce a vigorous immunity that must be qualitatively different (i.e., induce protective asymptomatic but not pathogenic symptomatic responses) and/or produce quantitatively higher protective responses than the natural suboptimal immunity detected in symptomatic individuals.

The presence of new humanized HLA-Tg animal models should greatly facilitate future vaccine development. In particular, the new humanized HLA-Tg rabbit model, which has spontaneous reactivation, produces recurrent HSV similar to the clinical disease and expresses human, rather than rabbit, HLA class I molecules, should greatly facilitate preclinical studies related to therapeutic herpes vaccines.

The next-generation vaccines will use needle-free mucosal application in which the epitopes stimulate the mucosal immune system. We recently found that synthetic peptide epitopes extended with an agonist of Toll-like receptor-2, that are abundantly expressed by DCs and epithelial cells of the vaginal and ocular mucosa, can lead to induction of protective immunity against herpes [98,99]. Thus, mucosal (topical ocular or intravaginal) immunization with self-adjuncting lipid-tailed peptides, bearing asymptomatic epitopes, appears to have attractive practical and immunological features.

Key issues

- Herpes simplex virus (HSV)-1 and HSV-2 infections cause prevalent, lifelong genital, dermal and ocular infections, with a spectrum of clinical manifestations including cold sores, genital ulceration, corneal blindness and encephalitis.

- One of the hallmarks of HSV infection is the establishment of a lifelong latent infection in sensory neurons of the trigeminal ganglia and sacral ganglia. An effective immunotherapy must produce an optimal immunity that can decrease HSV reactivation from latency and/or decrease virus replication following a reactivation event. Such a vaccine would be a highly useful cost-effective method of significantly reducing ocular and genital herpes. Such immunotherapies should induce a vigorous immunity that must be qualitatively different and/or quantitatively higher than the natural suboptimal immunity detected in seropositive symptomatic individuals. We expect that this can be accomplished by including asymptomatic epitopes while excluding symptomatic epitopes from the vaccine.
- while symptomatic and asymptomatic patients have similar virus-shedding rates, it is not known why virus reactivation tends to be asymptomatic in some individuals and symptomatic in others, or why the frequency and severity of recurrences vary among symptomatic patients.
- A needle-free mucosal vaccine stimulating the ocular and genital mucosal immune system would be beneficial for global delivery of a herpes vaccine. Such a lipopeptide-based herpes vaccine strategy is a promising approach, and the first wave of preclinical and clinical vaccine trials for many other infectious diseases has shown that lipopeptides are efficient, safe and can be scaled-up to good manufacturing practice levels by modern methods of peptide synthesis. In addition, a self-adjuvanting multivalent vaccine approach, designed to stimulate multiple layers of the body's immune cells, such as cytotoxic or killer CD8⁺ T cells and CD4⁺ T-helper cells, should help overcome most limitations of currently used monovalent vaccines.
- New 'humanized' HLA-transgenic mice and rabbit models should greatly facilitate future vaccine development. In particular, humanized HLA-transgenic rabbits would provide a better model, rather than normal rabbit, to study the spontaneous HSV reactivation and to assess the protective efficacy of human HLA-restricted epitopes, rather than rabbit MHC-restricted epitopes.
- Since genital herpes increases HIV-1 transmission by up to six-times, a vaccine that reduces genital herpes would be expected to also reduce the spread of HIV. Thus, development of a herpes vaccine may be an excellent alternative approach to fight the devastating AIDS epidemic.

Acknowledgments

Financial & competing interests disclosure

Studies performed by the authors (Gargi Dasgupta, Aziz Alami Chentoufi, Anthony B Nesburn, Steven L Wechsler and Lbachir BenMohamed), and reported herein were initiated and supported by research grants EY14900, EY14017 and EY09392 from the National Eye Institute, NIH by the Discovery Eye Foundation, and Research to Prevent Blindness grants. Lbachir BenMohamed and Anthony B Nesburn are founders of a recently incorporated biotechnology company (Micro Antigen Technologies, LLC), which is involved in making vaccines and immunotherapeutic agents against herpes. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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Figure 1. Selecting protective asymptomatic CD4 and CD8 epitopes from any HSV-1 target antigen

Step 1 indicates selection of promiscuous CD4 and CD8 T-cell epitopes from HSV tegument protein *UL25* by computational algorithms. Step 2 shows further screening of T-cell epitopes based on their high-affinity *in vitro* binding with soluble HLA class I and class II molecules. Step 3 indicates further selection of CD4 and CD8 T-cell epitopes based on their *in vitro* T-cell antigenicity using T cells from HSV-seropositive individuals. Step 4 shows the most antigenic CD4 and CD8 epitopes are divided into asymptomatic (green) and symptomatic (red) epitopes as recognized by T cells from HSV-seropositive asymptomatic and symptomatic patients. From this point onward, all symptomatic epitopes will be rejected from further studies (see symbol 'X'). Step 5 shows the *in vivo* screening of all asymptomatic CD4 and CD8 epitopes in terms of their T-cell immunogenicity is tested in 'humanized' HLA double-transgenic mice. Step 6 shows the final selection of best protective asymptomatic CD4 and CD8 epitopes as tested in HLA double-transgenic mice. Step 7 shows the chemical ligation of the best selected protective asymptomatic CD4 and CD8 epitopes along with a lipid moiety (palmitic acid molecule) at the N-terminus. The inset shows the ultimate construct of a multivalent lipopeptide (LipoVac) bearing several pairs of CD4–CD8 epitopes from HSV tegument protein *UL25* that are synthesized in tandem with GPGPG sequences (spacers) and then covalently linked at the N-terminal with a lysine (K) that is precoupled with one PAM. HSV: Herpes simplex virus; PAM: Palmitic acid moiety.

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Figure 2. Operating cellular mechanisms for mucosal immunization with lipopeptide vaccines

Lipopeptide antigens applied to intravaginal mucosal epithelium are taken up by mucosal resident antigen-presenting cells (APCs), especially DCs/pDCs, which are activated and migrate out of the mucosal inductive sites into the mucosal effector sites of draining lymph nodes where antigen presentation to T cells occurs, resulting in a strong local mucosal immune response. The mucosal immunization with a multivalent lipopeptide vaccine targeting TLR-2 induces an immune mechanism that bridges innate immunity to the ‘asymptomatic’ epitope-specific adaptive T-cell response. DCs are activated through recognition of the lipopeptide by TLR-2 receptors molecules. This triggered activation of the MyD88-dependent pathway and leads to the production of inflammatory cytokines (IL-12, IL-6 and TNF- α), and to phenotypic maturation of APCs (i.e., upregulation of costimulatory and MHC molecules on DCs surface). The multiepitope lipopeptide vaccine is captured, processed and presented by MHC molecules on the matured DCs to T lymphocytes (signal 1: MHC/TCR interaction). This is not sufficient to activate naive T lymphocytes, which need an additional signal from costimulatory molecules (signal 2: B7/CD28), of which expression on DCs is induced by TLR-2 stimulation. Activated T lymphocytes become further differentiated to effector T lymphocytes by stimulation with cytokines such as IL-12.

DC: Dendritic cell; HSV: Herpes simplex virus; HTL: Helper T lymphocyte; MyD88: Myeloid differentiation primary response gene 88; pDC: Plasmacytoid dendritic cell; TCR: T-cell receptor; TLR: Toll-like receptor.