

Recent Progress in Herpes Simplex Virus Immunobiology and Vaccine Research

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INTRODUCTION	96
ETIOLOGIC AGENT	96
HERPES SIMPLEX VIRUS CELLULAR REPLICATION CYCLE AND IMMUNE RESPONSE	97
PATHOGENESIS AND POINTS OF INTERACTION WITH INNATE AND ACQUIRED IMMUNITY	98
IMMUNE EVASION	99
ACQUIRED IMMUNE RESPONSE TO HERPES SIMPLEX VIRUS	100
CD4 T-Cell Responses	100
CD8 T-Cell Responses	101
Antibody Responses	101
Type-Common and Type-Specific Responses	102
VACCINE DEVELOPMENT	102
Peptide Vaccines	103
Subunit Vaccines	103
Killed-Virus Vaccines	104
Fractionated-Virus Vaccines	104
Discontinuously Replicating Viruses	105
Replication-Competent Live-Virus Vaccines	105
POTENTIAL TOXICITIES OF VACCINES	106
IMPACT OF SPECIFIC IMMUNITY ON CELL AND GENE THERAPY	106
UNANSWERED QUESTIONS AND PROSPECTS	106
ACKNOWLEDGMENTS	107
REFERENCES	107

INTRODUCTION

Medically serious complications of herpes simplex virus (HSV) infection are rare but constitute a significant burden, given the high rates of HSV seropositivity in the population (58). Many prophylactic and therapeutic vaccination approaches have been explored for the prevention or treatment of HSV infection. Thorough reviews have included a historical perspective on HSV vaccines and descriptions of preclinical work (28, 41, 42, 71, 98, 180, 254, 255, 291, 292). The basic virology, pathogenesis, epidemiology, and clinical syndromes due to HSV infections are also the subjects of recent excellent reviews (218, 292). This review emphasizes vaccines reaching clinical trials in humans and recent findings relevant to the immunobiology of HSV. Both acquired and innate immune responses are discussed; while classic vaccines influence only acquired immunity, it has been increasingly realized that adjuvants affect the outcome of vaccination in large part by influencing innate immunity.

ETIOLOGIC AGENT

The DNA genomes of HSVs contain about 85 open reading frames. Five of the open reading frames are diploid. Initiation

at internal methionine residues, mRNA splice variants, autocatalysis, and extensive posttranslational modifications such as phosphorylation, ribosylation, nucleotidylation, ubiquitination, and glycosylation add complexity to the proteome. Approximately half of the genes of HSV-1 are dispensable for replication in cultured cell lines (218). Fewer studies have been performed for HSV-2, but it is likely that most of the homologous HSV-2 genes are similarly dispensable or required. Deletion mutants with lesions in essential genes can also usually be propagated *in vitro* using *trans* complementation. Many HSV genes, especially those that are dispensable *in vitro*, are involved in immune evasion and pathogenesis. Both essential and nonessential genes are targets for modification in whole-virus vaccine formats.

HSV-1 and HSV-2 are members of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genera *Simplexvirus* (219). Their genomes are relatively stable compared with those of RNA viruses such as human immunodeficiency virus type 1 (HIV-1) or hepatitis C virus (HCV) (23, 54). HSV-2 has an inherently higher mutation rate than HSV-1 (225). Mutant strains can readily be selected *in vivo* by antiviral drug therapy (80). While patient-derived strains have specific nucleotide sequences, few data are available concerning variability within specific T- or B-cell epitopes (40, 100, 269).

Given the overall stability of the genome, it has been assumed that the viral strain chosen as the genetic data source for a subunit vaccine or as the parental strain for whole-virus

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approaches is relatively unimportant. Recent evidence that inactivated (or live) virus, or isolated viral proteins, can activate programs of innate immunity (4, 202) indicate that strain selection may still be important even if the epitopes recognized by the acquired immune system are relatively constant. Also, examples of variations in neutralizing epitopes among wild-type HSV-2 isolates have been documented (29). It is not known if immune escape variants arise in response to acquired immune responses and possibly reascend the axon, superinfect the ganglia, and reestablish latency, leading to endogenous reinfection. The related issue of exogenous reinfection is reviewed below. Mutant strains with a temporary, local replication advantage might also be more likely to be shed and transmitted. With the recent definition of many CD4 and CD8 epitopes and their HLA-restricting alleles (120, 122, 138), this hypothesis can be addressed.

HERPES SIMPLEX VIRUS CELLULAR REPLICATION CYCLE AND IMMUNE RESPONSE

The HSV replication cycle is central to understanding immunity to HSV and vaccine design. Interactions between HSV-encoded glycoproteins and cellular glycosaminoglycans, such as heparan sulfate, are involved in virion binding to the cell surface (234). The HSV envelope glycoproteins gB, gD, and gH-gL are required for HSV binding and entry into cells. The neutralizing activity of antibodies directed against these proteins (83, 88, 104, 105, 187, 208) was one of the major rationales for the use of HSV glycoproteins as immunogens for HSV subunit vaccines.

After initial binding, interaction between gD and one of several high-affinity receptors is required for viral entry (45, 247, 248). The first receptor protein to be characterized was HveA (herpesvirus entry mediator A), a trimeric transmembrane tumor necrosis factor (TNF) receptor family member also known as TNFR14 or HVEM (191). Physiologic ligands of HveA include the TNF-like molecules LIGHT and lymphotoxin- α 3 (168, 227). HveA can mediate costimulatory or proapoptotic (248) signals through its cytoplasmic domain (110, 162).

HveA is expressed by lymphoid and monocyte-dendritic lineage cells (75, 140, 191, 222). These cell types are permissive for HSV entry and replication *in vitro* (183, 215, 295), and some are present in lesions (63). Formal studies demonstrating that infection of lymphoid and myeloid cells is dependent on HveA have not been reported. While blood leukocyte cultures are occasionally positive in neonates or immunocompromised subjects (256), leukocytes are not currently appreciated as major targets of productive HSV infection *in vivo*. However, *in vitro* infections can be established in these cells (183) and modulation of the cellular functional activities by HSV could still influence local immune responses.

The second class of gD receptors to be described are several members of the immunoglobulin superfamily termed nectins, including nectin-1 α (HveC). These transmembrane proteins may serve as homophilic adhesion molecules and localize to cadherin-based cell junctions. The nectins occur as several isoforms based on mRNA processing. A third type of receptor is generated by specific sulfation of heparan sulfate by certain 3-*O*-sulfotransferases (234). The relative importance of these

classes of receptors *in vivo* is uncertain, but the expression of HveC by sensory neurons (96, 167) implicates this receptor in the pathogenesis of neuronal infection.

Elements within gD essential for interaction with HveA and the nectins have been identified (226, 227, 289). Reciprocal studies have defined host structures that interact with gD (45, 164, 248), and a crystal structure of gD bound to HveA (47) has been solved. Not unsurprisingly, a gD domain recognized by potent neutralizing antibodies is essential for interaction with these receptors (290). These recent insights raise the possibility that improved neutralizing antibodies might be elicited by subunit vaccines containing gD or other glycoproteins that present optimized neutralizing epitopes (290). Neutralizing epitopes might potentially include fusion intermediate structures formed during viral entry, as recently defined for HIV-1 (141). Receptor-blocking compounds based on gD interactions have already shown proof of principle *in vitro*.

Compelling evidence for the protective effect of antibodies *in humans* is available from studies of exposed neonates (37). If cervicovaginal HSV infection is present, the risk of serious neonatal infection after vaginal delivery is much lower if the mother has recurrent as opposed to primary HSV infection and is therefore able to provide the neonate with antibodies. The risk is not zero, however, and because more vaginal deliveries occur during recurrent than primary HSV infection, a considerable proportion of cases of neonatal herpes do occur as a result of recurrent infection.

Because HSV appears to enter using proteins that function in cellular communication and adhesion, it is also possible that vaccination could elicit primary antibodies, or anti-idiotypic antibodies, with pathologic effects. However, no such events have been noted in a series of subunit vaccine trials which have enrolled over 10,000 subjects. Homozygous mutations of HveC are known to be associated with congenital abnormalities (265), and overexpression of the HveA ligand, LIGHT, leads to abnormal lymphoid tissue (233). Further research into the normal physiological functions of HSV receptors and ligands may allow rational prediction of the toxicities of vaccines or therapeutics directed at inhibiting HSV entry.

Anti-gH antibodies also have potent neutralizing activity (208). The exact role of gH and the identity of the host structures that it interacts with (if any) during viral entry with are unknown. gH forms an obligatory complex with gL (287). Deletion of gH (82) is the basis of the DISC series of discontinuously replicating HSV strains that have been tested as human vaccines (see below). These gH-deleted viruses must be grown on complementing cell lines. After successful entry into, and replication in, wild-type cells, gH-deficient progeny virions are incapable of completing entry into a second generation of cells.

After envelope fusion, the tegument and nucleocapsid are delivered to the cytoplasm. These "virion input proteins" gain access to the HLA class I pathway of antigen processing to CD8 T cells without a requirement for *de novo* synthesis (120, 272). Viral genes are then expressed in a temporally regulated program. Immediate-early proteins are expressed shortly after viral entry. Some, such as ICP4, are required for subsequent events in replication, so that cells infected with an ICP4 deletion mutant will express only the other immediate-early proteins and can be used to probe HSV-specific cellular immune responses (120). Others, such as ICP27, are associated with

high-titer replication and animal virulence and have been deleted in candidate replication-competent vaccine strains (173). The US12 gene encoding ICP47 is expressed with immediate-early kinetics. ICP47 is an inhibitor of the transporter associated with antigen processing (106, 297), a mandatory cellular intermediate in the presentation of most viral epitopes to CD8 T cells. Deletion of ICP47 leads to decreased neurovirulence in mice (95) and increased recognition of infected cells by human CD8⁺ CTL (272). Viruses with deletions of multiple immediate-early genes have also been studied and have proven immunogenic in animal vaccination studies (35).

Immediate-early gene products in turn activate the transcription of early genes, which are expressed prior to DNA replication. Deletion of UL29, an early gene encoding the DNA binding protein ICP8, prevents DNA replication and true late-gene expression, while deletion of UL39, an early gene encoding the large subunit of ribonucleotide reductase, is associated with decreased virulence. An HSV-2 strain with a deletion in ICP8 has been used as a live attenuated vaccine and has been shown to attenuate experimental challenge with virulent HSV-2 (64). Double deletions within HSV-2 of both UL29 (ICP8) and UL5, which encodes a second protein required for DNA replication, have been constructed to increase safety for possible vaccine use (65, 66). In general, the breadth and magnitude of anti-HSV immune responses were found, in mice, to increase as viruses expressing additional temporal subsets of HSV proteins were used as vaccines (195). Late genes are expressed mostly after DNA replication and are required for viral assembly and egress. Mutations that reduce or block viral assembly or egress can be complemented by engineered host cells (68, 84). Other than gH-deleted strains (82), these viruses have seen little investigation as vaccines. Deletion of a late gene such as the one encoding gH has the theoretical advantage of expressing an almost full set of viral proteins. Knockout of genes in multiple kinetic classes has also been investigated. For example, lesions in the late *whs* gene (UL41, see below), which has immune evasion properties, increase the immunogenicity of ICP8-deleted HSV-1 in mice (91).

PATHOGENESIS AND POINTS OF INTERACTION WITH INNATE AND ACQUIRED IMMUNITY

HSV is thought to be transmitted from person to person as cell-free virus. Abrasions help HSV gain access to living cells below the keratinized debris on skin surfaces. Theoretically, local antibodies could neutralize virus prior to cell entry. In this scenario, no initial replication would occur, preventing both neuronal infection and horizontal transmission. This form of sterilizing immunity has been achieved in mice but requires vaginal immunization with live HSV (205). More typically, vaccines which fully protect from morbidity and mortality in animals still allow local replication at genital mucosal sites of inoculation (135). While HSV-specific antibodies are detectable in the cervical secretions in HSV-infected persons (11) and can be elicited by vaccination (12, 205), it is doubtful if sterilizing immunity can be achieved in humans by current vaccine approaches.

Subsequent to epithelial infection, HSV enters sensory nerve endings and ascends in a retrograde manner to neuronal

cell bodies in ganglia. It is not known if a level of immunity exists that differentiates between peripheral sterilizing immunity (205) and protection against initial entry into sensory neurons. The neurons become productively infected, and virus is transmitted across synapses. This phenomenon is thought to account for the HSV DNA that is widespread in the human central and peripheral nervous system (94, 112), although other explanations (hematogenous spread) are also possible. Resolution of lytic ganglionic infection is dependent on (236) and temporally associated with (249) the infiltration of CD8-bearing cells. At the same time, the transcription and expression of HLA class I increases, even for neurons, which are typically negative for these proteins (209). Control of ganglionic infection does not seem to be associated with cytotoxic T-lymphocyte (CTL) killing of neurons, implicating cytokine-mediated nonlytic halt of replication (165, 210). Recently, interest in the role of NK cells in the control of primary HSV infection has been renewed by the mapping of a susceptibility locus in mice to within or near an NK cell receptor locus (211), although the role of NK cells in the control of primary HSV in humans is unclear.

A further consequence of ganglionic infection is the establishment of latency in a variable number of neurons. Latency is associated with transcription of an RNA species termed LAT (latency-associated transcript). While the major LAT contains open reading frames (218), expression of the predicted proteins has been difficult to document in vivo. Recently, it has been appreciated that the levels of cytokines of lymphoid origin are persistently elevated in HSV-infected ganglia (in animals) (50). Acyclovir treatment reduces this local immune activation (97). Additionally, low levels of lytic gene expression can be documented in "latently" infected ganglia by reverse transcription-PCR (51, 133) and may drive local activation of antigen-specific T cells or innate immunity cells. Reactivation of latent HSV from isolated ganglia *ex vivo* is promoted by anti-CD8 or anti-gamma interferon (IFN- γ) treatments (149, 150), implying that resident CD8 cells and/or lymphocyte-derived cytokines contribute to the control of reactivation. Taken together, these data imply that acquired immunity in general, and CD8 T cells in particular, may be important in both initial control and suppression of reactivation at the level of the ganglia.

Prevention of the establishment of latency is difficult, although many vaccines have been shown in animal models to reduce the levels of acute ganglionic replication and/or the establishment of latency, as assessed by explant cultures or molecular methods (18, 48, 93, 192, 250, 283). It is not known how the mechanisms of immunologic control of acute ganglionic replication, the establishment of ganglionic latency, and reactivation are related. Dose-ranging studies in mice and guinea pigs have demonstrated that the peripheral inoculum, the extent of initial ganglionic replication, the number of latently infected neurons, and the frequency of reactivation are all directly related (146, 228, 229). Data on these issues are not available for experiments with humans. The animal data do give rise to optimism that vaccines that reduce, without eliminating, initial peripheral or ganglionic replication may still have a positive impact on the natural history of the chronic phase of infection.

After reactivation from latency, viral components travel

down the axon and assemble for transmission to epithelial cells (108, 189). Antibodies have been shown in an elegant human two-chamber ex vivo model to inhibit transmission from dorsal root ganglion neurons to keratinocytes (187). Polyclonal and monoclonal antibodies to gD and gB were active, while antibodies to gC, gE, and gG were not. These data were interpreted to be consistent with transit of free virus between neuron and keratinocyte. Both IFN- α and IFN- γ are also active in this model if administered to the keratinocytes early enough after inoculation of the dorsal root ganglion neurons (1842). Possibly, vaccines that elicit T cells capable of rapid homing to incipient lesions might lead to early, local IFN- γ secretion. While HSV-specific T cells are approximately 100-fold enriched in lesions compared with their levels in blood (118), little is known concerning the expression of candidate skin or mucosal homing receptors by these cells.

Reactivation of HSV can lead to either subclinical shedding or symptoms and lesions. Because most sexual transmission occurs during asymptomatic shedding (176, 178), it is critical to measure the effect of proposed preventative or therapeutic immunologic interventions on subclinical shedding. Among immunocompetent persons, rates of subclinical and clinical reactivations are directly related (285), consistent with a model in which similar mechanisms are involved in their control. Therefore, full evaluation of the public health benefits of candidate HSV preventative and therapeutic vaccines requires measurement of their effects on subclinical shedding.

When lesions occur, there is a brisk infiltration of NK and CD4 cells followed by CD8 T cells (63, 123). High levels of β -chemokines and both Th1 and Th2 T-cell cytokines are present (185, 278). Antigen-specific CTL activity typically peaks after antigen-specific CD4 proliferative activity, and is correlated with viral clearance. Both CD4 and CD8 cells contribute to this CTL activity (123). Subclinical shedding in the absence of lesions or symptoms may be the outcome of particularly early or effective local cellular immune responses, but no experimental data support this hypothesis.

IMMUNE EVASION

Chronic pathogens coexist with ongoing innate and acquired immune responses. Microbial agents have developed means of subverting host responses, as recently reviewed for herpesviruses (2, 152, 276). Some of the immune evasion functions of HSV are mentioned above. Studies of the immune correlates of disease severity, and the process of choosing a vaccine compound and format, must take immune evasion into account.

Dendritic cells (DC) are important for priming cellular immune responses. Specialized DC reside in both the skin (Langerhans' cells) and the dermis (151). HSV has profound effects on myeloid-lineage DC derived in vitro by treating monocytes with cytokines. These cells express HSV receptors (222) and can be productively infected with HSV depending on their maturation status (134, 183). HSV infection reduces the maturation, expression of costimulatory molecules, and antigen-presenting capacity of these DC (134, 222). CD83 may be specifically degraded in HSV-infected DC (134, 222). Interestingly, DC infected with the HSV-1 DISC vaccine strain (see below) could prime naive T-cell responses to HSV in vitro, a fairly stringent test of DC function (107). Thus far, no group

has identified the viral function(s) responsible for inhibition of DC function.

HSV is a profound inducer of IFN- α by unfractionated human peripheral blood mononuclear cells (PBMC) (224). This innate response influences acquired immunity, for example, by enhancing T-cell survival and supporting Th1-like responses (161, 217). Herpetic vesicle fluid contains very high levels of IFN (199). A DC variously termed plasmacytoid or pDC2, with a characteristic CD4⁺ CD11c⁻ CD123⁺ phenotype in humans, can be purified from PBMC. These cells respond to HSV by secreting large amounts of IFN- α (235). Cells with this phenotype can accumulate in cutaneous blister fluid (232). The viral and cellular structures involved in the induction of IFN- α are undefined, although gD and specific chemokine receptors were implicated in a study using whole PBMC (4). It has recently been demonstrated that resiquimod and imiquimod, small molecules which induce IFN- α secretion (1, 30, 33, 43, 79, 279) and have clinical activity against HSV (253), act via Toll-like receptor 7 (TLR7) (102). pDC2 expresses TLR7, and studies may soon determine whether the innate IFN- α response to HSV also uses this pathway.

HSV evades IFN- α action in several ways. The *vhs* protein, encoded by the UL41 gene and also delivered preformed on cell infection as a virion component, has endoribonuclease activity (153). *vhs* antagonism of the antiviral effect of IFN- α (266) may reflect accelerated turnover of IFN- α -induced transcripts. A more specific effect has been shown for the product of the $\gamma_134.5$ gene, which in *trans*-complementation assays with IFN receptor-, PKR-, and RNase L-deficient mice, controls HSV virulence through a PKR-dependent but RNase L-independent pathway (145). The $\gamma_134.5$ protein inhibits IFN-induced phosphorylation of eIF-2 α by redirecting protein phosphatase 1 to dephosphorylate eIF-2 α . By doing so, $\gamma_134.5$ reduces the shutoff of host protein synthesis normally caused by eIF-2 α phosphorylation. Viruses with the $\gamma_134.5$ gene deleted have extremely attenuated neurovirulence in animals (53), and deletion of both copies of this diploid gene is a rational component of the design of live attenuated vaccines (250).

As mentioned above, HSV can also infect a variety of immunocompetent cells. HSV infection of human T cells can lead to their lysis by HSV-specific CTL (215). This phenomenon, termed "fratricide," could influence the homeostasis of the CTL response to HSV. Exposure of monocytes to HSV causes the release of multiple cytokines (157, 200–202). These actions are not entirely dependent on live virus, and some can be mediated by soluble gD (4, 202). The molecules released by HSV-infected monocytes may have deleterious effects in animal models of HSV eye infection (116), although their net contribution to pathogenesis is uncertain.

HSV inhibits antigen processing and presentation in both the HLA class I and class II pathways. Inhibition of HLA class I antigen presentation by interaction of the US12 gene product, ICP47, with the transporter associated with antigen processing has been thoroughly described (106, 297). Cell types display differing sensitivity to this effect in vitro: fibroblasts (127, 272) and keratinocytes (127) are sensitive, while B cells (127) and T cells (215) are less sensitive. The *vhs* gene (UL41) is also involved in evasion of HLA class I-restricted responses. Infection of immature monocyte-derived DC with the discon-

tinuously replicating (gH-deleted) HSV-1 down-regulated class I, while wild-type HSV-1 did not (183, 222). Cells infected with *vhs* knockout viruses are recognized by CD8 CTL better than are cells infected with wild-type virus (272). IFN- γ can overcome HLA class I down-regulation in keratinocytes and fibroblasts in vitro (62, 120, 272), and high levels of IFN- γ are present in symptomatic HSV lesions (63, 275, 278). The dynamics and net effect of HSV and IFN- γ on class I expression have not been well studied in genital lesions. Limited human data from HSV encephalitis show net up-regulation of HLA class I in vivo (245).

Inhibition of TAP shortly after infection, by the immediate-early protein ICP47, would be predicted to favor CD8 recognition of virion input and immediate-early proteins. The available data (120, 186, 271) are consistent with this hypothesis. Restoration of HLA class expression on keratinocytes by IFN- γ secreted by infiltrating leukocytes may be required for any CD8 CTL recognition of infected keratinocytes. The spectrum of HSV antigens capable of being recognized may also be broadened in vivo by local cytokine effects. Knockout of ICP47 might increase the immunogenicity of a live or even discontinuously replicating HSV vaccine, although this has not yet been tested.

Less is known concerning HSV evasion of CD4⁺ T cells. The immediate-early protein of HSV-1, ICP22, inhibits antigen presentation to CD4 T-cell clones in vitro. HSV-infected or ICP22-transfected EBV-transformed B cells were found to inefficiently present antigenic epitopes to CD4 T-cell clones (22). The mechanisms involved are unknown, and these cells may not be representative of functional antigen-presenting cells (APC) in vitro. HSV was observed not to reduce HLA DR levels on monocyte-derived DC, although key costimulatory molecules were down-regulated (183), as discussed above.

ACQUIRED IMMUNE RESPONSE TO HERPES SIMPLEX VIRUS

CD4 T-Cell Responses

TCR $\alpha\beta$ ⁺ CD4⁺ cells are strongly stimulated in vivo by HSV infection. Responses are generally undetectable in HSV seronegative persons. Recently, Posavad and coworkers reported a group of HSV-seronegative persons with proliferative responses to whole HSV-2 antigen (263; F. Struyf, E. Keyaerts, C. Posavad, L. Corey, M. Van Ranst, and P. G. Spear, Proc. 25th Int. Herpesvirus Workshop, abstr. 2.35, 2000). Whether this represents true acquired immunity to HSV-2, as reported for HIV-1 and HCV in exposed but seronegative populations (115, 231), remains unknown.

The integrated CD4 memory response to HSV-1 appears to occupy about 0.2% of circulating CD4 T cells when measured by intracellular cytokine flow cytometry for IFN- γ (7). Results of similar magnitude were obtained by enzyme-linked immunospot (230) and limiting-dilution (213) assays. To measure responder cell frequencies for specific HSV-2 peptides, we combined secondary in vitro restimulation of carboxyfluorescein succinimidyl ester (CFSE)-labeled PBMC with HLA class II tetramer staining. This technique led to the estimate that 0.002% of circulating CD4 cells responded to a specific DQB1*0602-restricted epitope in HSV-2 VP16 (139).

CD4 responses are typically assessed in vaccine trials or natural history studies by performing simple [³H]thymidine incorporation proliferation assays, using whole PBMC as the source of APC and responders and using killed HSV as antigen. Limiting-dilution variants of this assay provide some degree of quantitation (143, 299). The predominant cell type secreting IFN- γ in response to HSV-1 antigen is the CD4 lymphocyte (60). However, caution is required in ascribing proliferative or cytokine responses to inactivated HSV antigen solely to CD4⁺ responders. TCR $\gamma\delta$ -bearing cells in the PBMC can proliferate to HSV; these responses are quite variable from person to person and do not segregate with HSV seropositivity (155, 156). Occasional CD8 T-cell clones that proliferate and secrete cytokines in response to killed HSV have been documented (111, 124). It has recently been appreciated that cross-presentation of the cytomegalovirus tegument protein, pp65, in apoptotic bodies has the ability to restimulate pp65-specific CD8 T-cells (5, 268). It is therefore also possible that the typical UV-inactivated HSV preparations used in typical "CD4" lymphoproliferation experiments also stimulate CD8 memory cells.

Given these caveats on proliferation and cytokine secretion studies, no consistent correlations between CD4 responses and disease severity have been observed in natural history studies. A small study indicates that brisk spontaneous (61) IFN- γ secretion correlates with a longer time to the next symptomatic recurrence of HSV-1. Comparison of Th1 (IFN- γ and interleukin-2 [IL-2]) and Th2 (IL-4 and IL-10) responses by PBMC between HSV-1-infected persons with and without a history of symptomatic infection failed to detect statistically significant differences, although trends were present consistent with a protective effect of strong Th1 responses (251). In phase II studies of prophylactic vaccination with gB2 and gD2, the vaccine-induced CD4 responses were as high as those measured in specimens from persons with natural HSV-2 infection (143). However, this vaccine was not protective against infection (57). Moreover, in a cross-sectional study of HIV-1- and HSV-2-infected men, the clinical severity of recurrent HSV-2 infection did not correlate with HSV-2-specific CD4 cell number as measured by the limiting-dilution assay (214).

HSV-specific CD4⁺ T-cell clones are heterogeneous with regard to CTL activity (296), but broad CD4 CTL activity has not been measured as a correlate of disease severity. CD4 CTL are capable of killing infected, IFN- γ -treated keratinocytes in vitro (182), and HSV-2-specific CD4 CTL are present in the human cervix (125) and in herpetic skin lesions (121, 123). Some, but not all, research groups have reported that CD4 cells are required for the protection of immune mice from vaginal HSV-2 challenge (188, 206). These data, along with the important roles of CD4 cells in supporting B-cell (294) and CD8 T-cell function, make it likely that a successful vaccine will elicit CD4 responses.

The CD4 response to HSV in humans is broadly directed. Targets include immediate-early (D. M. Koelle et al., unpublished data), early (dUTPase [122, 126]), and late proteins. At the structural level, virion proteins from the envelope (gB, gC, gD, gE, and gH [121, 126, 181, 274, 288, 300]), tegument (VP11/12, VP13/14, VP16, VP22, and the UL21 gene product [122, 280]), and capsid (VP5 [126]) are all recognized, as are nonstructural proteins that are only present within infected

cells (dUTPase and ICP8 [246]). These studies with defined antigens and T-cell clones reinforce earlier studies with fractionated viruses and PBMC (114). The large number of proven epitopes in VP16 and VP22 (122, 124, 138) is likely to reflect the intensive investigation of these proteins rather than bona fide immunodominance. A relatively large proportion of HSV-2-specific CD4 clones, including lesion-infiltrating clones, have been HLA DQ or DP restricted. Since lesional keratinocytes primarily up-regulate HLA DR (63), while Langerhans' cells express high levels of HLA DR, DP, and DQ (32), the broad use of class II-restricting loci may indicate that memory CD4 T cells are restimulated by professional APC *in vivo*.

CD8 T-Cell Responses

Investigation of antigen-specific CD8 responses has been difficult for both experimental and biological reasons. HSV-specific CD8 T cells occur at low frequency in PBMC and require secondary *in vitro* restimulation with antigen prior to the use of ^51Cr release cytotoxicity assays. The immune evasion mechanisms targeting the CD8 response, mentioned above, complicate restimulation, as does the need for autologous or HLA-matched APC. The most frequently used systems involve the use of autologous HSV-infected lymphoid cells as stimulators (treated to reduce HSV infectivity), CD8 cell selection prior to stimulation or readout, and autologous immortalized B cells as target cells. IFN- γ -treated keratinocytes are also useful as APC (62, 120, 181, 182, 186). It will be important to determine how the results are influenced by the type of APC used.

Several lines of evidence indicate that CD8 responses are functionally important. A cross-sectional study of HSV-2- and HIV-coinfected men, using a limiting-dilution variant of the above system, showed an inverse correlation between CD8 responder number and severity of genital herpes lesions (214). Infiltration of CD8 $^+$ cells occurs after the infiltration of CD4 $^+$ cells in recurrent HSV lesions (63). We found that HSV-specific CD8 T- cells were locally enriched in recurrent genital HSV-2 lesions (118, 127). In subsequent serial lesion biopsy studies, the infiltration of CD8 T- cells and CTL, but not CD4 or NK cells, was found to correlate temporally with the clearance of infectious virus (123). Data from murine models concerning ganglionic infection and CD8 were mentioned above.

The data available to date suggest that the CD8 $^+$ T-cell response to HSV is more narrowly focused than is the CD4 $^+$ response. Whether this is due to methodological issues or to a true biological difference is not clear. Early studies isolated single HLA class I-restricted CD8 clones specific for gB2 and gD2 (271, 272) but could not define the specificity of most CD8 CTL clones. A keratinocyte APC model revealed a high population prevalence of PBMC CD8 CTL responses to the immediate-early proteins ICP4 and ICP27 (186). These studies used *in vitro* restimulation methods, which could potentially favor the detection of responses to certain proteins.

To avoid secondary *in vitro* restimulation, we have used the *in situ* lesional enrichment of CD8 CTL. HSV-2-specific CD8 CTL clones isolated from lesions were cocultivated with Cos-7 cells cotransfected with the cDNA encoding the restricting HLA class I heavy-chain molecule and a library of HSV-2

DNA. T-cell activation was detected by IFN- γ secretion using an enzyme-linked immunosorbent assay (ELISA). While the novel specificities were uncovered using Cos-7 as APC, in each case the HSV-2 reactivity was initially detected using the human Epstein-Barr virus lymphoblastoid cell line (EBV-LCL) as APC and in most instances was also confirmed using skin-derived fibroblasts and/or keratinocytes as APC (120). With these methods, epitopes recognized by CD8 $^+$ T cells were uncovered in the immediate-early protein ICP0 and the tegument proteins VP13/14 (encoded by UL47) and VP22 (encoded by UL49) (120). Limited population prevalence studies using HLA class I peptide tetramers revealed that most HLA-appropriate persons had responses to UL47 (120). For the tegument-specific clones, protein transferred into cells on virion entry was sufficient to sensitize target cells to lysis. Further work is required to rank HSV-2 antigens in a hierarchy of immunodominance.

Antibody Responses

Neutralizing antibodies to HSV were documented in the 1930s (reviewed in reference 292) and formed the basis for early viral typing methods. Antibodies also cooperate with cells, including polymorphonuclear leukocytes, monocytes, and NK-like cells, in mediating antibody-dependent cell-mediated cytotoxicity (128). As noted above, the natural history of infection after neonatal exposure implies that antibody alone can be protective against infection and disease in some situations. Another indication of the functional importance of antibodies is the development of HSV-encoded immune evasion functions directed at the humoral response.

The HSV-encoded gE-gI high-affinity Fc receptor expressed by infected cells may absorb and inactivate anti-HSV antibodies. Animal pathogenesis experiments with viral FcR knockouts clearly show that this function is associated with virulence (193). In this analysis, HSV-specific antibodies do not protect because they are "neutralized" by the virus. Friedman has suggested that immunization strategies which generate anti-gE or anti-gI antibodies, which block this FcR function, might enhance the activity of vaccine-induced neutralizing antibodies (H. M. Friedman, Letter, JAMA 283:746, 2000).

Serum and local antibody responses are typically broad, reacting with envelope glycoproteins and tegument, and capsid proteins (10, 11). Immunoblots using whole virus antigen have a very complex pattern. To date, no consistent correlations between levels of antibodies, their subclasses, their functional activities, or their fine specificities and the severity of HSV infection have been demonstrated. Limited data suggest that symptomatic HSV-infected persons may have higher levels of antibody than asymptomatic but seropositive persons (251), consistent with a model in which antibody levels are driven by antigen load.

Cervical antibodies to HSV-2 are mostly immunoglobulin G (IgG), although HSV-specific IgA is also detectable (11, 12, 14). Immunized IgA knockout mice were not particularly susceptible to HSV-2 in a murine vaginal challenge model (220). The greater activity of therapeutic (260) and prophylactic HSV-2 vaccines in women than in men raises the possibility that the induction of mucosal antibody responses may be clinically important.

Vaccines can induce neutralizing antibodies. The Chiron gB2/gD2 vaccine used with alum (below) was able to elicit HSV-2 neutralizing antibodies in HSV-seronegative subjects, but the levels were lower than those measured in HSV-2 infected persons (259). Among HSV-1-infected subjects, this vaccine regimen increased HSV-2 neutralizing-antibody titers to levels above those seen in natural HSV-2 infection. Among HSV-2-infected subjects, vaccination raised levels significantly above baseline (258). Use of MF59 adjuvant in HSV-seronegative subjects produced neutralizing-antibody levels at or above the levels measured in HSV-2-infected persons (57, 143). However, this vaccine did not protect against infection, and subgroup analyses failed to show any correlation between neutralizing-antibody titers and protection against sexual HSV-2 acquisition (57). Antibody data from the GlaxoSmith-Kline (GSK) gD2-MPL vaccine, which did protect subsets of subjects from HSV-2 infection and disease, may clarify the possible role of antibody in vaccine-induced protection against HSV-2.

Complement in normal serum has potent virus-neutralizing activity. The HSV-1 gC binds several components of complement and reduces the antiviral activity of complement. This effect is at least in part antibody independent (87, 154). Complement can also enhance antibody-dependent lysis of infected cells (223). Blockade of the complement receptor activities of gC has also been proposed as a measure to enhance the antiviral effects of antibody (154).

Type-Common and Type-Specific Responses

The influence of preexisting immunity to HSV-1 (type-common responses) on the pathogenesis and natural history of HSV-2 infection is highly relevant to HSV-2 vaccine design. If HSV-1 protects against HSV-2, type-common responses in HSV-1-infected persons become rational benchmarks for vaccine-induced immunity. Type-specific seroprevalence data are quite unclear and inconsistent in this area. If prior HSV-1 infection protected against HSV-2 acquisition, geographic areas with a high seroprevalence of HSV-1 might have relatively low seroprevalences of HSV-2 infection. HSV-1 is invariably acquired during early childhood in many locations. For example, despite the nearly universal presence of HSV-1 antibodies, HSV-2 seroprevalences in Africa are among the highest in the world (92, 284). Similar data have been obtained in Mexico (55). In contrast, in other geographic areas, such as Japan, the seroprevalence of HSV-1 is moderately high but the seroprevalence of HSV-2 is very low (99).

Small, prospective studies of couples suggested that prior HSV-1 infection protects against HSV-2 acquisition (39, 178). In contrast, prospective study of 6,107 pregnant women showed no difference in HSV-2 seroconversion between HSV-seronegative and HSV-1-seropositive persons (38). Observation of 1,135 HSV-2-seronegative persons receiving a placebo vaccine also failed to show a protective effect of prior HSV-1 infection (57). Curiously, HSV-1-seropositive recipients of placebo did have a lower attack rate for HSV-2 than did HSV-1-seronegative persons in the GSK trial (257), with results reaching statistical significance in women, but not men.

While the data are unclear on whether prior HSV-1 protects against HSV-2 infection, HSV-1-infected persons have shorter

and milder symptoms associated with the acquisition of HSV-2 infection (56) and are more likely to have asymptomatic HSV-2 seroconversions (144) than are HSV-seronegative persons. However, the subsequent rates of symptomatic recurrences (24) and of asymptomatic shedding (285) are similar. These data warn that prophylactic vaccines that ameliorate symptomatic genital herpes, but do not limit infection, may have only limited public health benefit.

Type-common responses elicited by HSV-2 infection protect against HSV-1 infection (144). Given the serious medical sequelae of HSV-1 infection (292), administration of an active HSV-2 vaccine to HSV-seronegative persons might have clinical benefit by reducing HSV-1 infection and/or disease. In addition, since up to 30% of first clinical episodes of genital herpes are caused by HSV-1 (52, 58), administration of an "HSV-2-based" vaccine with cross-protection against HSV-1 would be desirable.

Type-common responses also have a practical influence on HSV-2 vaccine design and protocols for administration. Systemic reactogenicity of the Chiron gB2/gD2/MF59 was higher in the HSV-1-infected persons than in seronegative controls in a dose-finding phase II study (143). It is possible that cross-reactivity may limit the administration of potent vaccines to HSV-1-infected persons.

Definition of T-cell epitopes permits the direct assessment of the type specificity of clonal CD8 and CD4 responses. As expected, many of the CD4 T-cell clones specific for gB and gD described in the 1980s recognized type-common epitopes, since these proteins are >85% identical at the amino acid level (72, 169, 170). However, we have found more recently that the CD4 response to HSV-2 recognizes a large number of polypeptides (see below). More than 50% of the CD4 epitopes found to date are type specific for HSV-2 (118, 121, 122, 138). Similarly, the great majority of the known CD8 epitopes in HSV-2, including the only described epitope in the vaccine compound gD2 (271), are type specific. Recent efforts by our group to clone HSV-specific CD8 CTL from blood and herpetic lesions have yielded almost exclusively HSV-2 type-specific responses (118, 120, 123, 212, 213; Koelle et al., unpublished). Both the type specificity of the cellular immune response to HSV-2 and the degree to which prior HSV-1 infection protects from HSV-2 infection and disease require further study.

VACCINE DEVELOPMENT

Experimental vaccine formats have included peptides, proteins, mixtures of viral proteins, whole and split killed virus, replication-defective viruses, and attenuated replication-competent viruses. The advantages and disadvantages of these formats have been reviewed (255). Delivery of peptide and protein antigens has been performed in a variety of formats including recombinant heterologous viruses and bacteria and as DNA predicted to encode these antigens. Many adjuvants have been explored with these various forms of antigen, and a large number of animal species, routes, schedules, and doses of vaccine and variety of virus challenges have been evaluated. Major histocompatibility complex-inbred mice have been preferred for their uniformity and the related ability to analyze peptide-specific responses. After vaginal inoculation, guinea pigs experience locally recurrent disease that mimics some

features of human genital herpes. Therefore, the “natural history” of modern vaccines entering clinical trials typically includes extensive work in these models, which will not be reviewed in detail. We review selected recent human and animal trials, moving from simpler to more complex antigens and highlighting demonstrations of immunologic principles which may move into human trials.

Peptide Vaccines

The hypothesis that immune responses to a single epitope can protect against disease has been tested in major histocompatibility complex haplotype-identical inbred mice. When administered in a suitable context, immunization with a single immunodominant CD8 CTL epitope (31) or neutralizing epitope (179) can be protective. These systems are of interest in that adjuvants such as T-helper epitopes (220) and heat shock proteins (137), “string-of-pearls” sets of epitopes expressed as minigenes (298), and expression formats such as recombinant viruses (31, 302) and bacteria (49) can be quantitatively compared. The outbred nature of the human population obviously complicates the development of peptide-based vaccines, especially since immunodominant and disease severity-associated peptide-specific responses have not yet been defined. A phase I trial of immunization with a type-common, HLA A*0201-restricted epitope in gB2 (272) and a heat shock protein adjuvant has recently begun. This study is designed to determine if the peptide “concept” can elicit high levels of CD8⁺ T cells to an HSV antigen. Both HSV-seronegative and HSV-2-seropositive subjects will be studied.

Subunit Vaccines

This type of vaccine presents a more complex antigen to the immune system, potentially including CD4, CD8, and B-cell epitopes. Recombinant protein formats have been extensively tested in humans. Conceptually similar, the introduction of complete or near-complete HSV open reading frames into heterologous viruses (3, 26, 76, 159, 163), bacteria (81, 136), or expression vectors for DNA vaccination (76–78, 89, 194, 237–240), has been extensively tested in animals. As with peptides, subunit vaccines in these formats have allowed comparison of a large variety of adjuvants and vaccine formats. A phase I trial of a DNA vaccine using the gene for gD of HSV-2 has been completed, and immunogenicity analyses are under way.

Two similar subunit vaccines have recently completed phase III clinical trials. The Chiron vaccine contained gB2, truncated at amino acid 696, and gD2, truncated at amino acid 302, expressed in transfected CHO cells. The truncations of cytoplasmic tail and transmembrane domains were made to help the proteins exit the producing cells, allowing the manufacturing process to yield large amounts of protein. Phase I studies with gD2 and alum adjuvant established that this vaccine could both induce ELISA and neutralizing antibodies in seronegative subjects and boost preexisting responses in HSV-1- and also HSV-2-infected subjects. Maximal neutralizing-antibody levels in seronegative subjects were somewhat below baseline neutralizing-antibody levels in HSV-2-infected persons. Lymphoproliferative responses to gD2 were also induced (259).

A novel adjuvant containing MF59 (see below) and the

muramyl dipeptide derivative MTP-PE (198) was then tested with recombinant gD2 and gB2. This adjuvant and gD2 were active in prophylactic and therapeutic guinea pig models of genital HSV-2 (42). While this vaccine preparation was immunogenic in humans, it had a high level of local and systemic reactivity, particularly in HSV-1-infected subjects (C. L. Dekker, S. F. Adair, R. Sekulovich, N. Niland, and R. L. Burke, Prog. Abstr. 32nd Intersci. Conf. Antimicrob. Agents Chemother, abstr. 1431, 1992). Phase III studies, therefore, used MF59 alone as the adjuvant.

Adjuvant MF59 is a submicron-sized oil-in-water emulsion containing squalene, sorbitan monoleate (Tween 80), and sorbitan trioleate (Span 85) (197). In randomized human trials with HBV and cytomegalovirus antigens, MF59 was superior to alum with regard to induction of antibodies (101, 207). Labeled MF59 is internalized by macrophage-like and DC-like cells after intramuscular injection and is transported to draining lymph nodes (73, 74). Some data indicate that MF59 may produce a Th2-like CD4 response (241, 277).

The pivotal phase III experiments compared injection of 30 µg of truncated gB2 and gD2 with MF59 at 0, 1, and 6 months to injection of MF59 alone in 1:1 randomized double-blind trials. Subjects were monitored for 1 year after the last vaccination. One trial administered vaccine to the HSV-2-seronegative partner in stable couples disparate for HSV-2 infection status as assessed by serologic testing. The study population in the second trial were sexually transmitted infection clinic attendees with multiple partners or a history of a sexually transmitted infection.

The primary end point was seroconversion to HSV-2 as assessed by sensitive and specific immunoblotting (8). To clarify testing, type-common gB- and gD-specific antibodies were reduced by absorption from the serum of HSV-1-infected subjects. Seroconversions were confirmed by gG-based assays. Neutralizing antibodies and ELISA antibodies to the administered glycoproteins were also tested.

Vaccination appeared to have a partial and transient protective effect against HSV-2 seroconversion, which was most apparent during the initial 150 days of the trial, in women, and in HSV-1-seronegative persons. However, there was no overall protection regardless of sex or HSV-1 serostatus. While the study was not powered to uncover differences in HSV disease, there were no differences in the proportions of asymptomatic and symptomatic seroconversions between vaccine and placebo recipients in any subject group. There was also no difference in the duration of first-episode genital HSV-2 disease or the recurrence rate between vaccine and placebo recipients.

Overall, the peak neutralizing-antibody and ELISA titers exceeded those observed in natural HSV-2 infection. In a nested case-control study, immunized HSV-2 seroconverters were compared to matched, immunized nonseroconverters. There was no correlation between the peak levels of vaccine-induced neutralizing or anti-glycoprotein antibodies and seroconversion to HSV-2.

GSK has conducted trials with a vaccine containing a similar, truncated form of gD of HSV-2. The vaccine was administered at a dose of 20 µg intramuscularly at 0, 1, and 6 months. The protein was compounded with an adjuvant containing alum and 3-*O*-deacylated monophosphoryl lipid A (3d-MPL). MPL is derived from the gram-negative bacterium *Salmonella en-*

terica serovar Minnesota R595 (21). Forms of MPL have been reported in human studies to favor the induction of Th1-like CD4 responses and delayed-type hypersensitivity responses (M. Koutsoukos, G. Leroux, P. Vandepapeliere, M. Slaoui, and P. Pala, Program Abstr. 34th Intersci. Conf. Antimicrob. Agents Chemother., abstr. M. 1994; G. Leroux-Roels, E. Moreau, I. Desombere, O. Crayne, M. Francotte, P. Pala, M. Slaoui, and P. Vandepapeliere, Program Abstr. 34th Intersci. Conf. Antimicrob. Agents Chemother., abstr. H57 1994) and to be superior to alum for the induction of antibodies to hepatitis B surface antigen (270). There are no human data available concerning the induction of primary CD8 responses by 3d-MPL. MPL, based on its structural similarity to the lipid A component of lipopolysaccharide, may provide a "danger signal" to APC by interaction with CD14 and/or toll-like receptors to enhance immunologic priming.

The GSK vaccine results have recently been reported, (257) and suggest approximately 75% efficacy in the prevention of clinically apparent genital herpes. Only HSV-seronegative women were protected. Protection against HSV-2 infection among HSV-seronegative women was on the order of 40%. No statistically significant efficacy was seen in men, although the number of cases of genital herpes disease and HSV-2 seroconversion were small. It is unclear what accounts for the differences in the results of the Chiron and GSK trials. The gD2 proteins were very similar. Different adjuvants were used. No direct comparisons of the immune responses of vaccines containing MPL or its derivatives versus MF59 are available, and detailed immunogenicity data from the GSK vaccine are not yet available.

Subunit vaccines containing truncated HSV-2 glycoproteins have also been evaluated as immunotherapy in adults with symptomatic genital HSV-2 infection. Rational design of HSV-2 immunotherapy is difficult because we do not have consistent immune correlates of disease severity among naturally infected persons. Two doses of 100 μ g of gD2 with alum were administered 2 months apart in a blinded, placebo-controlled trial. A statistically significant reduction in the number of genital herpes recurrences was detected during the 12-month observation period. Neutralizing-antibody and lymphoproliferative responses were increased in vaccine recipients, but no correlations were noted between immune responses and vaccine efficacy among the treated group. In a follow-up study, a smaller dose (10 μ g) of both gD2 and gB2 was administered with MF59 adjuvant (see above). While there were suggestions of clinical activity, especially with regard to the severity of the first postvaccination recurrence, there was no overall difference in the monthly rates of HSV recurrences between the vaccine and MF59-alone control groups. Again, no relationship between baseline or vaccine-induced antibody levels and vaccine efficacy was noted (258, 260).

Killed-Virus Vaccines

Many variations of the killed-virus vaccine format have provided protection in animals. Early vaccines were made by phenol treatment of infected animal tissue or UV light treatment of cell culture-derived virus. These preparations were evaluated as immunotherapy; unfortunately, as reviewed by Whitley, many reports did not include placebo controls. Placebo-con-

trolled studies failed to establish long-term benefit (292), although some immunotherapeutic studies have shown short-term benefit similar in magnitude to that provided by suppressive acyclovir (166). We now know that both asymptomatic (119) and symptomatic (25) genital HSV-2 decrease over time, so that some improvement is part of the natural history of infection. Many studies were performed before accurate type-specific serologic testing or typing of viral isolates became available, and not all subjects had culture-confirmed disease, placing some doubt on the assignment of subjects. Subject-reported disease severity, without clinician examination or cultures during suspected recurrences, was used in many studies as the primary clinical end point.

An update on experience using this type of vaccine to prevent genital herpes was provided by Skinner et al. (242). Cells infected with a mixture of clinical HSV-2 isolates were sonicated and clarified. The resulting supernatant, which was likely to contain virions and viral protein, was treated with formaldehyde to make the vaccine. The dosage, number of administrations, and adjuvants varied. While the vaccination appeared safe, no conclusions about efficacy are possible from the available data.

Fractionated-Virus Vaccines

Several groups have prepared HSV vaccines by subjecting infected cells to procedures to inactivate virus and to partially purify subsets of viral proteins. Randomized, blinded, placebo-controlled trials were conducted in the mid-1980s by Merck with a mixture of envelope glycoproteins. HSV-2-infected cells were treated with detergent and DNase to inactivate virus, and glycoproteins were enriched by binding to a plant lectin. The vaccine contained gD2, gB2, and other HSV-2 glycoproteins (13). The bound fraction was further inactivated with formalin and compounded with alum. Most seronegative vaccinees developed neutralizing and antibody-dependent cell-mediated cytotoxicity antibodies and lymphoproliferative responses to HSV-2, but HSV-1-seropositive vaccinees had no increase in preexisting responses (177). The HSV-reactive T-cell clones recovered from vaccinated, initially HSV-seronegative donors were mostly CD4⁺ and CD8⁻; a subset of these clones had CTL activity (299). A dose of 50 μ g was administered at weeks 0, 4, and 22 in a blinded, placebo-controlled trial enrolling both HSV-uninfected and HSV-1-infected subjects. No protection from HSV-2 infection or disease was noted in a double-blind, placebo-controlled trial of 161 subjects (175).

A vaccine enriched in HSV-2 envelope glycoproteins by detergent extraction and sucrose density centrifugation was studied by Cappel et al. in the 1970s and 1980s. The vaccine was immunogenic and appeared to decrease the severity of recurrent HSV-2 disease (46), but it did not undergo further development. Experience with a preparation emphasizing viral proteins expressed in the cytoplasm of infected cells has been recently updated by Skinner et al. (243). The preparation of a recent HSV-1 vaccine involved the reduction of nuclear material by detergent lysis, treatment with formaldehyde, centrifugation to remove virions, and harvest of a protein fraction of the remaining material by acetone precipitation. A 300- μ g quantity of this material was injected three times with alum adjuvant into subjects with clinical diagnoses of genital herpes

in a randomized, double-blind, placebo-controlled trial. There was a trend toward modest increases in neutralizing and total antibodies and HSV-stimulated proliferation (bulk PBMC) stimulation indices in the vaccinees. While safe, there was no strong evidence that vaccination had clinical benefit.

Discontinuously Replicating Viruses

The deletion of one or more genes required for productive viral replication was discussed in an earlier section in the context of the viral replication life cycle. This type of HSV strain is typically grown in a complementing cell line genetically engineered to provide the required function in *trans*. When viruses with a deletion of UL22, the late gene encoding gH, infect a noncomplementing cell, the progeny virions can exit the cell but cannot infect a secondary cell. This type of HSV strain has been termed DISC (for "discontinuously replicating virus"). Other, conceptually similar HSV-2 deletion strains developed by Knipe and coworkers (192, 195) and Aurelian et al. (18), with blocks at a variety of points in the replication cycle, are discussed earlier in this review and have not yet entered human trials for HSV indications. All of these vaccine candidates share the potential disadvantage that they will not periodically restimulate immunity due to recurrent, peripheral lytic replication.

Replication-deficient vaccines elicit a classic CD8 CTL in mice after a single injection (85). Both HSV-1 (172) and HSV-2 (34) DISC viruses were protective in a guinea pig vaginal challenge model of HSV-2 infection. Immunotherapy experiments with the HSV-1 DISC virus in HSV-2-infected guinea pigs showed a trend toward improvement (171). With regard to safety, the HSV-2 DISC virus was avirulent in the normally susceptible BALB/c and nude athymic mouse strains. The establishment of neuronal latency could be detected after high-dose vaccination, but no reactivation from latency of the vaccine strain was noted.

The replication-deficient gH-deleted HSV-2 vaccine has entered clinical trials and has been safe and immunogenic. No serious adverse effects were noted during receipt of two doses of 2×10^3 to 3×10^5 PFU 8 weeks apart, regardless of HSV-1 or HSV-2 serostatus. No live virus was recoverable from sites of inoculation. Among HSV-seronegative subjects, dose-dependent induction of PBMC [3 H]thymidine incorporation in response to inactivated HSV antigen, typically ascribed to CD4 T-helper responses, was noted 4 weeks after a single infection and was detectable 16 weeks after the second infection. Responses were not increased in HSV-seropositive subjects. IFN- γ secretion was also induced in HSV-seronegative persons in a dose-related fashion, and IL-5, as an indicator of Th2 responses, was also induced in this population. No increases in cytokine production were detected in HSV-infected persons. De novo antibody responses were not noted in seronegative persons, and increases in preexisting titers were not seen in HSV-infected subjects. CTL responses were noted in one of six subjects receiving the highest dose but were not characterized as CD4 versus CD8 mediated. Follow-up studies used up to three doses at up to 7×10^6 PFU/dose. The higher-dose regimens induced antibody responses. In addition, proliferative (probably CD4) and cytokine secretion responses were induced and most of the higher dose recipients acquired CTL

activity. The phenotypes of the CTL were not evaluated (J. K. Hickling, S. E. Chisholm, I. A. Duncan, E. J. Taylor, C. Boswell, C. S. McLean, J. Uttridge, J. S. Roberts, A. Tomasi, L. Stanberry, D. J. Bernstein, M. E. G. Bournsnel, and S. C. Inglis, Proc. 8th Int. Congr. Infect. Dis., abstr. 20.008, 1998.)

A large phase 2 trial of HSV-2 DISC as an immunotherapeutic agent was performed. The study population included 483 HSV-2 seropositive adults with symptomatic infection. Recent analyses of data showed no clinical improvement (Xenova Group; <http://www.cantab.co.uk/pr2001101001.html>).

Replication-Competent Live-Virus Vaccines

The replication-competent live-virus vaccine approach has the advantages of stimulating a broad (antibody, CD4, and CD8) response and presenting a complex mixture of epitopes from the entire genome, lacking only those encoded by the purposely deleted genes. If the virus can establish latency and reactivate without harming the host, endogenous boosting may occur. Experience from experiments with varicella virus indicates that a single vaccination can be effective and that responses are quite durable (6). Disadvantages of live-virus vaccination include the inherent neurovirulence of HSV, the possibility that vaccine strains could reactivate and either recombine with wild-type virus or be transmitted to immunocompromised persons, the potential instability of the genotype of the vaccine strain during production, and interference with serologic testing for HSV infection.

Which genes should be deleted in a live attenuated vaccine? As comprehensively reviewed by Roizman and Knipe (218), many HSV-1 genes that are nonessential in culture alter virulence in animal models. Fewer HSV-2 genes have been studied, and there are almost certainly type-specific effects. In addition, neither murine acute lethality, or reactivation models, nor the guinea pig vaginal model, closely mimics the pathogenesis of HSV in humans. The effect of alterations in thymidine kinase provide an example of species and virus type-specific effects. Thymidine kinase-deficient mutants of HSV-1 have reduced acute lethality in mice, and while these strains can establish latency, they reactivate *in vitro* very poorly (113). However, thymidine kinase-deficient strains of HSV-2 can cause severe acute disease in mice and guinea pigs and can reactivate in guinea pigs similarly to wild-type strains (27). A case of recurrent human genital HSV-2 disease caused by thymidine kinase-deficient, mouse-irulent strain has been reported (267). Mutations in thymidine kinase, in isolation, clearly do not attenuate HSV-2 sufficiently for human vaccines.

Deletion of a portion of the ribonucleotide reductase large-subunit gene (RR1 protein, ICP10 of HSV-2, UL39 gene) with protein kinase activity yields an HSV-2 strain with attenuated replication *in vivo* and *in vitro* (18). This tegument (244) protein can self-phosphorylate; the cellular target(s) of the protein kinase is unknown (218). Interestingly, the kinase domain seems to have been captured from a cellular kinase expressed in keratinocytes (19). Removal of the kinase domain reduces the transforming activity of HSV-2 (reviewed in reference 16). Human studies with RR1 mutants have not been reported.

A second live, attenuated HSV strain developed for vaccine use is RAV 9395 (250). This virus is based on HSV-2 strain G and contains deletions of both copies of the virulence factor

γ_1 34.5 mentioned above, UL55, and UL56. Clinical results have not been reported for this agent. Attenuated HSV with deletions in multiple genes are also being developed for local therapy of cancer (59).

The most extensive experience in humans with an attenuated live HSV vaccine is with strain R7020, created by Roizman and coworkers. This virus, based on HSV-1 strain F, is attenuated by a deletion extending from UL54 (encoding ICP27) through the promoter region of ICP4. A portion of the unique short region of HSV-2 encoding gD2, gG2, gI2, and a part of gE2 was inserted in this deletion to make R7020. This virus was very strongly attenuated in rodents and primates (173, 174). In a dose escalation study, local reactions were noted in HSV-1-infected persons. A dose-dependent induction of antibodies occurred in HSV-seronegative subjects (M. Cadoz, M. Micoud, J. M. Seigneurin, M. R. Mallaret, C. Baccard, P. Morand, B. Meignier, R. Whitley, and B. Roizman, Program Abstr. 32nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 341, 1992), but the virus was thought to be poorly immunogenic overall, and development has been stopped (291).

POTENTIAL TOXICITIES OF VACCINES

As noted above, administration of subunit glycoprotein vaccines to HSV-seropositive persons has been associated with local pain and induration and with fever, especially when potent adjuvants have been used (260). This has generally been ascribed to restimulation of antigen-specific T cells or to the formation of immune complexes, with subsequent release of cytokines and inflammation, although reactions have also been noted in HSV-seronegative persons (257). Unless vaccination is preceded by serologic testing, an expensive and imperfect procedure (9), it is likely that any mass vaccination program would expose HSV-infected persons to vaccine. The competing requirements for high antigenicity and low reactogenicity may be difficult to satisfy in this population.

Over the last decade, a role for HSV-specific immunity has been hypothesized for two medical disorders, HSV-associated erythema multiforme (HAEM), and herpetic interstitial keratitis (HSK). No toxicity related to these diseases has been recognized in trials of over 10,000 persons, but brief review is warranted, given the increasing use of potent adjuvants and targeting of cellular immunity in contemporary vaccination trials. HAEM refers to episodic erythema multiforme skin lesions that occur in a pattern that is temporally, although not spatially, correlated with typical recurrent HSV infection (109). An estimated 50 to 80% of cases of recurrent erythema multiforme are HSV-associated (109). A causal role for recurrent HSV infection has been strongly suggested by the clinical improvement that occurs when suppressive doses of nucleoside antivirals are given (147). The pathogenesis is controversial. Evidence of HSV protein (129, 158, 196), DNA (15, 36, 131, 190), and RNA (129) in HAEM lesions has been obtained. However, live HSV has seldom been recovered in culture (131), and inclusions typical of lytic HSV replication are not seen. If immunity to HSV proteins is involved in the pathogenesis of HAEM, as implied by some data (17, 130, 131, 204), caution is warranted in therapeutic or prophylactic vaccination of individuals with a history of this disorder.

Corneal HSK is a serious HSV-1-related ocular disorder.

Because HSK is responsive to corticosteroids and cyclosporin and occurs in animals after clearance of all infectious virus, its pathogenesis is thought to include tissue damage by a dysregulated inflammatory response. Athymic mice fail to develop HSK, implicating T cells. Tissue and draining lymph nodes contain HSV-specific CD4 T cells and are enriched in Th1-like cytokines (261). Detailed, elegant animal models have shown a possible role for cross-reactive CD4 T cells recognizing HSV-1 UL6 and a corneal autoantigen (20, 203, 301), although contrasting data have also been presented (69, 70, 90). CD4 clones reactive with HSV-1, and in some cases cross-reactive with HSV-2, have been obtained from human HSK specimens (124, 281, 282). It is therefore reasonable to carefully monitor for exacerbations of HSV-1 ocular inflammatory conditions in persons receiving highly antigenic HSV-2 vaccines.

IMPACT OF SPECIFIC IMMUNITY ON CELL AND GENE THERAPY

Replication-competent and -incompetent HSV strains have entered human clinical trials for several conditions including cancer. Some protocols include repeated administration of recombinant viruses or cells that express HSV-encoded proteins. Preexisting immunity to HSV, perhaps boosted by administration of gene or cell therapy products, might limit the duration of the expression of therapeutic genes inserted into recombinant HSV, the antitumor activity of specialized HSV constructs injected into tumors, or the longevity of cells expressing HSV gene products. Similarly, primary immune responses to HSV antigens in seronegative persons might influence the biological activity of these types of therapies. Administration of cloned CD8 T cells which express the HSV-1 thymidine kinase protein has been documented to stimulate thymidine kinase-specific CD8 CTL responses, which may be responsible for the short biological survival of these cells after passive transfer to patients (216). Seroconversion to HSV-1 has been reported after the local injection of recombinant HSV-1 into a brain tumor (160). Further research is required to determine if preexisting or primary immunity to HSV or HSV-encoded proteins will have a significant impact on these promising areas.

UNANSWERED QUESTIONS AND PROSPECTS

Most pathogens which have been controlled with vaccinations elicit immune responses after natural, wild-type infections that are sufficient to lessen the severity and transmissibility of subsequent infection with the same viral type. The rationale for prophylactic vaccination against HSV begins with the assumption that natural infection protects against exogenous reinfection with the homologous viral type, or at least ameliorates it. Molecular fingerprinting of serial genital isolates has occasionally yielded more than one HSV-2 strain (40, 221). On some occasions, each of two or more distinct strains have been recovered more than once, consistent with the establishment of latency of more than one strain (264). While these subjects could have been inoculated with multiple strains prior to the development of immunity, it is likely that exogenous reinfection can occur. Some data indicate that this is rare (142, 221), but more research is needed to clarify this important point. We lack sufficient data to evaluate how acquired

immune responses, be they type specific to HSV-2 or the summation of type-specific and type-common responses in the case of HSV-1- and HSV-2-coinfected persons, influence the acute and long-term outcomes of exposure to a second strain of HSV-2. As a corollary, it is not clear if vaccines that elicit responses matching or even exceeding the levels seen after natural infection will prove adequate for prophylactic vaccines.

“Original antigenic sin,” in which prior immunity to a virus (HSV-1 in this case) changes or limits immune responses to a vaccine for a related virus (HSV-2), could influence the efficacy of HSV-2 vaccines in HSV-1-infected persons (117). Detailed analyses of epitope-specific responses are required to determine if this occurs in human HSV infection. The durability of immune responses to an HSV-2 vaccine is also important. Intermittent sexual exposure over a relatively long period (decades) is likely. Even some live-virus vaccines, such as the older measles vaccines, provided only temporary protection. Vaccination for HSV-2 would most logically be performed prior to sexual activity. However, preadolescents are harder to vaccinate than are infants and school-entry-age children. If a vaccine were found to give type-common protection against both HSV-1 and HSV-2, early childhood vaccination would be optimal. The durability of responses and the need for booster doses will have to be carefully assessed for any vaccine reaching licensure.

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