Evidence for Differences in Immunologic and Pathogenesis Properties of Herpes Simplex Virus 2 Strains From the United States and South Africa

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Background. Genital infection with herpes simplex virus 2 (HSV-2) is linked to an increased risk of infection with human immunodeficiency virus (HIV) in areas such as Sub-Saharan Africa. Thus, an effective genital herpes vaccine would be an important weapon in the fight against HIV/AIDS.

Methods. To test whether a current vaccine candidate can protect against HSV-2 from Sub-Saharan Africa, we examined the ability of an HSV-2 vaccine strain, dl5-29, and other HSV-2 replication-defective mutant strains to protect against genital challenge with US or South African strains in a murine model.

Results. Immunization with dl5-29 reduces infection by both viruses but is significantly more efficacious against the US virus than against the African virus. Furthermore, another US vaccine strain was more efficacious against US than against African viruses, and the converse was observed for the parallel African vaccine strain. Nevertheless, protection against the African viruses was significantly less with all vaccines used in this study.

Conclusions. We conclude that there may be differences in protective epitopes and pathogenesis between the US and African strains that raise the need for increased doses of the existing vaccine candidate or an HSV-2 vaccine strain based on viruses from that region.
mutant viruses remain as promising HSV-2 vaccine candidates because the HSV-2 dl5-29 replication-defective mutant viral vaccine strain can induce protective immunity against heterologous HSV-2 in mice [17], protective and therapeutic immunity against HSV-2 in guinea pigs [18] and protective immunity against ocular HSV-1 infection in mice [19]. The HSV-2 dl5-29 mutant virus contains deletions of the essential UL15 and UL29 genes, the UL5 gene encoding a component of the helicase-primase complex and UL29 encoding infected cell protein 8 (ICP8), the single-stranded DNA binding protein.

Because little is known about the genetic and epitope diversity of HSV-2, we wanted to test whether the US dl5-29 virus could protect against a South African HSV-2 virus. To further analyze the potential effect of HSV-2 genetic variation on vaccine efficacy, we compared the ability of 2 replication-defective HSV-2 viruses, one derived from a US virus and another derived from an African isolate, to protect mice against HSV-2 infection with viruses both geographically related and unrelated to those of the vaccine viruses.

MATERIALS AND METHODS

Cells and Viruses
Vero cells were used for propagation and titration of wildtype (WT) virus stocks and for analysis of viral protein expression [20]. The V5-29 complementing cell line [21] was used for propagation and titration of replication-defective viruses.

HSV-2 strain 186 was isolated from a genital lesion from an individual attending a sexually transmitted disease clinic in Houston, Texas [22], in the 1960s. The 186syn-1 virus was plaque purified from that virus [20]. The HSV-2 strain G low-passage virus, isolated in Chicago in the 1960s [23], was obtained from Bernard Roizman and passaged <4 times in our laboratory. The HSV-2 89-390 virus was isolated in Boston in 1989 from a patient with genital herpes attending the Infectious Disease Clinic at Beth Israel Hospital unpublished result; [24] and was passed twice in Vero cells and then twice at Harvard Medical School. The HSV-2 SD90, SD66, and SD15 viruses were isolated from genital ulcer swab samples from 3 HIV-1–negative men attending a sexually transmitted disease clinic in Carletonville, South Africa, in 1995 [25]. Viral cultures were grown at the Centers for Disease Control and Prevention, and supernatant samples were transferred to Harvard Medical School. The primary isolates were passed 3 times on Vero cells at Harvard Medical School to prepare stocks for these experiments. SD90-3P is a clonal virus derived from SD90 through 3 successive plaque purifications on Vero cells.

The HSV-2 5BlacZ replication-defective mutant virus was derived from 186syn+1 and contains a UL29-lacZ gene fusion in place of the essential UL29 ORF [26]. The SD90-8LacZ mutant virus was produced by the same method. Vaccine and challenge stocks were prepared as whole-cell lysates [27].

Animal Studies
Animal housing and experiments were conducted according to protocols approved by the Harvard Medical Area Standing Committee on Animals. For immunogenicity studies, 6-week-old female C57Bl/6 mice (Taconic Farms) were injected subcutaneously in the rear flank on days 0 and 28 with the indicated dose of vaccine virus. For challenge studies, 6-week-old female Balb/c mice (Taconic Farms) were injected subcutaneously in the rear flank on days 0 and 28 with the indicated dose of vaccine virus. On days 48 and 55, mice were injected subcutaneously with 3 mg of medroxyprogesterone (Sigma). On day 56, they were infected intravaginally with WT HSV-2 viruses at a dose equivalent to 50 times the 50% lethal dose [27].

Neutralizing Antibody Titers
Neutralizing antibody titers were determined using a standard plaque reduction assay [28]. The neutralizing antibody titer is reported as the reciprocal of the highest dilution of serum resulting in at least a 50% reduction in the number of plaques, compared with those incubated with serum samples from mock-immunized mice. Neutralizing titers were determined in 3 independent experiments, each in triplicate.

Major Histocompatibility Complex Class I Pentamer Staining of HSV gB-Specific CD8+ T Cells
Blood samples were collected via tail vein bleed, and peripheral blood mononuclear cells were stained with anti-CD8 and major histocompatibility complex class I (MHC-I) pentamers (Proimmune) [28]. Results are expressed as the percentage of CD8a positive cells staining positive for the gB-specific MHC-I pentamer.

Intracellular Cytokine Staining
Splenocytes were collected from C57Bl/6 mice to analyze the HSV specific CD8+ cellular immune response against the H2Kb-specific HSV gB SSIEFARL peptide or the negative control H2Kb-specific OVA SIINFEKL peptide [28]. To measure CD4+ cellular immune responses, splenocytes from mice were collected and infected with WT viruses at a multiplicity of infection of 1.0 and incubated overnight at 37°C. Infected stimulator splenocytes were then added to experimental splenocytes at a ratio of 1:1. Stimulated splenocytes were incubated at 37°C for 2 h, brefeldin A was added, and cells were incubated for 4 h. Negative controls were incubated with uninfected splenocytes. Samples were stained and analyzed via flow cytometry. Results are expressed as the percentage of cells staining positively for CD8a or CD4 that also stain positive for interferon γ when specifically stimulated with the gB peptide or infected cell
spleenocytes. Results from negative controls have been subtracted from the experimental values listed.

**Assays of Challenge Infection and Disease**

Genital swab samples were acquired daily after the challenge, and shed virus was determined by standard plaque assay on Vero cells [28]. Mice were observed daily for signs of genital lesions and systemic illness. The severity of disease was scored as follows: 0 indicated no sign of disease; 1, slight genital erythema and edema; 2, moderate genital inflammation; 3, purulent genital lesions; and 4, hind-limb paralysis [27]. Mice were euthanized with carbon dioxide at the first sign of paralysis.

**Statistical Analysis**

Statistical analysis of the cellular immune responses and levels of viral shedding after challenge were performed using Mann-Whitney-Wilcoxon 2-sample test. Analysis of protection from paralysis and final analysis of the percentage reduction in disease correlates were performed using the Fisher exact test.

**RESULTS**

Studies reported elsewhere have shown that the dl5-29 mutant virus could induce immunity against challenge infection in mice and guinea pigs with the US HSV-2 G and 333 strains [16, 17, 28]. For this study we used a panel of 3 HSV-2 strains originating in the United States, strains G, 89-390, and 186, and a panel of 3 HSV-2 strains originating in South Africa, strains SD15, SD66, and SD90 (Table 1). The 6 WT viruses replicated to similar levels in Vero cells (not shown), and they showed only slightly different levels of virulence in mice, as determined by the ability to cause hind-limb paralysis after intravaginal infection (Table 1).

We first compared the ability of dl5-29 immunization to protect against SD90-3P, a cloned version of the SD90 isolate, versus its ability to protect against the US HSV-2 G strain. We immunized groups of female Balb/c mice with various doses of dl5-29 virus and later challenged the mice intravaginally with SD90-3P or HSV-2 G. Mice immunized with 10⁴ plaque-forming units (PFU) of dl5-29 and challenged with SD90-3P or G virus showed clinical disease signs similar to that of mock-immunized mice (Figure 1A). Mice immunized with 10⁵ PFU of dl5-29 and challenged with the HSV-2 G strain showed significantly fewer clinical symptoms than mice challenged with SD90-3P (P < .001). Mice immunized with 10⁶ PFU of dl5-29 showed almost no discernible disease symptoms when challenged with SD90-3P or G virus.

Levels of viral shedding also correlated inversely with the immunization dose (Figure 1B). Mice immunized with a dose of 10⁴ PFU of dl5-29 showed essentially no reduction in viral shedding when challenged with SD90-3P but a limited reduction in shedding when challenged with G. Mice immunized with 10⁵ PFU showed significantly reduced levels of viral shedding when challenged with SD90-3P or G virus, with a greater reduction for G than for SD9-3P challenge (P < .01 at each day). An immunization dose of 10⁶ PFU significantly reduced levels of viral shedding in mice challenged with either virus, with a greater reduction for G than for SD90-3P challenge (P < .02 every day except day 5). Therefore, SD90-3P virus shedding was reduced by immunization with dl5-29, but significantly less than shedding with G virus.

All mice immunized with 10⁴ PFU of dl5-29 exhibited signs of hind-limb paralysis by 10 days after challenge, similar to mock-immunized mice (Figure 1C). In contrast, immunization doses of 10⁵ or 10⁶ PFU resulted in reduced paralysis in mice challenged with either SD90-3P or the G strain. Although all mice immunized with a dose of 10⁶ PFU were protected, those immunized with 10⁵ PFU and challenged with the G strain were better protected than those challenged with SD90-3P. Mice challenged with SD90-3P showed a protection rate of 14%, compared with 64% for those challenged with the G strain (P < .001). The calculated dose of dl5-29 needed for 50% protection was 5-fold higher against SD90-3P than against the G strain (3 × 10⁵ vs 6 × 10⁴ PFU). Therefore, protection provided by dl5-29 was stronger against the HSV-2 G strain than it was against SD90-3P.

**Table 1. Herpes Simplex Virus 2 Strains Used in Study**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Origin of parental isolate</th>
<th>Genotype</th>
<th>Paralysis dose50*</th>
</tr>
</thead>
<tbody>
<tr>
<td>186syn⁺-1</td>
<td>United States</td>
<td>Wild type</td>
<td>2.4 × 10⁴</td>
</tr>
<tr>
<td>G strain</td>
<td>United States</td>
<td>Wild type</td>
<td>6.0 × 10⁴</td>
</tr>
<tr>
<td>89-390</td>
<td>United States</td>
<td>Wild type</td>
<td>5.0 × 10⁴</td>
</tr>
<tr>
<td>SD90-3P</td>
<td>South Africa</td>
<td>Wild type</td>
<td>3.0 × 10⁴</td>
</tr>
<tr>
<td>SD15</td>
<td>South Africa</td>
<td>Wild type</td>
<td>5.0 × 10⁴</td>
</tr>
<tr>
<td>SD66</td>
<td>South Africa</td>
<td>Wild type</td>
<td>1.0 × 10⁴</td>
</tr>
<tr>
<td>dl5-29</td>
<td>United States</td>
<td>U₅, U₂₉ deletions</td>
<td>Not determined</td>
</tr>
<tr>
<td>5B-lacZ</td>
<td>United States</td>
<td>U₂₉-lacZ fusion</td>
<td>Not determined</td>
</tr>
<tr>
<td>SD90-8LacZ</td>
<td>South Africa</td>
<td>U₂₉-lacZ fusion</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

**NOTE.** *Dose in plaque-forming units needed for 50% paralysis in Balb/c genital challenge model.
The previous results raised the possibility that there were strain-specific differences in the protective epitopes of these viruses. To test this hypothesis, we assessed the ability of replication-defective mutants derived from US and African viruses to protect against 3 US and 3 African WT viruses. The HSV-2 5BlacZ replication-defective mutant virus, derived from the WT HSV-2 virus 186syn⁻¹⁻1, contains the UL29-lacZ fusion gene in the UL29 locus [26]. The SD90-8LacZ mutant virus contains the same mutation in the SD90-3P genome. Both of these viruses formed plaques only on complementing cell lines that expressed ICP8 (not shown), consistent with previous description of the 5BlacZ virus [26]. To compare the patterns and levels of protein expression by the 2 mutant viruses, we mock-infected or infected Vero cells with the HSV-2 strains 186syn⁻¹⁻1, 5BlacZ, SD90-3P, or SD90-8LacZ. The levels of viral protein synthesis were similar for 5BlacZ and SD90-8LacZ, and both lacked the ICP8 band and showed the more slowly migrating ICP8-lacZ fusion protein band (Figure 2).

To compare the humoral immune responses elicited by the 2 viruses, we immunized groups of Balb/c mice subcutaneously with 5BlacZ or SD90-8LacZ virus. Serum samples were prepared and pooled, and HSV-2 neutralizing antibody titers were determined. Mice immunized with 5BlacZ or SD90-8LacZ mutant viruses showed similar neutralizing antibody titers against 186syn⁻¹⁻1 virus (titer = 50), G virus (100), SD90-3P virus (50), SD15 virus (50), SD66 virus (50), and 89-390 virus (50 for 5BlacZ and 100 for SD90-LacZ).

To quantify the HSV-specific T cell responses induced by the 2 mutant viruses, we immunized groups of C57Bl/6 mice with 5BlacZ or SD90-8LacZ. Primary CD8⁺ T cell responses as analyzed by MHC-I pentamer staining peaked on day 7 after immunization, and mice immunized with either virus showed similar levels of HSV-specific circulating CD8⁺ T cells (P > .445) (Figure 3). Secondary CD8⁺ T cell responses as analyzed with intracellular cytokine staining showed no significant difference between mice immunized with 5BlacZ and those immunized with SD90-8LacZ (P > .594) (Figure 4A). Secondary CD4⁺ T cell responses as analyzed with intracellular cytokine staining were similar in mice immunized with 5BlacZ and those immunized with SD90-8LacZ (Figure 4B).

To compare the protective immunity induced by the 5BlacZ or SD90-8LacZ viruses against genital challenge infection with different HSV-2 strains, we immunized groups of female Balb/c mice either with uninfected cell lysate (control) or with 5BlacZ or SD90-8LacZ viruses. Mice were challenged intravaginally with the indicated WT HSV-2 viruses. Analysis of disease scores showed that the US-derived 5BlacZ vaccine virus protected consistently better than the African vaccine against US challenge.

**Figure 1.** The dl5-29 vaccine strain protects mice against intravaginal infection with herpes simplex virus 2 (HSV-2). Groups of mice (n = 14) were either mock-immunized or immunized with dl5-29 at a dose of 10^6, 10^5, or 10^4 plaque-forming units (PFU) at weeks 0 and 4 and then challenged at week 8 with either HSV-Z SD90-3P virus or HSV-2 G virus. A, Clinical disease score, as described in Materials and Methods. B, Viral shedding. Results are shown as means ± standard errors of the mean. Dotted line indicates limit of detection of 2 PFU. C, Protection from paralysis.

**Properties of HSV-2 Strains From the United States and South Africa**
viruses, and the African-derived SD90-8LacZ vaccine protected better against African viruses than against US viruses, except for the African virus SD66 (Figure 5A). The differences reached statistical significance in mice challenged with 89–390 or SD90-8LacZ, respectively ($P < .03$) (Figure 5A). When these results were pooled by origin of the challenge virus, the US vaccine was significantly more protective against US viruses, and the African vaccine was significantly more protective against African viruses ($P < .03$) (Figure 5B). Analysis of viral shedding also indicated that the US vaccine, 5BlacZ, protected better against US challenge viruses and that SD90-8LacZ protected better against African virus challenge viruses (Figure 5C). Although this trend held true for all the
challenge viruses except the G strain, statistically significant differences were seen only in mice challenged with homologous viruses (ie, 5BlacZ vs 186syn\(^{-1}\) vs 5BlacZ and SD90-8LacZ vs SD90-3P; \(P < .005\)). When the results were grouped by the geographic origin of the challenge virus, the US-derived vaccine, 5BlacZ, was significantly more protective against US viruses, and the African vaccine, SD90-8LacZ, was more protective against African viruses (\(P = .01\)) (Figure 5D).

When protection from paralysis was analyzed according to the geographic origin of challenge viruses, 5BlacZ tended to be more protective against US viruses whereas SD90-8LacZ was more protective against the African viruses. These differences reached statistical significance in mice challenged with 186syn\(^{-1}\) (\(P = .05\)) (Figure 5E). When protection from paralysis for all US challenge viruses was compared with that for all African challenge viruses, we observed that 5BlacZ was significantly more protective against challenge by US viruses than SD90-8LacZ (\(P = .020\)). Although SD90-8LacZ showed a trend toward being more protective against challenge by viruses of African origin than against those of US origin, the difference in protection rates did not reach statistical significance (\(P = .292\)) (Figure 5F). In total, we observed that the African SD90-8LacZ induced better protection against the African viruses, and the US 5BlacZ induced better protection against the US viruses. Further analysis of the challenge infections by the US or African viruses showed that challenge with the African viruses caused less reduction in overall disease (\(P < .001\)), viral shedding (\(P = .051\)), or paralysis (\(P = .016\)) than challenge with the US WT viruses (Figure 6). Therefore, we conclude it was more difficult to induce protection against the African viruses.

**DISCUSSION**

There is a great need for a genital herpes vaccine to prevent herpetic disease, and this need is especially high in certain geographic regions such as Sub-Saharan Africa, where there is a coepidemic of genital herpes and HIV. Although other studies have shown that HSV-2 has limited genetic diversity, we have limited information about the HSV-2 strains that are prevalent in Sub-Saharan Africa. In the current study we investigated the ability of a current vaccine candidate based on a US viral strain to protect against genital challenge in mice with HSV-2 strains originating in the US or South Africa. We found that dl5-29 immunization reduced genital infection with an African HSV-2
Evidence for Immunologic Differences Between US and African HSV-2 Strains

Because the optimal reduction in viral replication and disease occurred when the geographic origins of vaccine strain and challenge virus were matched, we interpreted this to mean that are components of the immune responses that are specific for each of the groups of viruses and that they are important for protection. We observed that the CD8+ T cell responses to the major gB epitope in C57Bl/6 mice were equivalent for the 2 vaccine strains, but the important CD8+ T cell responses could be different in Balb/C mice. We observed that the HSV-specific CD4+ T cell responses in C57Bl/6 mice were equivalent for the 2 vaccine strains, but these could also differ in Balb/C mice. Finally, the neutralizing antibody responses induced by the 2 vaccine strains were equivalent. Therefore, at this stage we are unclear what the key immunologic differences are, but the immune protection appears to be partially specific for the viruses from the US versus Africa. Earlier studies have shown that CD4+ T cells play the dominant role in immune protection induced by 5BlacZ virus in this murine genital infection model [30]; therefore, there may be differences in the levels or specificities of the CD4+ T cell responses that we have not yet elucidated.

The correlates of protection against HSV-2 in humans have not yet been determined, because vaccine protection against HSV-2 has not been achieved. However, several lines of evidence indicate that CD8+ T cells are important in controlling HSV-2 infection or disease in humans [31]. Therefore, the mouse model may not predict behavior of the vaccine in the human situation, but protection in this model is more dependent on cellular immunity than that in the guinea pig model [15]; therefore, it is appropriate to do the initial studies of HSV vaccines in this model.

It is difficult to match the challenge viruses perfectly; however, the panels for both the US and the South African challenge viruses consisted of the WT parent virus for the vaccine strain and 2 low passage HSV-2 strains. These results are important in highlighting the need for viral vaccine strains to be based on the HSV-2 genetic background of strains from that region. Studies of additional isolates are needed to determine the generality of this finding and the optimal vaccine strain and vaccine dose for each region. We hypothesize that the best protection against current HSV-2 strains in Sub-Saharan Africa will be with higher doses of the current vaccine candidate or a vaccine strain constructed in an HSV-2 genetic background currently circulating in Sub-Saharan Africa. We are reconstructing the dl5-29 mutations in additional strains from South Africa to determine the optimal genetic background for a genital herpes vaccine strain for use in South Africa.

Evidence for Pathogenesis Differences Between US and African HSV-2 Strains

In addition to the immunologic differences between the US and African strains, the African strains were particularly virulent, in that both vaccines were less effective against them than against the US viruses. The levels of humoral and cellular immune responses induced by the US 5BlacZ vaccine strain were equivalent to those induced by the African SD90-8lacZ vaccine strain; therefore, we conclude that the differences were due to differences in virulence of the US and African viruses. Further studies are needed to determine the mechanism(s) of the increased pathogenicity of the African strains, such as increased spread in the nervous system, increased replication in neurons, or increased inflammatory disease in the central nervous system. Although the 3 African viruses were isolated from HIV-negative individuals, immunosuppression due to AIDS may have allowed the rapid evolution of HSV-2 in this area to a more virulent form. The high prevalence of genital herpes in Sub-Saharan Africa may be due to the rapid spread of strains such as these with increased virulence. These results emphasize the need for further studies of the genetic diversity of HSV-2 strains from different geographic regions. The only HSV-2 genome completely sequenced is HSV-2 HG-52 [32], which is a laboratory strain and highly attenuated in mice [33]. This phenotype is not representative of other HSV-2 strains; thus, there is an urgent need for a complete HSV-2 genomic sequence from a clinical isolate to use as the framework on which to perform comparisons with sequences from other clinical isolates.

In summary, the results of this work raise the need for more detailed studies of the pathogenesis, genetic diversity, and
epitope composition of low-passage HSV-2 isolates from around the world. The reduction in rates of genital herpes disease could have a great impact on global public health in reducing both the herpetic disease and the risk of HIV infection.

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**References**