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Disruption of the U_L41 gene in the herpes simplex virus 2 *dl*5-29 mutant increases its immunogenicity and protective capacity in a murine model of genital herpes

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Abstract

The herpes simplex virus 2 *dl*5-29 replication-defective mutant virus has been shown to induce protective immunity in mice and both prophylactic and therapeutic immunity in guinea pigs. In an attempt to improve the efficacy of *dl*5-29 we disrupted its U_L41 gene, producing the triple mutant virus *dl*5-29-41L. *dl*5-29-41L has a decreased ability to inhibit host cell protein synthesis and a reduced cytopathic effect on cultured cells. When used to immunize mice, *dl*5-29-41L elicited significantly stronger neutralizing antibody responses and significantly stronger $CD4^+$ and $CD8^+$ cellular immune responses than *dl*5-29. The enhanced immune responses corresponded with increased protective capacity in a murine model of genital herpes. The protective immunity elicited by either virus was very durable, protecting mice for at least 7 months. Furthermore, we show that cell lysate preparations of both viruses were significantly more efficacious than the corresponding extracellular virus preparations.

Keywords: Replication-defective virus; Herpes; HSV; Vaccine

Introduction

Herpes simplex virus 2 (HSV-2) is a common human pathogen, infecting approximately 17% of the adult population in the US (Xu et al., 2006), for which there is currently no broadly effective vaccine. HSV-2 initiates infection in the epithelium and then quickly spreads to and establishes latency within local sensory neurons. The latent virus can later reactivate to cause disease, a process that continues throughout the life span of the host. A variety of clinical syndromes are caused by HSV infection, including life-threatening infections of neonates and immunocompromised individuals such as those with HIV/AIDS. There is a growing body of epidemiological and biological evidence that HSV-2 infection is strongly linked with the HIV epidemic (Freeman et al., 2006). It has been shown that a preexisting HSV-2 infection greatly increases the likelihood of HIV-1 acquisition (Reynolds et al., 2003; Wald and Link, 2002), and individuals co-infected with HSV-2 and HIV-1 tend to show higher HIV-1 viral loads (Schacker et al., 2002). Perhaps most notably, a recent study has shown that an anti-HSV therapeutic can reduce both the genital and plasma HIV-1 RNA levels in dual infected individuals (Nagot et al., 2007), and this reduction in HIV-1 viral RNA loads may lead to reduced transmission rates and decrease progression to AIDS. These studies suggest that an effective HSV-2 vaccine would not only be useful against HSV-2 disease, but also may provide an effective means of reducing HIV-1 infection. The large disease burden caused by HSV-2 and the contributory role of genital HSV-2 disease to the HIV/AIDS pandemic drive the efforts to find an effective HSV-2 vaccine (Jones and Knipe, 2003).

Various approaches have been undertaken to produce a vaccine against HSV-2, yet only one has shown any protective efficacy in humans (Stanberry et al., 2002) and none has shown efficacy within the general population (Corey et al., 1999;

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Koelle and Corey, 2003; Stanberry, 2004; Stanberry et al., 2002). It is likely that an effective vaccine against HSV-2 will be required to elicit both humoral as well as cellular immunity, while retaining a high safety profile that would allow for vaccination of individuals who are or may become immunocompromised. Replication-defective viruses may possess both of these desired properties (Dudek and Knipe, 2006).

The HSV-2 replication-defective mutant virus *dl*5-29 contains deletions of both the U_L5 and U_L29 genes. The U_L5 gene encodes a component of the viral helicase–primase complex and U_L29 encodes ICP8, the viral single-stranded DNA binding protein. Both of these gene products are essential for viral DNA replication and deletion of either gene renders a virus replication-defective. Therefore, *dl5-29* can infect a normal cell and express a vast majority of viral proteins, but it is unable to replicate its genome and produce infectious progeny so the viral life cycle is interrupted (Da Costa et al., 2000). *dl*5-29 has previously been shown to induce protective immunity in mice (Da Costa et al., 1999) and has shown both protective and therapeutic efficacy in guinea pigs (Hoshino et al., 2005). Based on the immunogenic and protection studies in mice and guinea pigs, *dl*5-29 has been chosen to enter clinical trials. We have undertaken studies to further modify *dl*5-29 genetically because comparison of additional viral mutants may not only provide better vaccine candidates but may also provide information that helps define the mechanisms of immune protection and immune correlates.

HSV-1 and HSV-2 both contain a gene, U_L41, which encodes the virion host shut-off (vhs) function. This gene product causes the degradation of both host and viral mRNA, leading to the shutdown of host cell protein synthesis. Early in infection the high level of viral transcription overcomes this effect, allowing the virus to continue replicating, while at late time points during the replication cycle the vhs activity may be inhibited by its binding to VP16 (Lam et al., 1996). Disruption of U_L41 has been shown to increase the immunogenicity of replication-competent (Walker and Leib, 1998) and replication-defective HSV-1 viruses (Geiss et al., 2000), but the immunogenicity of U_L41 disrupted HSV-2 mutant viruses has not been determined. HSV-2 is known to have a significantly greater effect on host cell protein synthesis than that of HSV-1 (Fenwick et al., 1979) and this effect has been shown to be vhs-specific (Everly and Read, 1997). Therefore, we wished to determine the effect of disrupting U_L41 on viral immunogenicity in the context of a replication-defective HSV-2 mutant virus, the *dl*5-29 candidate vaccine virus. Here we characterize this new virus, *dl*5-29-41L, and compare its immunogenicity and protective capacity with that of *dl*5-29 in a murine model.

Results

Construction and characterization of the dl5-29-41L mutant virus

To generate a triple mutant virus with the U_L41 gene inactivated, we inserted a LacZ expression cassette into a *Sac*II–*Sac*II deletion within the U_L41 gene locus in a plasmid and then introduced the insertion into the *dl*5-29 viral genome via homologous recombination as described previously (Da Costa et al., 1997) (Fig. 1). Similar disruptions of the U_L41 gene have been shown to disrupt Vhs function (Strelow and Leib, 1995).

To determine if disruption of U_L41 in our *dl*5-29 mutant virus affects the ability of the virus to shut off host protein synthesis, we examined host and viral protein synthesis in Vero and mouse embryo fibroblasts (MEF) cell lines by mock-infecting or infecting with *dl*5-29, *dl*5-29-41L or the wild-type HSV-2 strain 186 syn$^+$-1 virus (Fig. 2). Cells infected with *dl*5-29-41L showed a total host protein expression pattern most similar to that of mock-infected cells (Fig. 2, lanes 1 and 5; illustrative host protein bands are marked by asterisks), while still producing HSV-2 viral proteins similar in both specificity and intensity to that of *dl*5-29 (Fig. 2, lanes 3 and 7; representative viral bands marked by arrows). The lack of host cell protein shut-off was also apparent as delayed and decreased cytopathic effect on infected cells during a productive infection of the V5-29 complementing cell line (data not shown).

dl5-29-41L elicits stronger humoral immune responses than dl5-29

To compare immune responses elicited by the two viruses, we immunized Balb/c mice subcutaneously with two doses of 10^4, 10^5 or 10^6 pfu of *dl*5-29 or *dl*5-29-41L at an interval of 3 weeks. At 7 days post-immunization mice were bled via the tail vein, and serum samples from each immunization group were collected and pooled.

HSV-2 specific antibodies

The total serum IgG response against HSV-2 antigen was measured via ELISA using sera pooled from each immunization group of eight mice (Table 1). As an extracellular supernatant viral immunogen preparation, *dl*5-29-41L elicited 2-fold higher total IgG titers than *dl*5-29, although the antibody titers observed when mice were immunized with whole cell lysate viral immunogen preparations of either *dl*5-29 or *dl*5-29-41L

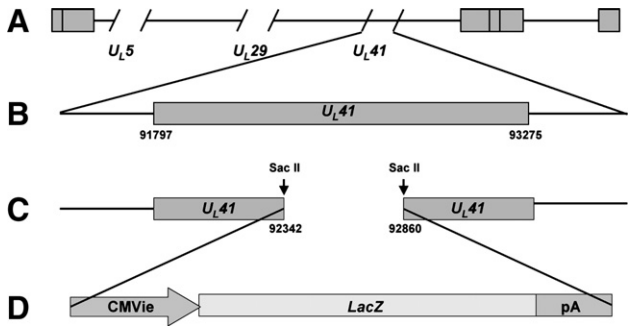

Fig. 1. Diagram of the *dl*5-29-41L genome. Line A represents the *dl*5-29 genome containing deletions in the U_L5 and U_L29 genes. Line B shows an expanded view of the U_L41 gene and bounding sequences. Line C indicates the section of U_L41 that was removed by *Sac*II digestion. Line D illustrates the mammalian LacZ expression cassette that was inserted into the 518 bp deletion within U_L41 created by the *Sac*II deletion. Nucleotide numbers correspond to the HSV-2 HG-52 viral genome.

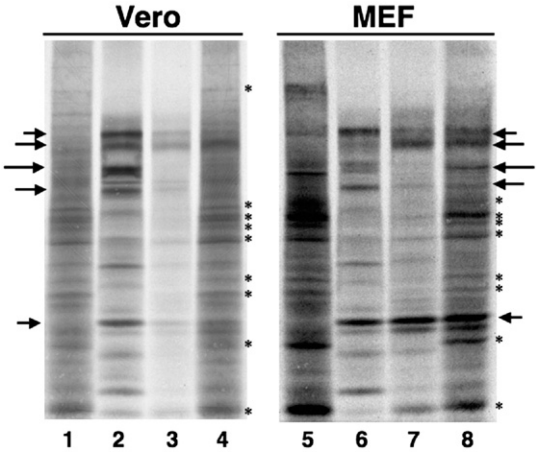

Fig. 2. Mutant *dl*5-29-41L has a reduced ability to inhibit host cell protein synthesis. Vero cells (left panel) and mouse embryo fibroblasts (right panel) were either mock infected (lanes 1, 5) or infected with wild-type HSV-2 (lanes 2, 6), *dl*5-29 (lanes 3, 7) or *dl*5-29-41L (lanes 4, 8) viruses at an MOI of 10 for 6 h. At 30 min before harvesting cells were labeled with ^{35}S-methionine. Proteins were resolved on a 9.25% DATD cross-linked gel and the gel was subjected to audioradiography. The resulting autoradiogram is shown. Major host protein bands are denoted by asterisks while representative viral proteins are marked by arrows.

were equal. There was a substantial increase in total antibody titers elicited in mice immunized with cell lysate immunogen (12,800 for either *dl*5-29 or *dl*5-29-41L) over those elicited by supernatant viruses (200 for *dl*5-29 and 400 for *dl*5-29-41L). The difference in total IgG titers most likely reflects the nature of the immunogens. The cell lysate viral immunogen preparation contains substantially more viral protein than supernatant virus and it was not unexpected that this immunogen raised much higher total virus-specific antibody titers than the extracellular supernatant viral preparation at equivalent doses.

Neutralizing antibody titers

Neutralizing antibody titers were determined using sera pooled from the indicated immunization groups of eight mice each. Balb/c mice immunized with *dl*5-29-41L showed an 8-fold increase in the neutralizing antibody titers as compared with animals immunized with *dl*5-29. The magnitudes of these responses were equal regardless of the immunogen preparation (Table 1). This indicated that the production of neutralizing antibody was proportional to the number of infectious viral particles used to immunize mice, not the total amount of antigen, as with the total HSV-2 specific IgG antibody titers measured by ELISA.

dl5-29-41L elicits stronger cellular immune responses than dl5-29

C57Bl/6 or Balb/c mice were immunized twice, subcutaneously, with 10^6 pfu of *dl*5-29 or *dl*5-29-41L at an interval of 3 weeks and HSV-specific T-cell responses were measured by two independent assays. CD8$^+$ T-cell responses were measured using the highly immunodominant H-2K^b restricted, HSV gB epitope, SSIEFARL, the only well-characterized HSV-specific CD8$^+$ T-cell epitope (Wallace et al., 1999). These responses were analyzed using both MHC-I pentamer (ProImmune, Oxford, UK) staining and intracellular cytokine staining. The CD4$^+$ T-cell response was measured using the recently identified H-2^d restricted, HSV-2 gD-specific CTL epitope KPPYTSTLLPPELSD (Cooper et al., 2006) by intracellular cytokine staining.

MHC-I pentamer staining

HSV gB-SSIEFARL peptide-specific, circulating CD8$^+$ T-cells in immunized C57Bl/6 mice were quantified using MHC-I pentamers. *dl*5-29-41L consistently elicited a stronger response than *dl*5-29 with a statistically significant difference seen at the peak responses (Fig. 3A). The primary response peaked at 7 days post-immunization with *dl*5-29-41L showing a 2.1-fold higher response ($p \leq 0.05$) than *dl*5-29 when mice were immunized with lysate virus and 2.3-fold higher response ($p \leq 0.05$) elicited by *dl*5-29-41L over *dl*5-29 when mice were immunized with supernatant virus. After boosting at day 21 there was also a transient increase in the number of gB-specific CD8$^+$ T cells (Fig. 3A). The secondary responses peaked at 6 days post-boost with *dl*5-29-41L eliciting a 1.7-fold higher response ($p \leq 0.05$) in mice immunized with lysate virus and a 2.8-fold increase ($p \leq 0.05$) in mice immunized with supernatant virus. Raw data of representative samples from the MHC-I pentamer staining experiment are shown as dot plot graphs allowing visualization of the separation of cell populations (Supplementary Figure, panel A).

Intracellular cytokine staining

At 7 days post-boost, mice were sacrificed and splenocytes were collected. CD8$^+$ responses were measured in C57Bl/6 mice, and CD4$^+$ responses were measured using Balb/c mice. The percentages of CD8$^+$ splenocytes that specifically produced IFN-γ when stimulated with the HSV gB-specific peptide SSIEFARL were measured. This assay verified the MHC-I pentamer results in that *dl*5-29-41L induced a statistically significantly stronger CD8$^+$ T-cell response than *dl*5-29 regardless of the immunogen preparation, showing a 2.6-fold ($p \leq 0.05$) and 4.1-fold ($p \leq 0.05$) increase seen in mice immunized with lysate virus and supernatant virus, respectively (Fig. 3B). The

Table 1
Humoral immune responses induced by *dl*5-29 and *dl*5-29-41L

	Dose (pfu)	Total serum IgG titer[a]		Neutralizing Ab titer[b]	
		Ex. cell[c]	Lysate[d]	Ex. cell[c]	Lysate[d]
*dl*5-29	10^4	≤50	200	<25	<25
	10^5	≤50	800	<25	<25
	10^6	200	12,800	25	25
*dl*5-29-41L	10^4	≤50	200	<25	<25
	10^5	200	800	<25	<25
	10^6	400	12,800	200	200

[a] Measured by ELISA against total HSV-2 G strain virus antigen.
[b] Measured by plaque reduction assay against HSV-2 G strain.
[c] Extracellular supernatant virus immunogen.
[d] Cell lysate virus immunogen.

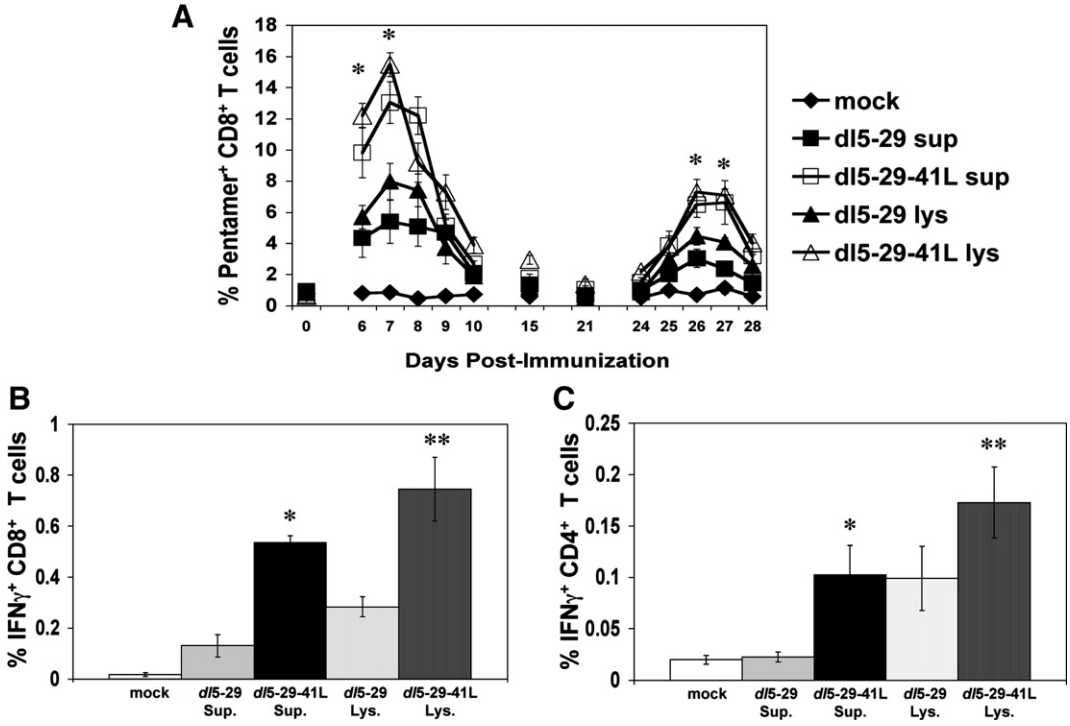

Fig. 3. *dl*5-29-41L virus elicits significantly stronger $CD4^+$ and $CD8^+$ cellular immune responses than *dl*5-29 virus. (A) MHC-I pentamer staining. C57Bl/6 mice were immunized with *dl*5-29, *dl*5-29-41L, uninfected V5-29 cell lysate or PBS + 15% glycerol. Whole blood was collected via tail vein bleed, red blood cells were lysed, and cells were stained with MHC-I pentamers specific for the $H2K^b$ restricted HSV-2 gB immunodominant epitope SSIEFARL and antibody against murine CD8a. Results are shown as the percentage of $CD8^+$ cells that are also positive for MHC-I pentamer staining ±SEM over time. *$p < 0.05$ for *dl*5-29 vs *dl*5-29-41L for both supernatant and lysate preparations.. (B) $CD8^+$ intracellular cytokine staining. C57Bl/6 mice were immunized twice with *dl*5-29, *dl*5-29-41L, uninfected V5-29 cell lysate or PBS + 15% glycerol. At 7 days after boosting, splenocytes were collected and stimulated with the gB-SSIEFARL peptide. Results are shown as the percentage of $CD8^+$ cells that express IFN-γ specifically after stimulation with the gB-SSIEFARL peptide ±SEM. *$p = 0.03$, **$p = 0.04$. (C) $CD4^+$intracellular cytokine staining. Balb/c mice were immunized twice with *dl*5-29, *dl*5-29-41L, uninfected V5-29 cell lysate or PBS + 15% glycerol. At 7 days after boosting, splenocytes were collected and stimulated with the HSV-2 gD Balb/c specific peptide-KPPYTSTLLPPELSD. Results are shown as the percentage of $CD4^+$ cells that express IFN-γ specifically after stimulation with the gD peptide ±SEM. *$p = 0.03$, **$p = 0.04$.

$CD4^+$ cellular immune response was measured using the HSV-2 gD-specific CTL epitope KPPYTSTLLPPELSD (Cooper et al., 2006). The percentage of $CD4^+$ splenocytes that specifically produced IFN-γ when stimulated with this peptide was also significantly stronger in mice immunized with *dl*5-29-41L than *dl*5-29, with a 1.7-fold ($p \leq 0.05$) or a 4.6-fold ($p \leq 0.05$) increase when mice were immunized with lysate or supernatant viral preparations respectively (Fig. 3C). Raw data of representative samples from the intracellular cytokine staining experiment are shown as dot plot graphs allowing visualization of the separation of cell populations (Supplementary Figure, panels B, C).

dl5-29-41L elicited stronger protective immunity than dl5-29 against an HSV-2 G strain challenge

To compare the protective immunity induced by the two viruses, we immunized 8 female Balb/c mice per group, twice, with either extracellular virus or lysate virus immunogens at 0 and 3 weeks with varying doses (10^4, 10^5 or 10^6 pfu/dose) of the *dl*5-29 or *dl*5-29-41L viruses. At 1 month post-boost, mice were challenged intravaginally with 5×10^5 pfu of the wild-type HSV-2 G strain, a dose equal to 50 times the lethal $dose_{50}$ (50 LD_{50}) (Fig. 4). As a quantitative measure of viral replication, we assessed the levels of viral shedding from the vaginal cavity. Vaginal swabs were taken every 24 h post-challenge for 1 week, and the amount of virus captured was assessed by plaque assay.

Clinical disease

Mice immunized with *dl*5-29-41L showed less severe signs of clinical disease than those immunized with *dl*5-29 for mice immunized with either supernatant virus (Fig. 5A) or lysate virus (Fig. 5B). Mice immunized with lysate virus preparations

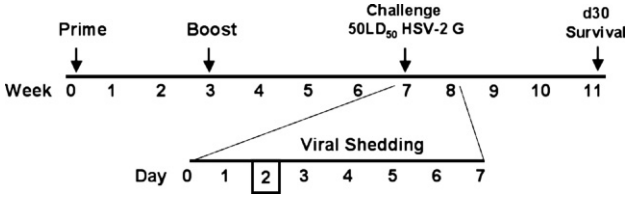

Fig. 4. Time line for immunization/challenge study. Balb/c mice were immunized subcutaneously, at 0 and 3 weeks, with varying doses of virus. At 4 weeks post-boost mice were challenged intravaginally with the equivalent of 50 times the lethal $dose_{50}$ (50 LD_{50}) of wild-type HSV-2 strain G. Vaginal swabs were collected daily for the first 5 days post-challenge to determine the level of viral shedding from the site of infection. Mice were followed for 30 days to determine protection rates.

of either *dl*5-29-41L at a dose of 10^5 pfu or *dl*5-29 or *dl*5-29-41L at a dose of 10^6 pfu showed no signs of clinical disease.

Protection from paralysis

There was a significant increase in the 30-day protection rates of mice immunized with *dl*5-29-41L over those immunized with *dl*5-29, this being the case whether mice were immunized with extracellular supernatant (Fig. 5C) or cell lysate viral (Fig. 5D) immunogens. The increased protection was quantified through calculation of protective dose 50 (PD_{50}) values for these immunogens, the dose of vaccine virus needed for the protection of 50% of the mice from paralysis. As extracellular virus immunogens, *dl*5-29-41L had a PD_{50} calculated to be 15× lower than that of *dl*5-29 (4×10^4 pfu vs. 6×10^5 pfu, respectively), while for the lysate viral immunogens, *dl*5-29-41L had a PD_{50} calculated to be 4× lower than that of *dl*5-29 (1×10^4 pfu vs. 4×10^4 pfu, respectively).

Shedding of challenge virus

Levels of viral shedding peaked at day 2 post-challenge and then declined steadily over the next 5 days. The effects of immunization on the duration of viral shedding paralleled the effects seen on peak viral shedding (data not shown). Peak levels of viral shedding are shown here as a function of immunogen dose (Fig. 5E). Mice immunized with *dl*5-29-41L showed a greater reduction in the amount of virus being shed from the vaginal tract when compared to mice immunized with *dl*5-29 at all doses administered. The magnitude of the difference in viral shedding increased with increasing doses of immunizing virus. Combining the results from both supernatant and lysate viral immunization groups, mice immunized with 10^4 pfu of *dl*5-29-41L showed an average of 2.4-fold less virus shed from the vaginal tract compared with those mice immunized with *dl*5-29, while mice immunized with 10^5 pfu showed a 6.1-fold average reduction in viral shedding and mice immunized with 10^6 pfu showed a 7.0-fold average reduction. Mice immunized with lysate viral immunogen showed a significantly reduced level of viral shedding as compared with mice immunized with supernatant virus regardless of dose. This difference peaked at over a 100-fold decrease in viral shedding in mice immunized with *dl*5-29-41L at a dose of 10^6 pfu. Thus, over a range of doses, *dl*5-29-41L elicited greater protective immunity than *dl*5-

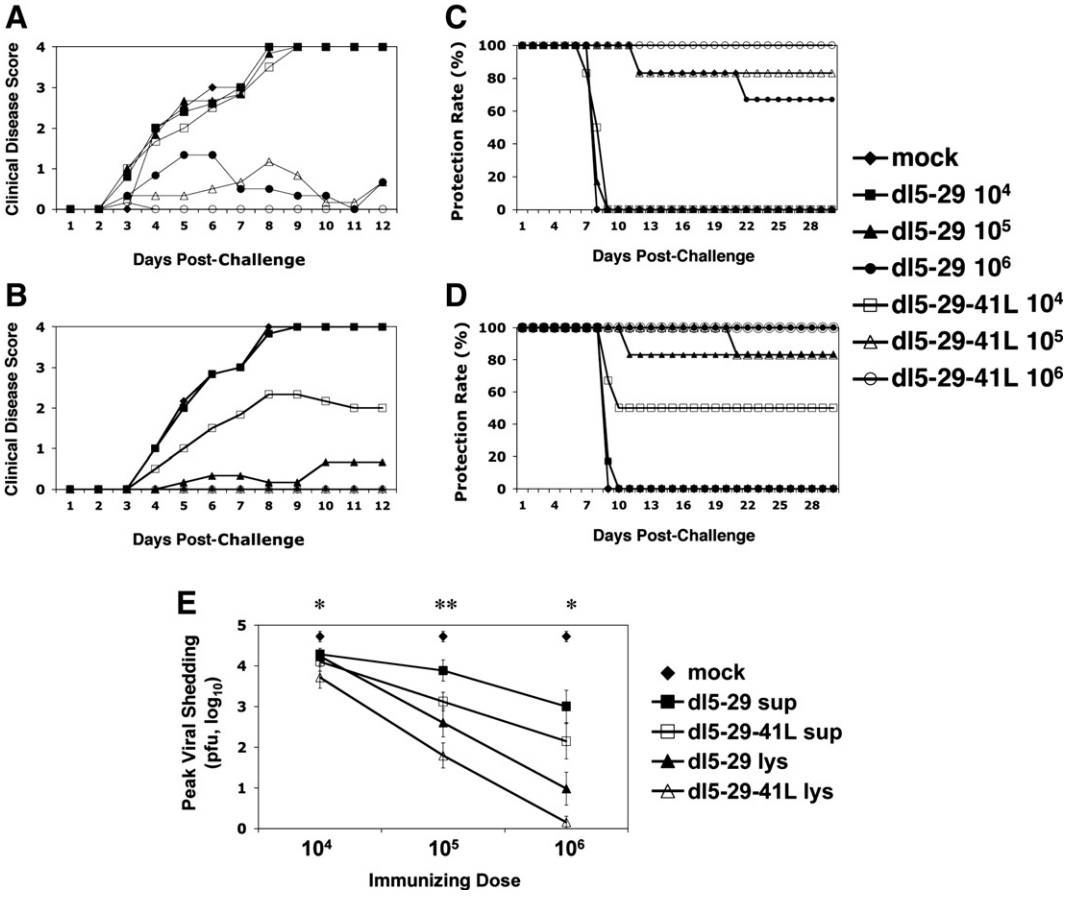

Fig. 5. Mice immunized with *dl*5-29-41L virus have lower clinical disease scores and higher rates of protection than those immunized with *dl*5-29 when challenged intravaginally with a highly lethal dose of wild-type HSV-2 strain G. (A) Clinical disease scores of mice immunized with extracellular viral immunogen. (B) Clinical disease scores of mice immunized with lysate viral immunogen. (C) Rates of protection from encephalitis in mice immunized with varying doses of extracellular supernatant viral immunogen. (D) Rates of protection from encephalitis in mice immunized with varying doses of whole cell lysate viral immunogen. (E) Viral shedding in the genital tract. Vaginal swabs were taken daily for 7 days post-challenge. Viral shedding was determined by standard plaque assay and the levels of virus shedding from the vaginal tract peaked at day 2 post-challenge. Shown here is the day 2 viral shedding for different doses of immunogen. *$p<0.05$ between mice immunized with lysate viruses. **$p<0.05$ between mice immunized with lysate viruses and between mice immunized with supernatant viruses.

29 and lysate viral preparations elicited greater protection than supernatant viral preparations.

Durability of immune responses elicited by dl5-29 and dl5-29-41L

To examine the durability of the immune responses, groups of female Balb/c mice were immunized twice, at weeks 0 and 3, with 10^5 pfu of *dl*5-29 or *dl*5-29-41L lysate virus and challenged intravaginally with 5×10^5 pfu (50 LD_{50}) of the wild-type HSV-2 G strain at 1, 4 or 7 months post-boost.

Immunogenicity

Mice were bled via tail vein at 1 week pre-challenge and the total serum IgG response against HSV-2 antigen was measured via ELISA. We detected no reduction in HSV-2 specific antibody titers over time with both viruses eliciting a titer of 12,800 (Table 2). The neutralizing antibody responses were determined as described earlier with *dl*5-29 and *dl*5-29-41L eliciting titers of 256 and 1024 respectively. These titers were also extremely durable, showing no loss of effectiveness over this 7-month time period (Table 2). Although the humoral responses elicited during the "acute infection" experiments, comparing multiple doses of immunogens, and the "durability of immunity" experiments were not of equal magnitude (Tables 1 and 2), the relative levels of the humoral responses elicited by *dl*5-29 and *dl*5-29-41L were similar in the two studies, with *dl*5-29-41L eliciting 4- to 8-fold higher neutralizing titers than *dl*5-29.

Shedding of challenge virus

Consistent with the previous results, when challenged at 1 month post-immunization, mice inoculated with *dl*5-29-41L showed 8.6-fold less ($p=0.02$) virus being shed from the vaginal tract as compared with mice immunized with *dl*5-29 (Fig. 6). Surprisingly, mice that were challenged at 4 months post-immunization no longer showed significant differences in the levels of viral shedding when immunized with *dl*5-29 or *dl*5-29-41L. This lack of difference in viral shedding was also seen when mice were challenged at 7 months after immunization. Although the levels of viral shedding did increase slightly with an increase in the duration of time between immunization and challenge, at 7 months after immunization there was still a highly significant difference in the levels of viral shedding between mice immunized with *dl*5-29 or *dl*5-29-41L and mock immunized mice ($p<0.00001$), indicating that the protective immune response was quite durable.

Table 2
Durability of humoral immune responses

	Immunogen	Time post-immunization		
		1 month	4 months	7 months
Total IgG titer[a]	Mock	100	100	100
	*dl*5-29	12,800	12,800	12,800
	*dl*5-29-41L	12,800	12,800	12,800
Neutralizing Ab titer[b]	Mock	<16	<16	<16
	*dl*5-29	256	256	256
	*dl*5-29-41L	1024	1024	1024

[a] Measured by ELISA against total HSV-2 G strain antigen.
[b] Measured by plaque reduction assay against HSV-2 G strain.

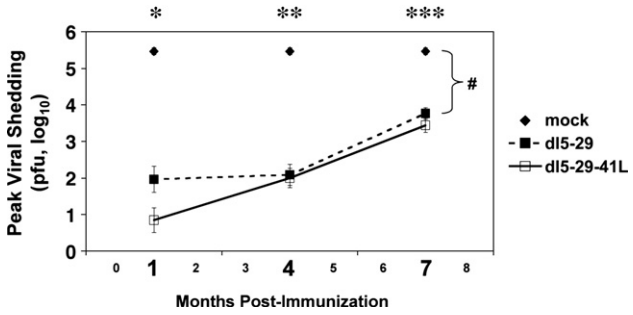

Fig. 6. Durability of protective immunity. Female Balb/c mice were immunized at 0 and 3 weeks with 10^5 pfu of lysate viral immunogen or mock-infected V5-29 cell lysate. Groups of 8 Balb/c mice were challenged intravaginally with 50 LD_{50} of the wild-type HSV-2 strain G at 1, 4 or 7 months after boost. Vaginal swabs were taken daily for 7 days post-challenge and viral shedding was determined by standard plaque assay. Shown here is the day 2 viral shedding ± SEM. *$p=0.02$, **$p=0.41$, ***$p=0.11$, $^{\#}p<0.00001$.

Protection from paralysis

When mice were challenged at 1 month post-immunization both *dl*5-29-41L and *dl*5-29 gave a 100% (8/8) protection rate. When challenged at 4 months post-immunization there was an insignificant difference ($p>0.1$) with the *dl*5-29-41L immunized group showing a 100% (8/8) protection rate, while the *dl*5-29 group had a protection rate of 75% (6/8). Both groups of mice showed 100% (8/8) protection rate when challenged at 7 months post-immunization. Therefore, both *dl*5-29 and *dl*5-29-41L induced protective immunity in mice that was durable for at least 7 months.

Discussion

The HSV-2 *dl*5-29 replication-defective mutant virus has been highly protective as a vaccine in both the mouse (Da Costa et al., 1999, 2000) and guinea pig genital herpes infection model systems (Hoshino et al., 2005). Inactivation of the U_L41 gene has been shown to increase the immunogenicity of replication-competent HSV-1 (Walker and Leib, 1998), as well as replication-defective HSV-1 viruses (Geiss et al., 2000), but the immunogenicity of U_L41 disrupted HSV-2 replication-defective mutant viruses has not been examined. Here we determined the effect of disrupting U_L41 on immunogenicity in the context of an HSV-2 replication-defective mutant virus, the *dl*5-29 candidate vaccine virus. We observed that *dl*5-29-4lL was more immunogenic than the *dl*5-29 double mutant, stimulating higher neutralizing antibody titers and higher $CD4^+$ and $CD8^+$ T-cell responses and, ultimately, providing higher levels of protection against a lethal challenge.

The correlate(s) of protection against HSV infection in humans have not been defined, but optimal protection most likely requires both the humoral and cellular arms of the immune system. Both $CD8^+$ and $CD4^+$ T cells can directly interfere with viral replication by killing virally infected cells, while $CD8^+$ T cells have also been shown to suppress HSV reactivation by

non-cytolytic means (Decman et al., 2005; Liu et al., 2001; Pereira et al., 2000). In both mice and humans HSV-specific $CD4^+$ and $CD8^+$ T lymphocytes are found at the site of viral replication, and infiltration of HSV-specific $CD8^+$ T cells and interferon-gamma (IFN-γ) production correlates with clearance of virus (Koelle et al., 1998; Liu et al., 2001, 2000).

The importance of antibody in protection against HSV is less well defined, but protection of neonates from HSV acquisition suggests that antibody can protect humans against central nervous system infection (Brown et al., 1991), and studies in animal models have also shown passive immunization both before or shortly after infection can beneficially affect the outcome of an HSV infection (Bourne et al., 2002; Morrison et al., 2001). Theoretically, strong antibody responses may be important in preventing primary infection of the mucosa or reducing the number of infecting HSV particles. Humoral immunity may also be important in reducing viral spread from the site of primary infection or recurrent outbreaks. While the improved humoral immune response is encouraging, the increase in cellular immunity (Fig. 3) is most significant as immunization studies have shown that the strong elicitation of a Th1 type cellular immune response is vital for protecting against and controlling HSV infections in animals (Milligan and Bernstein, 1995; Morrison and Knipe, 1997).

Potential mechanisms of increased immunogenicity of dl5-29-41L

Several mechanisms could explain the ability of *dl*5-29-41L to elicit stronger humoral and cellular immune responses. First, this could involve the ability of U_L41 to inhibit type-I interferon responses (Duerst and Morrison, 2004; Murphy et al., 2003), an important innate mechanism for effectively initiating an anti-viral response. Type-I interferons can act by directly stimulating an innate anti-viral response in host cells and by stimulating the initiation of T-cell responses (Nguyen et al., 2002). In the absence of the vhs function, infected cells may undergo a more robust innate response, allowing increased presentation of viral antigens. Second, lack of vhs function could lead to decreased viral cytopathic effects, allowing prolonged viral gene expression and increased antigen presentation, resulting in a more robust stimulation of an adaptive immune response. Third, U_L41 is required for HSV-specific inhibition of dendritic cell (DC) maturation (Prechtel et al., 2005; Samady et al., 2003), a key cell in stimulating and directing the development of adaptive immune responses. By relieving the vhs-specific inhibition of DC maturation, we may be allowing DCs to stimulate a more robust immune response. DCs have been shown to be efficient stimulators of $CD4^+$ and $CD8^+$ T lymphocytes and B lymphocytes (Adams et al., 2005); thus, they make a likely candidate for at least part of the increased humoral and cellular immunogenicity seen with *dl*5-29-41L. HSV infection of immature DCs has been shown to inhibit their maturation (Samady et al., 2003), while infection of mature DCs blocks their ability to become re-stimulated by antigen and inhibits their ability to migrate to lymphoid tissue (Prechtel et al., 2005). It has also been shown that viruses deficient in vhs do not inhibit DC maturation, activation and/or chemotaxis (Prechtel et al., 2005; Samady et al., 2003). Perhaps dendritic cells that have taken up or been infected by *dl*5-29-41L may be allowed to further mature and directly prime a stronger response than those infected by *dl*5-29. Alternatively, in the absence of the vhs function, infected cells will continue to express cellular proteins required to undergo the innate antiviral response of apoptosis. During HSV infection of several cell types, viral immediate early gene expression induces apoptosis, which is quickly inhibited by specific viral gene products (Roizman et al., 2007). Infection of DCs by wild-type HSV-2 causes them to undergo apoptosis, a process that does not appear to be efficiently inhibited by viral gene products (Jones et al., 2003; Pollara et al., 2005). These apoptotic DCs can be taken up by uninfected bystander DCs, which can then stimulate an immune response (Bosnjak et al., 2005). More efficient apoptosis of infected host cells, including dendritic cells, would increase the amount of material that bystander DCs can take up allowing for an increase in cross-presentation or indirect priming of an immune response, a theory supported by recent studies with HSV (Bosnjak et al., 2005) and Modified Vaccinia Ankara virus (Chahroudi et al., 2006).

Disruption of U_L41 increases the protective capacity of dl5-29

Most importantly, the increased immunogenicity caused by the disruption of U_L41 in *dl*5-29 led to an increase in protective efficacy. Protection of mice immunized with *dl*5-29-41L was significantly higher than those immunized with *dl*5-29, as measured by clinical disease symptoms, viral replication within the vaginal tract and protection against paralysis. Focusing on viral shedding, the most quantitative measure of protection in our model, disruption of U_L41 reduced the average level of viral shedding by up to 7.3-fold. The superior protective efficacy of *dl*5-29-41L was seen with both extracellular supernatant virus and whole cell lysate virus immunogen preparations. Directly comparing the efficacy of the different immunogen preparations, we found that lysate virus immunogen is significantly more protective than extracellular supernatant purified virus immunogen for both mutant viruses, *dl*5-29 and *dl*5-29-41L, with a 15-fold and 4-fold decrease in the PD_{50}, respectively. Although we did not observe any difference in neutralizing antibody titers between preparations of either virus, we did detect an increase in the $CD4^+$ and the $CD8^+$ cellular immune response elicited by the lysate immunogen preparations of both *dl*5-29 and *dl*5-29-41L, suggesting that the viral proteins present in the lysate preparation may add to the cellular immune response through cross-presentation mechanisms and/or that the high levels of viral and cellular proteins present in the inoculum act as an adjuvant, stimulating a stronger innate response leading to a more robust adaptive response.

Protective immunity elicited by dl5-29 and dl5-29-41L is durable

A successful vaccine must elicit immunity that is not only protective but also long-lived. We show that the protective

immunity elicited by both *dl*5-29 and *dl*5-29-41L is very durable. The protective efficacy of both mutant viruses persisted for at least 7 months post-immunization, protecting mice from lethal HSV-2 challenge with only a slight decline in the ability to reduce levels of viral shedding. Unexpectedly, animals that were challenged at 4 and 7 months after immunization with *dl*5-29-41L no longer showed a significant decrease in the levels of viral shedding from the site of infection over those animals immunized with *dl*5-29. Because the neutralizing antibody response did not wane over this time period, it can be concluded that it is not solely responsible for the initial increase in protection provided by *dl*5-29-41L over *dl*5-29 shortly after immunization or the subsequent waning of that protection. Cellular immunity was not measured at these later time points, and we can therefore not say anything about the longevity of these responses.

Conclusions

In summary, these results show that the disruption of the U_L41 gene in the context of a replication-defective HSV-2 mutant virus, *dl*5-29, significantly improves its immunogenicity and protective capacity in a murine HSV-2 infection model system. We show also that a cellular lysate viral immunogen preparation is significantly more protective than the more purified extracellular supernatant viral preparation, indicating a potential advantage for the inclusion of viral proteins in a replication-defective HSV-2 mutant virus vaccine formulation over that of a vaccine containing only highly purified virus. Finally, we show that the protective efficacy elicited by either *dl*5-29 or *dl*5-29-41L is not only robust but also durable, protecting mice from challenge for up to 7 months post-immunization. While *dl*5-29 has previously been shown to elicit impressive protective immunity in animal models (Da Costa et al., 1999; Hoshino et al., 2005), the degree to which this will translate in humans is unknown. Thus, any improvement in immunogenicity has the potential to improve its protective efficacy in humans. The significant improvement seen here warrants further study of U_L41 deficient replication-defective HSV-2 mutant viruses to evaluate their potential to protect humans prophylactically and therapeutically against HSV-2 infection.

Materials and methods

Plasmids

To produce the p1941 plasmid, a 2508 bp fragment of wild-type HSV-2 viral DNA encompassing the entire U_L41 gene locus flanked by *Nde*I and *Bam*HI restriction enzyme sites was cloned into the respective restriction enzyme sites on the pUC19 plasmid (Yanisch-Perron et al., 1985). The p1941L plasmid was then constructed by insertion of a LacZ expression cassette into a 518 bp deletion (corresponding to nucleotides 92,342–92,860 of HSV-2 strain HG-52) within the U_L41 gene locus that was created by a *Sac*II–*Sac*II digestion followed by blunting the ends via incubation with the Klenow fragment of *E. coli* DNA Polymerase I.

Cells and viruses

The wild-type HSV-2 G (Ejercito et al., 1968) and 186 syn^+-1 strains (Spang et al., 1983) were propagated on Vero cells (ATCC CCL-81). Vero cells were also used to measure virus neutralizing antibody titers. Vero and mouse embryo fibroblasts (ATCC SCRC-1008) cells were used to analyze protein expression patterns of various viruses. The *dl*5-29 and *dl*5-29-41L mutant viruses were grown and titered on V5-29 cells, a Vero derived cell line that expresses the HSV-1 U_L29 gene product, ICP8, and the HSV-2 U_L5 gene product when infected with HSV (Da Costa et al., 2000). The *dl*5-29-41L virus was produced by co-transfection of linearized p1941L plasmid together with sodium iodide gradient purified *dl*5-29 viral DNA. Triple mutant viruses, containing the LacZ expression cassette within the U_L41 gene locus, were screened for as blue plaques in the presence of X-gal as described previously (Da Costa et al., 1997). Whole cell lysate virus immunogen was obtained by collecting infected cells and resuspending them in 50% DMEM culture media and 50% sterile whole milk followed by a single freeze–thaw cycle and sonication to disrupt cellular membranes and other coagulates. Extracellular supernatant virus immunogen was collected by removal of cells and cellular debris from culture supernatants via low-speed centrifugation and virus was then concentrated by ultracentrifugation and resuspended in PBS–15% glycerol and sonicated.

Animal studies

Animal housing and experiments were conducted as per protocols approved by the Harvard Medical Area Standing Committee on Animals.

(i) *Immunogenicity studies.* Five-week-old female C57Bl/6 or Balb/c mice (Taconic Farms) were immunized twice, subcutaneously, 3 weeks apart, in the rear flank with 1×10^6 plaque forming units (pfu) in a 50 µl volume of either extracellular supernatant virus or cell lysate virus immunogen.

(ii) *Challenge studies.* Five-week-old female Balb/c mice (Taconic Farms) were immunized twice, subcutaneously, 3 weeks apart, in the rear flank with varying amounts of extracellular supernatant virus or cell lysate virus (10^4, 10^5 or 10^6 pfu) in a 50 µl volume. At 1, 4 or 7 months after the second immunization the vaginal cavity was pre-swabbed with a wet dacron swab (PurFybr, Munster, IN) and mice were challenged intravaginally with the wild-type HSV-2 G strain equivalent to 50 times the LD_{50} (5.0×10^5 pfu) in a 25 µl volume using a micropipettor as described (Morrison et al., 1998). Protective dose 50's (PD_{50}) were calculated using the Reed-Muench method.

Antibody titers

At 7 days post-immunization mice were bled via the tail vein, and sera were prepared using Microtainer tubes (BD

Biosciences, Franklin Lakes, NJ), pooled and stored at −20 °C. Total HSV-2 specific IgG antibody titers were determined by ELISA as described previously (Da Costa et al., 2000). Briefly, 96-well MaxiSorb plates (Nalge NUNC, Rochester NY) were coated with HSV-2 G strain viral lysate (Advanced Biotechnologies Inc., Columbia, MD) at 50 ng per well in 0.05 M carbonate–bicarbonate buffer (pH 9.6) (Sigma-Aldrich, St. Louis, MO) overnight at 4 °C. Plates were blocked with PBS containing 5% milk at 37 °C for 1 h, washed three times with PBS containing 0.05% Tween-20 (PBS T-20) and incubated with serial two-fold dilutions of mouse sera, in triplicate, for 4 h at 37 °C. Plates were then washed three times with PBS T-20 and incubated with goat anti-mouse IgG antibody conjugated with alkaline phosphatase (1:1000 in PBS T-20; Sigma-Aldrich) for 1 h at 37 °C. The plates were then washed three times with PBS T-20. Finally, SigmaFast *p*-nitrophenyl phosphate (Sigma-Aldrich) was used to develop a colored product, and the optical density was detected at 405 nm using a microplate reader (Molecular Devices, Union City, CA). Antibody titers are reported as the reciprocal of the final dilution that resulted in an absorbance value 0.2 higher than mock immunized mouse sera or, for mock immunized mouse sera, an absorbance value 0.2 higher than background. Total serum IgG titers were determined through three independent experiments, each time in triplicate.

Neutralizing antibody titers

Neutralizing antibody titers were determined using a standard plaque reduction assay. Mouse sera were heat-inactivated for 30 min at 56 °C, and neutralizing antibody titers were determined against the HSV-2 G strain. One hundred plaque-forming units of virus was incubated with two-fold serial dilutions of sera for 90 min at 37 °C in a 1:8 dilution of LowTox-H Rabbit complement (Cedarlane, Burlington, NC) in DMEM. The incubated mixtures were then added, in triplicate, to Vero cell monolayers in 6-well plates for a 1-h incubation at 37 °C. The inocula were removed and replaced with DMEM containing 1% bovine calf serum, penicillin/streptomycin and 0.5% pooled human immunoglobulin G (Massachusetts Public Health Biologic Laboratories, Boston, MA). After 48 h the plates were fixed with methanol, stained with Giemsa stain, and plaques were counted. 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, as compared to those incubated with sera from mock immunized mice. Neutralizing titers were determined through three independent experiments, each time in triplicate.

MHC-I pentamer staining of HSV gB specific $CD8^+$ T cells

Whole blood samples, approximately 35 μl, were collected via tail vein bleed. Red blood cells were lysed using RBC Lysis Buffer (eBioscience, San Diego, CA). White blood cells were resuspended in 20 μl PBS, 1% BSA, 0.1% sodium azide and incubated with 5 μl of HSV gB SSIEFARL peptide-specific MHC-I pentamers (Proimmune Ltd., Oxford, United Kingdom) at room temperature for 15 min. Cells were washed and then stained with antibodies against murine CD8a at 4 °C for 30 min. Samples were analyzed by flow cytometry. Results are expressed as the percentage of CD8a positive cells that stain positive for the gB-specific MHC-I pentamer.

Intracellular cytokine staining

Splenocytes from immunized C57Bl/6 or Balb/c mice were harvested at 7 days post-immunization. To analyze the $CD8^+$ cellular immune response, 1×10^6 cells were incubated with the $H2K^b$-specific HSV gB peptide SSIEFARL (10 ng/ml). Negative controls were stimulated with the $H2K^b$-specific OVA peptide SIINFEKL (10 ng/ml). To measure the $CD4^+$ cellular immune responses, splenocytes from Balb/c mice were incubated with the HSV gD peptide KPPYTSTLLPPELSD (10 ng/ml). The splenocytes were then placed in a 37 °C, 5% CO_2 humidified incubator for 1 h, at which point Brefeldin A was added, and cells were incubated for 4 h. Cells were stained with antibodies against murine CD8a or CD4 for 1 h then fixed with 1% formaldehyde in PBS, permeabilized in PBS + 0.2% saponin and stained with antibodies for IFN-γ for 16 h at 4 °C. Samples were analyzed via flow cytometry. Results are expressed as the percentage of cells staining positively for CD8a or CD4 that also stain positive for IFN-γ when stimulated with the gB or gD-specific peptides. Results from negative controls have been subtracted from the experimental values listed.

Assay of acute infection

Every 24 h post-intravaginal challenge the vaginal cavities of the mice were swabbed with pre-wetted dacron swabs (PurFybr) twice successively. The swabs were placed in 1 ml of assay medium (PBS, 0.1% Glucose, 1% FCS) and stored at −80 °C. Viral titers were determined by standard plaque assay.

Clinical observations

Wild-type HSV-2 infected mice were observed daily for signs of genital lesions and systemic illness. The severity of disease was scored as follows: 0 = no sign of disease; 1 = slight genital erythema and edema; 2 = moderate genital inflammation; 3 = purulent genital lesions; 4 = hind-limb paralysis (Morrison et al., 1998). Mice were euthanized using CO_2 at the first sign of paralysis.

Statistical analysis

All statistical analysis was performed using two-sided Student's *t*-tests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2007.10.014.

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