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Comparison of immunogenicity and protective efficacy of genital herpes vaccine candidates herpes simplex virus 2 *dl5-29* and *dl5-29-41L* in mice and guinea pigs

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Abstract

A replication-defective HSV-2 vaccine, *dl5-29*, which is deleted for two essential early genes, UL5 and UL29, is highly immunogenic and protective in mice and guinea pigs. In a prior study, a derivative of HSV-2 *dl5-29* termed *dl5-29-41L*, which has an additional deletion in UL41 (that encodes the virion-host shut-off protein), was more immunogenic and protective against challenge with wild-type HSV-2 in mice when compared with *dl5-29*. To determine if deletion of UL41 improves the efficacy of *dl5-29* in protecting guinea pigs from HSV-2, animals were immunized with *dl5-29*, *dl5-29-41L*, or PBS. The geometric mean neutralizing antibody titers from the *dl5-29* and *dl5-29-41L* recipients were comparable ($10^{1.97}$ and $10^{2.19}$, respectively, $p=0.15$). After intravaginal challenge with wild-type HSV-2, the *dl5-29-41L* and *dl5-29* recipients shed similar titers of HSV-2 from the vagina. Mean acute disease severity scores, numbers of recurrences during 3 months after infection, and latent viral loads in sacral ganglia were similar for *dl5-29* and *dl5-29-41L* (all p values >0.05). *dl5-29* and *dl5-29-41L* completely protected mice from lethal challenge with HSV-2 and induced virus-specific CD8⁺T cells in the spleens of the animals. Thus, *dl5-29* was as immunogenic and protective as *dl5-29-41L* under these conditions. *dl5-29* was at least 250,000-fold less virulent than parental virus by intracranial inoculation in healthy mice, and caused no disease in SCID mice. Both *dl5-29-41L* and *dl5-29* are equally effective and immunogenic in guinea pigs, and *dl5-29* is very safe in immunocompromised animals.

1. Introduction

Most cases of genital herpes are due to herpes simplex virus (HSV)-2 [1]. When compared to the period of 1976–1980, the seroprevalence of HSV-2 increased by 30% during the period from 1988 to 1994 [2]. In addition to genital herpes, transmission of HSV-2 to neonates causes severe life-threatening infections [3]. Recent studies suggest a link between genital herpes and increased rates of transmission [4,5] and elevated viral loads [6] of human immunodeficiency virus (HIV). Therefore, an effective HSV-2 vaccine is needed. One of the most well studied HSV-2 vaccines in animal models is recombinant glycoprotein D of HSV-2 (rgD2), which is

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effective in preventing genital herpes disease in mice and guinea pigs and is effective as a therapeutic vaccine in previously infected guinea pigs [7–9].

Several HSV-2 vaccines have recently been tested in humans for prevention or reduction of genital herpes disease. rgD2 given in an alum/monophosphoryl lipid A adjuvant reduced the rate of HSV-2 disease in women seronegative for HSV-1 and HSV-2 in two randomized, controlled clinical trials [10]. The vaccine, however, showed no efficacy in HSV-1 seropositive women or in men. Another HSV-2 vaccine, containing recombinant glycoprotein D and B in MF59 adjuvant, failed to prevent HSV-2 infection in two randomized, double-blind, placebo-controlled trials [11]. Recombinant HSV-2 glycoprotein D and B in MF59 adjuvant reduced the duration and severity of first recurrences, but not the overall frequency of recurrences, in patients with a history of recurrent genital herpes [12]. A disabled infectious single cycle HSV-2 vaccine, deleted for glycoprotein H, was ineffective as a therapeutic vaccine in previously infected persons [13].

Replication defective HSV-2, *dl5-29*, has been shown to be effective in mice [14–16]. Previously we compared the efficacy of *dl5-29* with rgD2 vaccine in Freund's adjuvant in guinea pigs and showed that *dl5-29* was as effective as rgD2 in reducing acute and recurrent HSV-2 genital disease. However, HSV-2 *dl5-29* induced higher neutralizing antibody titers in guinea pigs, and higher HSV-2 specific T cell responses in mice, compared with rgD2 [17]. HSV-2 *dl5-29* encodes a virion host shut-off protein (*vhs*), the product of *UL41*, which is toxic to infected cells by its ability to rapidly degrade mRNA. HSV-2 *dl5-29* unable to express *UL41* (*dl5-29-41L*) was more immunogenic and more effective than HSV-2 *dl5-29* in protecting mice from challenge with HSV-2 [18]. HSV-2 *dl5-29* also prevented ocular disease and reduced latent infection in mice challenged with HSV-1 [19]. In this study, we compared the immunogenicity and efficacy of HSV-2 *dl5-29-41L* with *dl5-29* in guinea pig and mouse models of genital herpes.

2. Methods

2.1 Viruses and cell lines

HSV-2 strain 333 was grown and titered in Vero cells. Replication-defective mutant HSV-2 *dl5-29*, derived from strain 186 Syn+1 [20], is deleted for the coding regions of *UL5* (a component of the helicase-primase complex) and *UL29* (which encodes ICP8, a single-strand DNA-binding protein) and was described previously [14,15]. Replication-defective HSV-2 *dl5-29-41L*, derived from *dl5-29*, is deleted for *UL5* and *UL29* and has the LacZ gene inserted in place of *UL41* (the virion-shutoff protein, *vhs*) as described previously [18]. HSV-2 *dl5-29* and *dl5-29-41L* were propagated in V529 cells which express the HSV-2 *UL5* and *UL29* proteins when infected with HSV-2 [14]. HSV-2 *dl5-29* and *dl5-29-41L* were prepared from infected cell lysates that were resuspended in 50% DMEM and 50% sterile milk, freeze-thawed, and sonicated as described previously [18] and used in animal studies. HSV-2 strain 186 Syn +1 is the parent virus of HSV-2 *dl5-29* and was used for intracranial inoculations or studies in immunocompromised mice.

2.2 Mouse infection models

For the vaginal infection model, six-week old Balb/c mice (Harlan Sprague Dawley, Indianapolis, IN) were immunized with PBS, 1×10^6 pfu of HSV-2 *dl5-29*, or 1×10^6 pfu of *dl5-29-41L* subcutaneously (s.c.) in the flank. Animals were boosted with the same doses of the same vaccines 3 weeks later. Three weeks after the last vaccination, mice were given 2 mg of hydroxyprogesterone (Depo Provera, Pharmacia & Upjohn, Kalamazoo, MI) s.c., and one week later mice were challenged with 1×10^6 pfu of HSV-2 strain 333 by inoculating 20 μ l of virus stock into the vaginal vault with a micropipette.

For the ocular infection model, six-week old Balb/c mice were immunized with PBS, 1×10^6 pfu of *dl5-29*, or 1×10^6 pfu of *dl5-29-41L* s.c. in the flank. Animals were boosted at 21 or 10 days with the same vaccines. Two weeks after the last vaccination, mice were anesthetized with 1.5 mg of ketamine and 0.15mg of xylazine (by intraperitoneal injection) and then challenged with 2 or 4×10^4 pfu of HSV-2 strain 333 onto the scarified corneas of both eyes.

For the intracranial infection model, four-week old Balb/c mice were anesthetized with 1.2 mg of ketamine and 0.12mg of xylazine. Mice were inoculated with 10^6 pfu of *dl5-29*, or 4 pfu or 20 pfu of HSV-2 strain 186 Syn+-1 by the intracranial route in a volume of 10 μ L.

For the immunocompromised infection model, six-week old severe combined immunodeficiency (SCID, CB17/IcrHsd-*scid*) mice (Harlan Sprague Dawley, Indianapolis, IN) were inoculated s.c. with either 10^6 pfu of *dl5-29* or 500 pfu of HSV-2 186 Syn+-1.

2.3. Guinea pig genital herpes model

Female Hartley guinea pigs 4 to 6 weeks old (Harlan Sprague Dawley) were immunized s.c. with PBS, 1×10^6 pfu of *dl5-29*, or 1×10^6 pfu of *dl5-29-41L*, on the back. Each vaccine was given 49 and 28 days before intravaginal challenge with 2×10^5 pfu of HSV-2 strain 333. Lesion severity after challenge was determined by direct examination of each animal daily for up to 90 days according to the severity scale of 0 for no lesions, 1 for erythema, 2 for single or a few small vesicles, 3 for large or fused vesicles, and 4 for ulcerated lesions [8]. Animals were sacrificed after day 90 and lumbosacral ganglia were harvested and stored at -20°C .

2.4. Titration of virus shedding

Vaginal fluid specimens were collected with dacron swabs [21] on days 2, 5, and 8 after challenge, placed in 1 ml of medium containing penicillin, streptomycin, and amphotericin B, and stored at -80°C . The titer of HSV-2 was determined by plaque assay on Vero cell monolayers.

2.5. Quantitative real-time PCR

Lumbosacral ganglia from individual latently infected guinea pigs were dissected, pooled, and rinsed in PBS. DNA was isolated using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). The number of copies of latent HSV-2 DNA was quantified by real-time PCR using the Taqman System, ABI 7700 Sequence Detector (PE Applied Biosystem, Foster City, CA) with primers and probes specific for HSV gD2 [22]. The sequence of the forward primer was, 5'-TCAGCGAGGATAACCTGGGA-3', the reverse primer was, 5'-GGGAGAGCGTACTTGCAGGA-3', and the probe was TAMRA-5'-CCAGTCGTTTATCTTCACTAGCCGCAGGTA-3'. Each reaction contained 100 ng of ganglia DNA. A standard curve with known amounts of plasmid DNA diluted in salmon sperm DNA was used to determine copy numbers. The detection limit of this PCR assay is ~6 copies per reaction with excellent linearity ($R > 0.94$) over 5 logs of DNA content. For statistical purposes, reactions yielding less than 6 copies of HSV-2 DNA were assumed to contain 3 copies. The geometric mean result for three independent experiments was determined.

2.6. Neutralizing antibody responses

Antibody titers for neutralization of HSV-2 strain 333 *in vitro* were determined using 0.1 ml of serum from each animal. HSV-2 (60 to 80 pfu) was incubated with serial 2-fold dilutions of mouse sera for 1 h on ice and added in triplicate to Vero cell monolayers in 12-well plates. Plates were incubated for 1 hr at 37°C , and the inocula were replaced with fresh medium containing 0.5% human IgG (Gammagard, Baxter, Westlake Village, CA) to prevent spread of cell-free virus. Two days later, cells were stained with crystal violet and plaques were

counted. Regression lines representing the best fit for the data were calculated using Microsoft Excel software and dilutions that reduced the numbers of plaques by 50% were calculated from the fitted lines.

2.7. Quantification of HSV-specific IFN- γ ⁺CD8⁺ T cells

Mouse spleens were gently crushed, splenocytes were passed through 100 μ m filters, and the cells were frozen in liquid nitrogen. To quantify HSV-2-specific IFN- γ ⁺CD8⁺ T cells in the spleen, 2×10^6 splenocytes were stimulated by cocultivation with 1×10^6 P815 cells infected 5 hr previously with 3×10^6 pfu of HSV 333, and then incubated for 5 hr in the presence of brefeldin A (Sigma, St. Louis MO). After washing, cells were stained with FITC-conjugated anti-CD8 and PerCP-conjugated anti-CD3 monoclonal antibodies (BD Pharmingen, San Diego, CA). After fixation, permeabilization with Cytotfix/Cytoperm Buffer (BD Pharmingen), and washing with Permash buffer (BD Pharmingen), the cells were stained with PE-conjugated anti-IFN- γ monoclonal antibody (BD Pharmingen). Data from 10,000–20,000 live CD8⁺CD3⁺ cells were collected by FACS Caliber (Becton Dickinson, Franklin Lakes, NJ).

HSV-specific IFN- γ ⁺ CD8⁺ T cells infiltrating trigeminal ganglia were detected and quantified as previously described [23]. Briefly, P815 cells were infected with HSV-2 strain 333 at a multiplicity of infection (moi) of 3 for 5 hr. Four to 6 trigeminal ganglia (TG) were pooled and dispersed in 2 mg/ml of collagenase type I (Sigma) for 1–1.5 hr and passed through a 40 μ m filter. TG cells were resuspended in Iscove's Dulbecco-modified medium supplemented with 10% of fetal bovine serum, 10 U/ml of IL-2, glutamine and antibiotics. Pooled TG suspensions were stimulated by coincubation with 5×10^5 P815 cells per TG in the presence of 10 μ g/ml of brefeldin A for 5 hrs. The cells were washed and stained with FITC-conjugated anti-CD8 and PerCP-conjugated anti-CD45 monoclonal antibody (BD Pharmingen). After washing, the cells were fixed, permeabilized with Cytotfix/Cytoperm Buffer, and washed with Permash buffer according to the manufacturer's protocol. Cells were stained with PE-conjugated anti-IFN- γ monoclonal antibody and analyzed on a FACSCaliber (Becton Dickinson, Franklin Lakes, NJ) using CellQuest software (Becton Dickinson). Forward and side scatter gates were set for TG cells by back gating from the CD45⁺CD8⁺ population.

Results

HSV-2 UL41 shuts off host protein synthesis in guinea pig cells

Previous studies of HSV-2 *dl5-29-41L* were performed in mice [18], and one of the genes deleted in *dl5-29-41L*, UL41, was previously shown to shut off host protein synthesis in mouse and human cells [24,25]. The ability of UL41 to shut down host protein synthesis has not been studied in guinea pig cells. Therefore, before comparing the effects of *dl5-29-41L* and *dl5-29* in a guinea pig model of genital herpes, we compared the host protein synthesis shut off activity of HSV-2 UL41 in guinea pig cells infected with the two viruses. As previously reported in mouse and monkey kidney (Vero) cells [18], HSV-2 protein expression (Figure 1, arrows) in guinea pig cells infected with *dl5-29-41L* was similar to that in cells infected with *dl5-29*, while host cell protein expression (Figure 1, asterisks) in cells infected with *dl5-29-41L* was more similar to mock-infected than *dl5-29*-infected cells. Thus, the host protein synthesis shut off activity of UL41 is active in guinea pig cells.

Guinea pigs vaccinated with *dl5-29-41L* show slightly higher HSV-2 neutralizing antibody titers than those vaccinated with *dl5-29*

Vaginal infection of guinea pigs with HSV-2 results in both acute genital herpes and spontaneous recurrent genital lesions which is similar to what is observed in humans. Previously we showed that guinea pigs vaccinated with *dl5-29* had significantly higher neutralizing antibody titers than those receiving gD2 [17]. Guinea pigs were vaccinated twice

(3 weeks apart) subcutaneously with PBS, 10^6 pfu of *dl5-29-41L*, or 10^6 pfu of *dl5-29*, and serum was obtained 3 weeks after the last vaccination. The HSV-2 specific neutralizing antibody titer induced by *dl5-29-41L* was slightly higher ($10^{2.19 \pm 0.07}$ [mean $\pm$ SE]) than that induced by *dl5-29* ($10^{1.97 \pm 0.11}$), although the difference was not statistically significant ($p=0.15$) (Figure 2). No HSV-2 neutralizing activity was detected in serum obtained from animals receiving PBS.

Vaccination with HSV-2 *dl5-29* or *dl5-29-41L* reduces shedding from the vaginal tract and reduces acute disease after challenge with wild-type virus in guinea pigs

Guinea pigs vaccinated twice with *dl5-29-41L*, *dl5-29* or PBS (as described above) were challenged intravaginally 4 weeks after the last vaccination with 2×10^5 pfu of wild type HSV-2 strain 333. In this model, acute disease is quantified by lesion scores during the first two weeks after challenge, and recurrent disease is measured by lesions that occur after two weeks.

Titers of HSV-2 shed on day 2 after challenge with wild-type virus in animals vaccinated with *dl5-29-41L* ($10^{3.3}$) were significantly lower than in animals that received PBS ($10^{5.0}$, $p<0.01$, Figure 3A). In contrast, the difference in titers between animals that received *dl5-29* ($10^{4.2}$) were not significant from those that received PBS ($p=0.06$), and the difference in titers between *dl5-29* and *dl5-29-41L* was not significant ($p=0.09$). On day 5 after challenge, HSV-2 shedding in the 3 groups was comparable. On day 8 after challenge, animals vaccinated with either *dl5-29-41L* or *dl5-29* had reduced vaginal shedding ($10^{0.7}$, $10^{0.8}$, respectively) compared to those receiving PBS ($10^{2.4}$, $p<0.01$), while the titers of HSV-2 shed in animals receiving *dl5-29-41L* were similar to those receiving *dl5-29* ($p=0.9$).

The average lesion score on the first four days after challenge with wild-type virus was similar among the vaccinated groups (Figure 3B). Lesion scores in the PBS group continued to increase until day 6, and then declined by day 11. In contrast, animals vaccinated with *dl5-29* or *dl5-29-41L* started to differ from PBS group by day 5 and showed marked declines in lesion scores by day 7. The lesion scores of vaccinated groups were lower compared to the PBS group throughout the acute period. The mean lesion scores during the acute period for animals receiving PBS were significantly higher than those vaccinated with *dl5-29* or *dl5-29-41L* ($p<0.01$), while the difference in lesion scores between animals receiving *dl5-29* and *dl5-29-41L* were not significant ($p=0.9$).

Vaccination with HSV-2 *dl5-29* or *dl5-29-41L* significantly reduces the rate of recurrent genital disease and reduces the latent viral load in guinea pigs

The cumulative number of genital herpes recurrences from days 15 to day 90 after challenge with wild-type HSV-2 was an average of 14.7 for animals receiving PBS compared with 3.4 for animals vaccinated with *dl5-29* and 3.3 for those vaccinated with *dl5-29-41L* (Figure 4A). While the difference between animals receiving PBS and either *dl5-29* or *dl5-29-41L* was significant ($p<0.01$), the difference between animals vaccinated with *dl5-29* and *dl5-29-41L* was not ($p=0.9$). Therefore *dl5-29* and *dl5-29-41L* were equally effective in reducing recurrences.

At 90 days after challenge with wild-type virus, sacral ganglia were removed, and the latent HSV-2 DNA load was measured using real-time PCR. The latent viral load was significantly higher in animals receiving PBS than in those receiving *dl5-29* or *dl5-29-41L* ($p<0.01$), while the difference in latent viral load between animals receiving *dl5-29* and *dl5-29-41L* was not significant ($p=0.8$) (Figure 4B).

HSV-2 *dl5-29* and *dl5-29-41L* completely protect mice from lethal vaginal challenge with HSV-2

HSV-2 does not cause recurrent genital disease in mice after vaginal infection; however, a high dose vaginal challenge is lethal. We have previously shown that subcutaneous vaccination with *dl5-29* or recombinant gD2 in Freund's adjuvant protects mice from a lethal vaginal challenge with HSV-2 strain 333 (10^6 pfu) [17]. HSV-2 *dl5-29-41L* has been shown to be more effective in protecting mice from vaginal infection with HSV-2 strain G (5×10^5 pfu) [18]. Therefore we wanted to determine the immunogenicity and protective efficacy of *dl5-29-41L* and *dl5-29* in mice challenged with HSV-2 strain 333. In the current study, two doses of *dl5-29* or *dl5-29-41L* (10^6 pfu given subcutaneously) completely protected mice from lethal vaginal challenge with HSV-2, while only 10% of mice in the control group survived after 12 days from challenge (Figure 5A). Both vaccines were still effective at a dose of 3×10^5 pfu; 90% of mice receiving *dl5-29* and 100% receiving *dl5-29-41L* survived vaginal challenge with HSV-2 (Figure 5B). At a challenge dose of 1×10^5 pfu, 30% of mice vaccinated with *dl5-29-41L* and 30% of animals receiving *dl5-29* survived HSV-2 infection (Figure 5B). There was a 3-day delay death in animals vaccinated with *dl5-29-41L* (40% survival by day 12) compared with those receiving *dl5-29* (40% survival by day 9) after challenge.

HSV-2 *dl5-29* and *dl5-29-41L* induce virus-specific CD8⁺T cells in the spleens of mice

Previously we showed that *dl5-29* induced HSV-specific cellular responses in the spleens of mice [17]. Spleens from mice vaccinated with 2 doses of *dl5-29-41L* showed $10^{3.6 \pm 0.1}$ (mean $\pm$ SE) HSV-specific IFN- γ producing CD8⁺T cells, animals vaccinated with *dl5-29* produced $10^{3.6 \pm 0.2}$ IFN- γ producing CD8⁺T cells, and animals that received PBS had $10^{2.5 \pm 0.1}$ IFN- γ producing CD8⁺T cells (Figure 6A). The difference in the number of IFN- γ producing CD8⁺T cells between animals receiving PBS and either *dl5-29-41L* or *dl5-29* was statistically significant ($p < 0.001$).

HSV-specific IFN- γ producing CD8⁺T cells accumulated more rapidly in the trigeminal ganglia (TG) of mice vaccinated with *dl5-29-41L* or *dl5-29* than those receiving PBS after corneal challenge with wild-type HSV-2 (2×10^4 pfu per mouse). In animals vaccinated with PBS, HSV-specific CD8⁺T cells were barely detected in TG before 5 days after infection ($10^{0.7 \pm 0.3}$ per TG and $10^{1.2 \pm 0.5}$ per TG on day 3 and day 5 after challenge [mean $\pm$ SE], respectively) (Figure 6B). In contrast, mice vaccinated with *dl5-29-41L* had $10^{1.6 \pm 0.2}$ and $10^{2.2 \pm 0.2}$ HSV-specific CD8⁺T cells per TG on day 3 and day 5 after challenge, respectively. Mice receiving *dl5-29* showed $10^{1.7 \pm 0.4}$ and $10^{2.1 \pm 0.1}$ virus-specific CD8⁺T cells per TG on day 3 and day 5 after challenge, respectively. The difference between animals receiving PBS and those vaccinated with either *dl5-29-41L* or *dl5-29* was statistically significant on day 3 ($p = 0.03$), but not on day 5 ($p = 0.12$). In a second experiment, HSV-specific IFN- γ producing CD8⁺T cells accumulated in the TG of mice vaccinated with *dl5-29-41L* or *dl5-29* more rapidly than those receiving PBS on day 5 and 7 after corneal challenge with wild-type HSV-2 (4×10^4 pfu per mouse); however, at day 13 after challenge, the numbers of HSV-specific IFN- γ producing CD8⁺T cells in TG were similar in the 3 groups (Figure 6C). In both sets of experiments (Figure 6B and 6C), the difference in the number of HSV-specific IFN- γ producing CD8⁺T cells that accumulated in the TG of mice vaccinated with *dl5-29-41L* or *dl5-29* was not statistically significant ($p > 0.9$). Differences in the numbers of HSV-specific IFN- γ producing CD8⁺T cells in TG between animals vaccinated with HSV-2 *dl5-29* compared with PBS were also noted at days 1 and 5 after challenge, but not day 14 after challenge as previously reported [17]. Therefore, *dl5-29-41L* or *dl5-29* are both able to prime the immune system to increase the production of HSV-specific IFN- γ producing CD8⁺T cells after challenge with wild-type virus.

HSV-2 *dl5-29* does not cause disease after intracranial inoculation of healthy mice or after subcutaneous inoculation of mice with severe combined immunodeficiency

To determine if HSV-2 *dl5-29* might have the potential to cause disease in the central nervous system, four-week old Balb/c mice were inoculated intracranially with 4 or 20 pfu of wild-type HSV-2 strain 186 Syn+-1, or 10^6 pfu of *dl5-29*. All mice infected with wild-type HSV-2 died within 2 weeks, while none of the mice infected with 10^6 pfu of *dl5-29* died over a 2 months observation period (Figure 7A).

To determine if HSV-2 *dl5-29* might cause disease in severely immunocompromised animals, SCID mice were inoculated subcutaneously with the virus. Inoculation of 500 pfu of wild-type HSV-2 resulted in death of 80% (4 of 5) of the animals by two weeks, while none of 12 mice inoculated with 10^6 pfu of *dl5-29* died after a two month observation period (Figure 7B). Therefore, *dl5-29* is highly attenuated even in immunocompromised mice.

Discussion

In this study, we found that HSV-2 *dl5-29* and *dl5-29-41L* were equally effective in reducing acute and recurrent disease in guinea pigs compared with animals that received PBS alone after challenge with wild-type HSV-2. HSV-2 *dl5-29* and *dl5-29-41L* were also equally effective in reducing virus shedding and in reducing the latent viral load in ganglia after challenge with wild-type HSV-2. Animals vaccinated with HSV-2 *dl5-29-41L* had somewhat higher levels of HSV-2 neutralizing antibody titers than those receiving *dl5-29*; however, the difference was not statistically significant.

Our prior study indicated that HSV-2 *dl5-29* was as effective as recombinant glycoprotein D2 (gD2) in reducing acute disease, recurrent disease, and the latent viral load in guinea pigs after challenge with wild-type virus; however, HSV-2 *dl5-29* reduced shedding and resulted in higher levels of neutralizing antibodies compared with gD2 [17]. The level of protection obtained with HSV-2 *dl5-29* for acute and recurrent disease was similar in the current study when compared with the prior study. The cumulative number of recurrences during 90 days of observation in animals vaccinated with HSV-2 *dl5-29* and challenged with wild-type virus was 1.7 per animal in the prior study and 3.4 per animal in the present study, while the number of recurrences in animals vaccinated with PBS was 14.0 per animal in the prior study and 14.7 per animal in the current study. Geometric mean HSV-2 neutralizing antibody titers induced by HSV-2 *dl5-29* were $10^{2.3}$ in the prior study and $10^{2.0}$ in the present study. Peak vaginal shedding in animals vaccinated with HSV-2 *dl5-29* and challenged with wild-type virus was $10^{3.3}$ pfu per animal in the prior study and $10^{4.2}$ pfu per animal in this study, while peak shedding in animals vaccinated with PBS was $10^{4.5}$ pfu per animal in the prior study and $10^{5.0}$ pfu per animal in the current study. Therefore, the efficacy of *dl5-29* is reasonably reproducible in the guinea pig genital herpes model.

We found that HSV-2 *dl5-29* and *dl5-29-41L* were equally effective in reducing mortality in mice after challenge with wild-type HSV-2 when compared with animals that received PBS alone. HSV-2 *dl5-29* and *dl5-29-41L* were also equally effective in inducing HSV-2 specific CD8⁺ T cells in mice after vaccination. Our results differ from those of Dudek et al [18] who showed that *dl5-29-41L* was more effective than *dl5-29* in reducing mortality and inducing HSV-2 specific CD8⁺T cells in mice. Some differences in these studies might explain the discordant results. First, Dudek et al. [18] found significant differences in HSV-gB epitope-specific CD8⁺T cells from C57Bl/6 mice between HSV-2 *dl5-29* and *dl5-29-41L* at days 6 and 7 after primary vaccination and at days 5 and 6 after the second vaccination; no significant difference was noted at 7 days after the second vaccination. In contrast we compared CD8⁺T cells that would recognize all HSV-2 antigens (i.e. antigen presenting cells were infected with wild-type HSV-2) from Balb/c mice for the two viruses at 14 days after the second vaccination

in mice. Second, Dudek et al [18] found that *dl5-29-41L* resulted in less severe clinical disease when used at a dose of 10^4 compared with *dl5-29* after challenge with HSV-2, while doses of 10^5 or 10^6 failed to show much difference in clinical disease. In contrast we used doses of 10^5 to 10^6 of HSV-2 *dl5-29* and *dl5-29-41L* followed by challenge with HSV-2 strain 333. Third, Dudek et al. [18] infected mice that had received two doses of hydroxyprogesterone with 5×10^5 pfu of HSV-2 strain G, while we infected mice that had received only one dose of hydroxyprogesterone with 1×10^6 pfu of HSV-2 strain 333. In our study, at the lowest dose of *dl5-29-41L* (1×10^5) we noted a three day delay in death when compared to same dose of *dl5-29* (Figure 5B). Thus, at even lower doses of vaccine, or a higher dose of challenge virus, we may have noted more of a difference in mortality between the two vaccines. Fourth, the difference in the results observed in mice in the prior study [18] and in guinea pigs in our study might be due to differences in the amount of vaccine required for effective protection against HSV-2 in the two different types of animals. If guinea pigs require a lower dose of vaccine to protect against HSV-2 than mice, then relatively large doses of the two vaccines in guinea pigs may have masked differences in effectiveness of the two vaccines in these animals. Thus, our results indicate that the differences between *dl5-29* and *dl5-29-41L* observed by Dudek et al [18] are not observed under all test situations.

Since HSV-2 vaccines might be inadvertently administered to persons who are not known to be immunocompromised, vaccine safety in immunocompromised hosts is essential. Since coinfection of HSV-2 and HIV has been shown to increase the risk of transmission of HIV, an HSV-2 vaccine might reduce rates of transmission of HIV in persons who are seronegative for HSV-2 [4]. Thus, it is important to evaluate the safety of HSV-2 *dl5-29* in highly immunocompromised animals and after intracerebral inoculation. We found a $>100,000$ fold difference in neurovirulence of *dl5-29* when compared with wild-type virus. One million pfu of *dl5-29* caused no disease after intracranial infection, while only 4 pfu of wild-type virus killed all mice in two weeks. Inoculation of SCID mice with 500 pfu of wild-type HSV-2 subcutaneously killed 80% of mice, whereas one million pfu of *dl5-29* caused no disease in any of the 12 mice tested. In conclusion, both HSV-2 *dl5-29* and *dl5-29-41L* are highly immunogenic and protective in mice and guinea pigs, and *dl5-29* is safe in highly immunocompromised mice. Therefore *dl5-29* or its derivative *dl5-29-41L* are excellent candidates for human clinical trials.

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Figure 1.

HSV-2 vhs shuts off host protein in guinea pig cells. Guinea pig embryonic fibroblasts (GPEF) were mock infected or infected with wild-type HSV-2, HSV-2 *dl5-29*, or *dl5-29-41L* at an MOI of 10 for 6h. Cells were labeled with ^{35}S -methionine and 30 min later, lysates were prepared and proteins were resolved on 9.25% DATD cross-linked gel and autoradiography was performed. Representative HSV-2 proteins are indicated with arrows (5, 6, 8 and gB represent ICP-5, ICP-6, ICP-8 and glycoprotein B, respectively) and major host cell proteins are indicated with asterisks.

Figure 2.

HSV-2 *dl5-29-41L* induces slightly higher neutralizing antibody titers in guinea pigs than *dl5-29*. Guinea pigs were vaccinated subcutaneously with 10^6 pfu of *dl5-29-41L* (open squares), 10^6 pfu of *dl5-29* (open circles), or PBS (closed diamonds) at 5 weeks and 2 weeks before serum was obtained. The dilutions of each serum that reduced the plaque numbers by 50% compared with no serum wells are shown. Short horizontal bars indicate geometric means and vertical bars indicate standard errors.

Figure 3.

HSV-2 *dl5-29* and *dl5-29-41L* protect guinea pigs from acute infection of HSV-2. (A) Guinea pigs were vaccinated subcutaneously with 10⁶ pfu with *dl5-29-41L* (closed bars), *dl5-29* (open bars), or PBS (stippled bars) 6 and 3 weeks before intravaginal challenge with wild type-HSV-2. The titer of HSV-2 shed from the vagina on day 2, 5 and 8 after challenge is shown. The mean titer in each group is shown with vertical lines above each bar indicating standard errors. (B) Mean lesion scores for animals vaccinated with *dl5-29-41L* (open squares), *dl5-29* (open circles), or PBS (solid diamonds) are shown from day 1 to 14 after challenge.

Figure 4.

HSV-2 *dl5-29* and *dl5-29-41L* reduce recurrent disease and latent viral load (A) Animals shown in Figure 3 were scored for reactivation events for 90 days after challenge. (B) The HSV-2 latent viral load in sacral ganglia was determined for each vaccinated group. Short horizontal bars indicate geometric means and vertical bars indicate standard errors.

Figure 5.

HSV-2 *dl5-29-41L* and *dl5-29* protect mice from challenge with wild-type HSV-2. (A) Mice were vaccinated subcutaneously with 10^6 pfu of *dl5-29-41L* (open squares, 10 mice), 10^6 pfu of *dl5-29* (open circles, 10 mice), or PBS (closed diamonds, 10 mice) at 7 and 4 weeks before intravaginal challenge with 10^6 pfu of wild-type HSV-2 (strain 333). All mice were given Depo Provera 1 week before challenge. (B) Mice were vaccinated subcutaneously with 3×10^5 pfu of *dl5-29-41L* (open squares, 10 mice), 3×10^5 pfu of *dl5-29* (open circles, 10 mice), 10^5 pfu of *dl5-29-41L* (closed squares, 10 mice), 10^5 pfu of *dl5-29* (closed circles, 10 mice), at 7 and 4 weeks before intravaginal challenge with 10^6 pfu of wild-type HSV-2 (strain 333). All mice were given Depo Provera 1 week before challenge.

Figure 6.

HSV-2 *dl5-29-41L* and *dl5-29* induce HSV-2 specific CD8⁺T cell responses. (A) Frequencies of IFN- γ producing CD8⁺T cells in spleen that respond to HSV-2 infected antigen-presenting cells. Mice were vaccinated with 10⁶ pfu of *dl5-29-41L* (open squares, 8 mice), 10⁶ pfu of *dl5-29* (open circles, 8 mice), or PBS (closed diamonds, 8 mice) at 5 and 2 weeks before assay. Short horizontal bars indicate geometric means and vertical bars indicate standard errors. (B, C) Number of IFN- γ producing CD8⁺T cells in TGs from mice vaccinated subcutaneously with 10⁶ pfu of *dl5-29-41L* (open squares), 10⁶ pfu of *dl5-29* (open circles), or PBS (closed diamonds) at 31 and 10 days before challenge with 2 × 10⁴ pfu of HSV-2 strain 333 (Panel B) or at 20 and 10 days before challenge with 4 × 10⁴ pfu of HSV-2 strain 333 (Panel C) onto the

scarified corneas of both eyes. The x axis shows the day after challenge when mice were sacrificed and blood was obtained for CD8⁺T cell responses. Each data point is the geometric mean of the number of IFN- γ producing CD8⁺T cells obtained from 4 mice (in which the 2 TGs were pooled together) and short vertical bars indicate standard errors.

Figure 7.

HSV-2 *dl5-29* is highly attenuated in healthy mice after intracranial inoculation or in immunocompromised animals inoculated subcutaneously. (A) Four week old Balb/c mice (5 animals per group) were inoculated with either HSV-2 *dl5-29* (10^6 pfu) or wild-type HSV-2 strain 186 Syn+1 (4 or 20 pfu/mouse) by the intracranial route and mortality was assessed. (B) Six week old SCID mice were inoculated subcutaneously with either *dl5-29* (10^6 pfu, 12 mice per group) or wild-type HSV-2 strain 186 Syn+1 (500 pfu, 5 mice per group) and mortality was measured.