Comparative Efficacy and Immunogenicity of Replication-Defective, Recombinant Glycoprotein, and DNA Vaccines for Herpes Simplex Virus 2 Infections in Mice and Guinea Pigs

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Many candidate vaccines are effective in animal models of genital herpes simplex virus type 2 (HSV-2) infection. Among them, clinical trials showed moderate protection from genital disease with recombinant HSV-2 glycoprotein D (gD2) in alum-mono-phosphoryl lipid A adjuvant only in HSV women seronegative for both HSV-1 and HSV-2, encouraging development of additional vaccine options. Therefore, we undertook direct comparative studies of the prophylactic and therapeutic efficacies and immunogeneicities of three different classes of candidate vaccines given in four regimens to two species of animals: recombinant gD2, a plasmid expressing gD2, and dl5-29, a replication-defective strain of HSV-2 with the essential genes UL5 and UL29 deleted. Both dl5-29 and gD2 were highly effective in attenuating acute and recurrent disease and reducing latent viral load, and both were superior to the plasmid vaccine alone or the plasmid vaccine followed by one dose of dl5-29. dl5-29 was also effective in treating established infections. Moreover, latent dl5-29 virus could not be detected by PCR in sacral ganglia from guinea pigs vaccinated intravaginally. Finally, dl5-29 was superior to gD2 in inducing higher neutralizing antibody titers and the more rapid accumulation of HSV-2-specific CD8+ T cells in trigeminal ganglia after challenge with wild-type virus. Given its efficacy, its deficiency for latency, and its ability to induce rapid, virus-specific CD8+ T-cell responses, the dl5-29 vaccine may be a good candidate for early-phase human trials.

Genital herpes is an epidemic sexually transmitted disease for which there are effective treatments but inadequate options for prevention. Herpes simplex virus type 2 (HSV-2), which infects upwards of 22% of adult Americans (14), causes most cases of genital herpes, with a growing minority of cases being due to HSV-1. In primary genital infection, these viruses replicate and spread to regional ganglia, where they establish latent infection. HSV-2 reactivates episodically to be shed without symptoms or to precipitate bouts of recurrent genital lesions. Asymptomatic shedding renders about 3% of seropositive people potentially infectious each day (38). Overall, about 5% of seronegative, sexually active young adults will acquire HSV-2 infections from persons with symptomatic or asymptomatic infections each year (21).

While genital herpes is physically, psychologically, and/or socially debilitating in many of those who acquire it, immunocompromised patients risk severe and disseminated disease. Moreover, active genital lesions increase the probability of acquiring and transmitting human immunodeficiency virus (37). In the aggregate, the burden of genital herpes has made acquiring and transmitting human immunodeficiency virus (37). In the aggregate, the burden of genital herpes has made

The limited clinical benefits of recombinant glycoproteins in adjuvants suitable for widespread human use have encouraged further exploration of candidate vaccines that might elicit more potent immune effector, and hence protective, responses while remaining well tolerated. In this regard, we undertook direct comparative studies of the prophylactic and therapeutic efficacies and immunogeneicities of three different classes of candidate vaccines given in four regimens in two species of animals. We examined recombinant gD2, a plasmid expressing gD2, and HSV-2 with the essential genes UL5 and UL29 deleted (9). This mutant virus, dl5-29, is both defective for replication and impaired for establishment of latency in mice (10). The series of experiments summarized here shows that among the candidate vaccines tested, dl5-29 is avirulent, impaired for latency, and as effective as gD2 in Freund’s adjuvant in preventing and treating genital herpes in guinea pigs while inducing the strongest neutralizing antibody and cellular immune responses.

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VIRUSES, cell lines, and candidate vaccines. HSV-2 strain 333 was grown in Vero cells, and the titers of the virus were determined. The replication-defective mutant dl5–29 of HSV-2 strain 186, with the coding regions of UL5 (which encodes a component of the helicase-primease complex) and UL29 (which encodes ICP8, a single-strand DNA-binding protein) deleted, was described previously (9, 10). A stock of dl5–29 was prepared under the FDA’s “Good Manufacturing Practices” after the virus was rederived at Avant Immunotherapeutics, Inc., Needham, Mass. The virus was rederived by transfection of purified dl5–29 viral DNA (provided by the laboratory of D. M. Knipe to Avant) into the Good Manufacturing Practices cell line V295, which stably produces HSV UL5 and UL29 genes. 

Truncated, recombinant gD2 expressed in CHO cells has been described previously (5, 29) and used in multiple human trials (8, 33) and was generously provided by Chiron Corp., Emeryville, Calif. The pgD2 plasmid vaccine was constructed by inserting the 1.22-kb coding region of gD2 from strain S, a region corresponding to bases 140110 to 142116 of the sequenced strain OH52, into the pcDNA3 plasmid vector under the control of the cytomegalovirus immediate-early promoter (Invitrogen, Carlsbad, Calif.). Efficient gD2 expression was confirmed by transient transfection of Vero cells with pgD2 followed by Western blotting (data not shown).

Animal studies. (i) Mouse vaginal infection model. Six-week-old BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.) were immunized with phosphate-buffered saline (PBS), 10^6 PFU of dl5–29, or heat-inactivated HSV-2 strain 333 virus (10^5 PFU for 5 min) subcutaneously (s.c.) in the flank or with 0.1 μg of the pgD2 plasmid vaccine in the footpad. They were boosted of the same vaccines at the same dosages 3 weeks later. Two weeks after the last vaccination, the mice were given s.c. 2 mg of medroxyprogesterone acetate (Depo-Provera; Pharmacia & Upjohn, Kalamazoo, Mich.). One week later, the mice were challenged intravaginally with 10^6 PFU of HSV-2 strain 333 by delivering 50 μl of virus stock with a micropipetter into the vagina of intact mice.

(ii) Mouse ocular infection model. Ten-week-old BALB/c mice were immunized with 10^6 PFU of dl5–29 or heat-inactivated dl5–29 virus s.c. in the flank or with 3 μg of gD2 in complete Freund’s adjuvant (CFA) intramuscularly (i.m.). They were given boosters of the same vaccines 10 days later, but incomplete Freund’s adjuvant (IFA) was used in place of CFA for mice given gD2. Control mice were vaccinated with PBS in CFA for the first dose and then with PBS in IFA for the second (PBS/CFA/IFA). Some mice were sacrificed, and their spleen cells were harvested for assays of cellular immune responses. Two and 5 weeks after the last vaccination, mice were challenged with 2 × 10^6 and 4 × 10^5 PFU of HSV-2 strain 333 onto the scuffed right cornea in the first and second experiments, respectively. At the indicated time points thereafter, animals were sacrificed for immune studies.

(iii) Guinea pig genital herpes model. This guinea pig genital model, unlike mouse models, manifests both acute disease and spontaneous recurrences, as described previously (9, 10). In studies using gpD2, 4- to 6-week-old female Hartley strain guinea pigs (Charles River Laboratories, Southbridge, Mass.) were immunized with PBS/CFA/IFA: 3 μg of recombinant gD2/CFA/IFA i.m. in the thigh. 10^6 PFU of dl5–29 s.c. on the back or, in one experiment, intravaginally, or 25 or 100 μg of the pgD2 plasmid in the footpad. Each vaccine was given on days 42 and 21 before intravaginal challenge on day 0 with 2 × 10^6 PFU of HSV-2 strain 333. The severity of postchallenge lesions was determined by direct examination of every animal daily for up to 90 days by use of a severity scale of 0 for no lesions, 1 for erythema only, 2 for single or few modest vesicles, 3 for large or fused vesicles, and 4 for ulcerated lesions (29). Animals were sacrificed after day 90, and lumbosacral ganglia were harvested and stored at −20°C.

For studies in which candidate vaccines were given immunotherapeutically, guinea pigs were first inoculated intravaginally on study day 0 with 2 × 10^6 PFU of HSV-2 strain 333. On days 7 and 15 after infection, the animals were administered PBS/CFA/IFA: 3 μg of recombinant gD2/CFA/IFA i.m. in the thigh, or 10^6 PFU of dl5–29 s.c. Lesion severity scores were assessed daily for up to 90 days.

Titration of viral shedding. Vaginal fluid specimens were collected with Dacon swabs as previously described (39) on days 2, 4, 6, 8, and 10 after challenge in the prophylaxis experiments and on days 2 and 6 of the immunotherapy experiment; placed in 1 ml of medium containing penicillin, streptomycin, gentamicin, and fungizone; and stored at 4°C; and stored at −80°C. The titers of HSV-2 were determined by plaque assay on Vero cell monolayers.

Quantitative real-time PCR. Lumbosacral ganglia from each latently infected guinea pig were dissected, pooled, and rinsed in PBS. DNA was isolated with the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.). The number of copies of latent HSV-2 DNA was quantified by real-time PCR using the Taqman system and an ABI 7700 sequence detector (PE Applied Biosystems, Foster City, Calif.) with primers and probes specific for gD2 (12). The forward primer was 5′-TACGGGAGGATACCTGGGGA-3′, and the reverse primer was 5′-GGAGAGGGCCTTGCCTGAGGA-3′. The probe was TAMRA-5′-CCAGTCTTTATCTCATTGCGCAGTA-3′ (where TAMRA is 6-carboxytetrahydro rhodamine). Each reaction included 100 ng of ganglion DNA. A standard curve based on known amounts of plasmid DNA diluted in salmon sperm DNA was used to determine copy numbers. The detection limit of this PCR assay proved to be about six copies per reaction (shown as dashed lines in the figures) with excellent linearity (R > 0.96) over 5 logs of DNA content. For statistical purposes, yielding fewer than 6 copies of DNA were assumed to contain 3 copies. The geometric mean results for three independent experiments were determined.

Anti-gD2 antibody responses. Blood was collected at 3 weeks after the last vaccination. Sera were separated and stored at −20°C. Titers of antibody to gD2 were measured by kinetic enzyme-linked immunosorbent assay. Briefly, Immuno-1 96-well plates (Dynex Technologies, Chantilly, Va.) were coated with 1 μg of gD2 per ml overnight at 4°C and then washed and blocked with buffer containing 2% bovine serum albumin. Sera diluted 1:1,000 (as determined in preliminary experiments) were added to each well in duplicate and incubated for 1 h at room temperature. Plates were washed with PBS containing 0.05% Tween 20 and incubated for 1 h with a 1:2,500 dilution of anti-guinea pig immunoglobulin G antibody conjugated with horseradish peroxidase (Sigma, St. Louis, Mo.). After the plates were washed, the increases in optical density per minute were measured and calculated for six time points at 30-s intervals, starting immediately after the addition of substrate (1 Step ABST; Pierce, Rockford, Ill.).

Neutralizing antibody response. Titters of antibodies that neutralize the infectivity of HSV-2 strain 333 were determined by using 0.1 ml of serum recovered from each animal. HSV-2 (100 PFU) was incubated with serial twofold dilutions of sera for 1 h and added in triplicate to Vero cell monolayers in six-well plates. The plates were incubated for 1 h at 37°C, and the inocula were replaced with fresh medium containing 0.5% human immunoglobulin G (Abbott Labs, Chicago, Ill.) to prevent diffusion of cell-free virus. Two days later, the plates were stained with crystal violet to visualize plaques. The plaques were counted. Regression lines were fitted to calculate the titer for the data were calculated with Microsoft Excel software, and dilutions that reduced the numbers of plaques by 50% were calculated from the fitted line.

Quantification of HSV-specific IFN-γ* CD8* T cells. HSV-specific gamma-interferon-positive (IFN-γ*) CD8* T cells infiltrating trigeminal ganglia (TG) were detected and quantified as previously described (18), with slight modification. Briefly, P815 cells were infected with HSV-2 strain 333 at a multiplicity of infection of 1 for 5 h. Four or six TG were pooled and dispersed in a 2-ml concentration of collagenase type I (Sigma) for 1 to 1.5 h and passed through a 100-μm-pore-size filter. TG cells were resuspended in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum, 10 U of interleukin-2 per ml, glutamine, and antibiotics. About 10^6 neurons and variable numbers of lymphocytes were recovered from each ganglion, depending on whether and when the animals were vaccinated or infected. Animals were sacrificed by cervical dislocation and by concussion in a 5-ml fluorescence-activated cell sorter (FACS) tube with 5 × 10^6 HSV-infected P815 cells per TG for another 5 h in the presence of 10 mg of brefeldin A per ml (Sigma). The cells were washed and stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD8 and peridinin chlorophyll protein (PerCP)-conjugated anti-CD45 monoclonal antibody (BD Pharmingen, San Diego, Calif.). After being washed again, the cells were fixed and permeabilized with Cytofix/Cytoperm buffer (BD Pharmingen) and washed with PermWash buffer (BD Pharmingen) according to the manufacturer’s protocol.

Cells were stained with phycoerythrin (PE)-conjugated anti-IFN-γ monoclonal antibody and analyzed promptly with a FACSCaliber flow cytometry system (Becton Dickinson, Franklin Lakes, N.J.) by using CellQuest software (Becton Dickinson). Forward- and side-scatter gates were set by back gating from the CD45* CD8* population. Data continued to be acquired until the events recorded decreased to fewer than 50% of the events acquired over the first 40,000 events. The population was analyzed for the percentage of CD8* T cells expressing IFN-γ by using the FACSDiva software (Becton Dickinson).
conjugated anti-CD3 monoclonal antibody (BD Pharmingen). After fixation and permeabilization, the cells were stained with PE-conjugated anti-IFN-γ monoclonal antibody. Data continued to be acquired until 10,000 to 20,000 events of live CD8+ CD3+ cells were collected by FACS.

RESULTS
dl5–29 prevents acute, recurrent, and latent infection in guinea pigs as effectively as a recombinant gD2 vaccine. Four candidate vaccine formulations—dl5–29, gD2(CFA/IFA), pgD2 plasmid, or plasmid followed by dl5–29 in a priming-booster fashion—were compared with PBS(CFA/IFA) in a series of studies with guinea pigs. On day 0, animals were challenged intravaginally with virulent HSV-2.

(i) Virus shedding. The titers of virus in vaginal secretions fell significantly in the days after infection for all vaccine and control groups, with no further virus detectable on day 10 (Fig. 1A; see legend for P values). The decline in viral shedding was most rapid among dl5–29 recipients, resulting in titers that were significantly less than those of control animals at selected times after infection.

(ii) Acute disease severity. Lesion scores for animals in three of the four vaccine formulation groups were significantly reduced relative to those seen in PBS recipients (Fig. 1B; see legend for P values): two doses of pgD2 failed to attenuate acute disease significantly. Two doses of dl5–29 and of gD2(CFA/IFA) prevented acute disease nearly completely and indistinguishably (P = 0.80). One dose of pgD2 followed by dl5–29 was also effective.

(iii) Recurrent genital lesions. The relative efficacy of each vaccine formulation in attenuating acute disease was predictive of subsequent rates of disease recurrence (Fig. 1C). The group mean cumulative numbers of recurrent genital lesions were

FIG. 1. Vaccination with dl5–29 or gD2 is most effective as prophylaxis for acute, latent, and recurrent infection in guinea pigs. Animals received the vaccines on days 21 and 42 before challenge. On day 0, they were challenged intravaginally with HSV-2 strain 333. (A) Virus shedding from the vaginal tract. Significant differences between groups are indicated by *n, •1, significantly lower titers on day 2 for dl5–29 recipients than for control animals and pgD2 recipients (P < 0.01); •2, significantly lower titers on day 4 for dl5–29 recipients than for dl5–29/pgD2 recipients (P = 0.02); •3, on day 6, significantly lower titers for dl5–29 recipients than for control mice (P = 0.02), pgD2 mice (P = 0.02), and gD2 recipients (P = 0.01); •4, on day 8, significantly higher titers for control mice than for dl5–29 and pgD2 mice (P = 0.03 each). (B) Group mean lesion scores during acute disease. Recipients of dl5–29 alone, gD2, and dl5–29/pgD2 had milder acute disease than animals given two doses of pgD2 DNA and control animals (each P < 0.01). (C) Mean cumulative numbers of recurrent lesions. The number of recurrences in recipients of dl5–29 alone, gD2, and dl5–29/pgD2 was significantly lower than that in unvaccinated, control guinea pigs (each, P < 0.001), while recurrences in pgD2 recipients remained higher than those in dl5–29 and gD2 recipients (P = 0.02 and 0.03, respectively). (D) Latent viral DNA loads in pooled sacral ganglia. The broken line indicates the limit of detection of this quantitative PCR assay (6 copies of viral DNA/100 ng of DNA). A viral load of <6 copies was assumed to be 3 copies of viral DNA/100 ng of DNA. The horizontal thick and vertical thin bars show the geometric mean numbers of viral genome copies and SEs, respectively. The viral load in control guinea pigs was significantly higher than that in recipients of dl5–29 alone (P = 0.001), dl5–29/pgD2 (P = 0.01), and gD2 (P < 0.001), while pgD2 recipients had higher viral loads than dl5–29 and gD2 recipients (P = 0.03 and 0.002, respectively). Differences between results for gD2 and dl5–29 recipients were not significant (P = 0.21).
greatly and indistinguishably \( (P = 0.95) \) reduced among dl5–29 and gD2 recipients. Two doses of pgD2, or one followed by dl5–29, were also effective, yet less so than PBS. Not only were the cumulative numbers of recurrences reduced by vaccination, but also the scores of the lesions that did arise, with the differences between each vaccine group and the control group achieving statistical significance during study days 61 to 90 (data not shown).

(iv) Latent viral DNA load. Real-time PCR was used to quantify the numbers of copies of latent HSV-2 genomes in sacral ganglia of guinea pigs sacrificed about 90 days after challenge (Fig. 1D). These levels were significantly lower in dl5–29, dl5–29/pgD2, and gD2 vaccinees than in pgD2 or PBS(CFA/IFA) recipients. One of 8 recipients of gD2(CFA/IFA) and 5 of 10 recipients of dl5–29 had detectable latent viral DNA, but the geometric mean copy numbers were statistically similar \( (P = 0.21) \). This finding suggested the possibility that the latent DNA in dl5–29 recipients may have included some of the vaccine virus itself.

dl5–29 is impaired for establishment of latency. We addressed the question of dl5–29 latency in an experiment in which the vaccine was administered intravaginally to guinea pigs without subsequent viral challenge (mock-infected group). Other groups were given two doses 3 weeks apart of either PBS (CFA/IFA), dl5–29 intravaginally, 3 \( \mu \)g of gD2(CFA/IFA), or 100 \( \mu \)g of pgD2 by the same routes as those in the prior study (see above), followed 3 weeks later by challenge with 2 \( \times 10^5 \) PFU of wild-type HSV-2. Because genital herpes infections in humans prevent most subsequent homotypic reinfections, we reasoned that dl5–29 given intravaginally would be efficacious.

The results indicated that intravaginal vaccination with dl5–29 was not very effective in reducing virus shedding from the vaginal tract or acute lesion scores. Viral shedding was reduced in gD2 and pgD2 recipients, as expected, with the differences in these results from those for control (PBS) guinea pigs being significant on days 2 and 8 after challenge (data not shown). The group mean acute lesion scores were 2.3, 1.7, 0.7, 1.0, and 0.0 for control, intravaginal dl5–29, pgD2, and gD2 recipients and mock-challenged animals, respectively. The scores for the dl5–29 recipients were lower than those for control animals, but not significantly \( (P = 0.84) \), whereas pgD2 and gD2 recipients had significantly milder acute disease than animals in the other two groups \( (P < 0.01) \).

Intravaginal vaccination reduced subsequent disease recurrence rates, but only modestly (Fig. 2A). The mean numbers of recurrences from days 15 to 80 were reduced in recipients of all vaccines relative to those in PBS(CFA/IFA) recipients, being 15.0, 7.0, 8.4, and 2.8 for the PBS, dl5–29 \( (P = 0.01) \), pgD2 \( (P = 0.04) \), and gD2 \( (P < 0.001) \) groups, respectively. The animals vaccinated vaginally with dl5–29 but then mock challenged experienced no acute or recurrent lesions, verifying the lack of virulence of dl5–29 in immunocompetent animals.

While intravaginal vaccination was not shown by this experiment to be an optimally effective means of infecting or protecting guinea pigs from severe viral challenge, it did verify findings from earlier tests in mice \( (10) \) that dl5–29 is impaired for establishment of latency (Fig. 2B). Compared with PBS (CFA/IFA) controls, recipients of recombinant gD2(CFA/IFA) had significantly reduced quantities of latent viral DNA, being undetectable in 4 out of 12 animals. Recipients of pgD2 DNA also had lower copy numbers of latent viral DNA than PBS vaccinees did but more than gD2 recipients did \( (P \) values in Fig. 2 legend). By contrast, none of the pooled sacral ganglia from eight animals that received 10\(^6\) PFU of dl5–29 vaginally

**FIG. 2.** When given intravaginally, the dl5–29 vaccine is partially protective but impaired for latency. All animals were vaccinated on days 21 and 42 before challenge. Animals were given PBS mixed with CFA or IFA, dl5–29 vaginally, pgD2, or gD2. Mock-challenged animals were given two doses of dl5–29 vaginally and then challenged with uninfected Vero cell lysate. On day 0, animals other than the mock-challenged group were challenged vaginally with HSV-2 strain 333. (A) Group mean cumulative numbers of recurrent lesions. Control animals experienced more recurrences than recipients of dl5–29 vaginally, pgD2, and gD2 \( (P = 0.01, 0.04, \text{ and } < 0.001, \text{ respectively}) \). dl5–29 vaccination was superior to vaccination with pgD2, but not significantly \( (P < 0.07) \), and was significantly superior to vaginal administration of dl5–29 \( (P < 0.01) \). Mock-challenged animals had no recurrences. (B) Latent viral DNA load in pooled sacral ganglia. Thick and thin bars indicate the geometric mean and SE, respectively, for each group. The viral load of gD2 recipients was significantly lower than that of PBS(CFA/IFA), vaginal dl5–29, and pgD2 recipients \( (P < 0.001, P = 0.001, \text{ and } P = 0.02, \text{ respectively}) \). Animals administered dl5–29 vaginally but mock challenged had no detectable latent viral DNA. Their geometric mean DNA load, assumed to be \( \leq 3 \) copies of viral DNA/100 ng of DNA, was significantly lower than that for all other groups \( (P < 0.001) \).
twice but that were not further challenged contained latent viral DNA that could be detected by real-time PCR.

**dl5–29 is an effective immunotherapeutic vaccine.** Prior studies showed recombinant gD2(CFA/IFA) or recombinant gD2 with interleukin-2 to be active as an immunotherapeutic agent for guinea pigs previously infected intravaginally with HSV-2 (1, 16, 17, 30). Moreover, preliminary human trials suggested that gD2 in alum may lessen the frequency of subsequent recurrences in patients with genital herpes (33, 34). To compare the therapeutic efficacies of dl5–29 and gD2, guinea pigs were infected intravaginally with virulent HSV-2 on day 0. On days 7 and 15 thereafter, they were given either PBS(CFA/IFA), gD2(CFA/IFA) i.m., or dl5–29.

To verify that the groups of animals had experienced comparably severe acute infections prior to vaccination, vaginal swabs were taken on days 2 and 6 and viral titers were determined. As expected, the mean titers of HSV-2 for the three study groups were similar (data not shown). Moreover, the mean acute lesion scores for the three groups were similar, certainly until the first vaccination on day 7 (Fig. 3A). Thereafter, the lesion severity in dl5–29 and gD2 recipients rose slightly relative to that of PBS recipients, reflecting either experimental variation or a transient immunopathological response to the vaccines. Nonetheless, the mean numbers of recurrent lesions for days 15 to 90 were reduced after vaccination, being 15.8, 12.1, and 10.5 for the PBS, gD2, and dl5–29 groups, respectively (Fig. 3B). Initially, the rates of recurrences in the gD2 and control groups were similar, but they diverged after day 50, although the difference did not attain statistical significance during the study interval. The recurrence rate among the dl5–29 recipients, however, diverged from the rates among the other groups very soon after vaccination and sustained its relative difference from the rate among control animals throughout the study period. The difference between the control and dl5–29 groups was significant (P = 0.04).

**Latent viral load correlates with acute lesion scores and disease recurrence rates.** Initial studies of guinea pigs showed that the latent viral load is predictive of the subsequent lesion recurrence rate (22). In the present series of three independent guinea pig experiments, we sought confirmation of that observation and extension to other correlates of recurrence rate. Here, by pooling the data for all vaccines and routes of administration, the latent viral loads correlated significantly with the acute lesion scores (Fig. 4A) and the numbers of recurrent lesions experienced by each animal (Fig. 4B). These correlations suggest that the severity of acute disease affects later outcomes such as the recurrence rate, which is itself dependent on the latent viral load.

**dl5–29 induces higher neutralizing antibody titers in guinea pigs than does recombinant gD2.** Overall, the dl5–29 vaccine given s.c. protected and treated guinea pigs as well as recombinant gD2(CFA/IFA).Studies of vaccine-induced immunogenicity were performed to better understand the basis for this efficacy. HSV-2-specific neutralizing antibody titers in sera harvested after vaccination and before challenge in the experiment shown in Fig. 1 were determined first. These geometric mean titers were significantly higher among dl5–29 recipients than for all other groups, including gD2 recipients (Table 1). By contrast with the results for neutralizing antibodies, the gD2-specific antibody titers, as measured by enzyme-linked immunosorbent assay, were 0.002 ± 0.001, 0.069 ± 0.022, 0.078 ± 0.014, 0.013 ± 0.005, and 0.920 ± 0.052 (mean optical density ± standard error [SE]) for recipients of PBS (CFA/IFA), dl5–29, pgD2 followed by dl5–29, pgD2 alone, and gD2, respectively. The specific antibody titers were significantly higher in gD2 recipients than in all other groups (P < 0.001). Overall, these serological assays imply that dl5–29 infection induces significant neutralizing antibody responses against numerous viral epitopes, while the recombinant protein vaccine induces high-level responses to the more limited set of gD2 epitopes.

**dl5–29 virus must be live to achieve protection in mice.** Da Costa et al. showed that dl5–29 expresses multiple viral antigens before the infectious process is aborted (9). To determine whether HSV-2 structural proteins or de novo proteins expressed by dl5–29 infection mediate protection, we vaccinated...
mice with heat-killed HSV-2 strain 333 and compared them with mice vaccinated with pgD2 or dl5-29 in a lethal challenge model. Heat-killed HSV-2, with its full complement of antigens, failed to protect any mice; live dl5-29 protected all of them; and pgD2 recipients were afforded partial protection (Fig. 5). Thus, the prophylactic efficacy of dl5-29 in mice, as in guinea pigs, is superior to that of a plasmid vaccine and seems likely to depend on de novo protein expression.

**HSV-2-specific IFN-γ⁺ CD8⁺ T-cell responses are greater after vaccination with dl5-29 than with gD2.** As a live virus, dl5-29 has a greater potential than recombinant gD2 to induce specific CD8⁺ T-cell responses, due to presentation of its antigens in the context of major histocompatibility complex class I molecules. To this end, we measured the frequencies of HSV-2-specific IFN-γ-producing CD8⁺ T cells in spleens (Fig. 6A) and TG (Fig. 6B) of mice by using flow cytometry and intracellular cytokine staining. Control mice were administered PBS(CFA/IFA). Vaccinated mice were given dl5-29 or gD2 (CFA/IFA), and their spleen cells were harvested at the indicated days after the second vaccination. From control mice, the frequency of splenic IFN-γ⁺ CD8⁺ T cells responding to uninfected P815 cells was 360 ± 11 (mean ± SE) per 10⁶ CD8⁺ T cells, while the frequency responding to HSV-2-infected P815 cells was essentially the same, being 470 ± 12 (Fig. 6A).

Among gD2 recipients, virus-specific responses were marginally, if at all, higher than those seen in control mice. By contrast, for dl5-29 recipients, 1,610 ± 13 to 1,670 ± 15 per 10⁶ splenic CD8⁺ T cells examined at days 7 and 14 produced IFN-γ in response to cocultivation with HSV-2-infected cells. These frequencies fell by day 32 after vaccination but remained higher than the peak levels observed in gD2 recipients. Overall, dl5-29 vaccination yielded significantly higher systemic HSV-2-specific CD8⁺ T-cell responses than gD2(CFA/IFA) at all time intervals studied (P < 0.01 by the Mann-Whitney U test).

We next investigated the regional responses induced by the vaccine. Because the numbers of HSV-2-specific CD8⁺ T cells were too low to be measured reliably in vaccinated mice, we examined whether systemic vaccination facilitated their more rapid trafficking to, and accumulation in, TG following unilateral corneal challenge with virulent virus. Of ipsilateral TG pooled 24 h after infection from control [vaccinated with PBS (CFA/IFA)], gD2-vaccinated, and dl5-29-vaccinated mice, 1.5, 0, and 7.1% of CD8⁺ T cells, respectively, were IFN-γ⁺. By

**TABLE 1. Geometric mean neutralizing antibody titers in vaccinated guinea pigs**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean titer ± SE</th>
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<tbody>
<tr>
<td>Control</td>
<td>&lt;10.0ᵃ</td>
</tr>
<tr>
<td>Vaccinated dl5-29</td>
<td>191.2 ± 51.9ᵇ</td>
</tr>
<tr>
<td>dl5-29/pgD2</td>
<td>43.8 ± 8.1</td>
</tr>
<tr>
<td>pgD2</td>
<td>22.7 ± 6.7</td>
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<tr>
<td>gD2</td>
<td>67.1 ± 18.9</td>
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ᵃ No control sera contained detectable neutralizing antibodies.
ᵇ Titers in dl5-29 recipients were significantly higher than those in control animals and in dl5-29/pgD2, pgD2, and gD2 recipients based on the Mann-Whitney U test (each, P < 0.01).
day 5 after infection, 0.6% of all CD8+ T cells from control mice, 0.5% from gD2 recipients, and 6.1% from dl5-29 recipients were also IFN-γ+ (Fig. 6B). By day 14 after infection, all groups showed substantial percentages and comparable numbers of IFN-γ+ CD8+ T cells in TG.

In a second study, mice were vaccinated twice 10 days apart with PBS, gD2, or dl5-29 and challenged with twice the dosage of wild-type HSV-2 used in the prior experiment. Based on our prior experience, unvaccinated mice needed to be given human immunoglobulin 24 h after infection in order for any of them to survive the more virulent challenge in this experiment (12). The numbers of infiltrating HSV-2-specific IFN-γ+ CD8+ T cells in all groups of mice were measured at days 4 and 14 after infection. Again, IFN-γ+ CD8+ T cells accumulated in TG from dl5-29 recipients earlier than in TG from mice in the other groups. For the PBS, gD2, and dl5-29 recipients, the percentages of CD8+ cells that were also IFN-γ+ were 0.9, 4.1, and 13.5%, respectively. By day 14, ganglia from all groups contained substantial numbers and percentages of IFN-γ+ CD8+ T cells, with 290 (28.7%), 114 (8.0%), and 352 (7.1%) per TG for control, gD2 recipient, and dl5-29 recipient mice, respectively.

**DISCUSSION**

The purpose of this study was to compare candidate DNA and replication-defective vaccines directly with recombinant gD2, the one reagent shown to have significant effects in some humans, for their relative immunogenicities and efficacies in preventing and treating HSV-2 infections in mice and guinea pigs. While each of these types of vaccines has been found to be effective in animal models (6, 9, 23–25, 27, 29, 30), few studies compared any of them directly (11, 28). Moreover, dl5-29 had not been studied previously in the guinea pig, the singular model in which acute disease, latency, and spontaneous recurrences characteristic of human infection are recapitulated and in which responsiveness to interventions has been predictive of antiviral drug efficacy in patients (4, 13). We found that a plasmid expressing gD2 at both 25-μg (Fig. 1) and 100-μg (Fig. 2) dosages was less effective in attenuating acute
and recurrent genital disease in guinea pigs than recombinant gD2(CFA/IFA) or dl5–29 and less effective than dl5–29 in protecting against lethal challenge in mice (Fig. 5). A regimen including one dose of dl5–29 given after a dose of pgD2 was more efficacious than two doses of pgD2 alone but still less efficacious than what was achieved with two doses each of gD2 or dl5–29 (Fig. 1). Administration of dl5–29 vaginally to naive guinea pigs resulted in no detectable signs or symptoms of genital disease but was only modestly protective against a subsequent wild-type challenge (Fig. 2B). Subcutaneous dl5–29, however, was as effective as gD2(CFA/IFA) in preventing and treating acute and recurrent genital disease. Moreover, dl5–29 protected against establishment of latency by wild-type virus (Fig. 1D) while itself being impaired for establishment of latency (Fig. 2B).

Because both dl5–29 given s.c. and gD2(CFA/IFA) given i.m. were highly effective, it was not possible in the present studies to discern whether one has the potential for meaningful prophylactic or therapeutic advantages over the other. Nonetheless, dl5–29 proved superior to gD2 in eliciting virus-specific immune responses (Table and Fig. 6). As revealed by Western blotting, the relative quantity of gD2 administered in the recombinant vaccine (3 μg/dose) exceeded considerably that expressed during a single, defective replication cycle of the dl5–29 inoculum (10^6 PFU) in Vero cells (data not shown). Thus, although gD2 elicited higher antigen-specific antibody responses, dl5–29 induced significantly higher neutralizing antibodies, indicating perhaps that it presented a far broader palette of neutralizing epitopes than gD2 alone or that humoral responses to these epitopes were elicited more efficiently when presented in the context of dl5–29 infection. Moreover, dl5–29 induced significantly more splenic HSV-specific CD8^+ T cells than gD2(CFA/IFA) did (Fig. 6A) and primed such cells to traffic more quickly to ganglia once they had been invaded by virulent challenge virus (Fig. 6B). While innate immunity and neutralizing antibodies may play the greatest role in limiting the spread of virus to and within sensory nerves (12), the early recruitment of HSV-specific CD8^+ T cells to ganglia may also be important for attenuating a primary infection. In mice, HSV-specific CD8^+ T cells can inhibit development of subsequent secondary skin lesions if they are transferred adaptively within 24 h of infection (36). Also, HSV-specific CD8^+ T cells begin to proliferate no sooner than 24 h after infection but undergo up to four rounds of cell division by day 2 after infection (7, 26). Thus, it would seem that animals previously primed for HSV-specific CD8^+ T cells by dl5–29 vaccination may be poised for more rapid clearance of virus from regional tissues and ganglia, resulting in less local inflammation, lesion formation, and neuronal cell death and ultimately a reduced latent viral load. Although we have not yet shown that the cellular responses induced by dl5–29 persist for months, the reduced rate of lesion recurrences in guinea pigs (Fig. 1C) may, at least in part, result from sustained vaccine-mediated immunity. Khanna et al. recently showed that HSV-specific CD8^+ T cells infiltrating ganglia block HSV reactivation from latency in a dose-dependent manner (18).

dl5–29 contains deletions that render it competent for replication only in cell lines that express the essential HSV early gene products UL5 and ICP8. The V529 cell line used here to prepare stocks of dl5–29 contains open reading frames 5 and 29 sequences smaller than those deleted from the virus so as to prevent their recombination into the dl5–29 stock. Theoretically, however, coinfection with wild-type HSV-2 could both complement the replication of dl5–29 and rescue its deleted sequences through a recombinatorial event, although such a recombinant should not be more virulent than the coinfecting wild-type virus itself. Moreover, the probability that cells in vivo would be dually infected with wild-type HSV-2 and dl5–29 is almost nonexistent, both because the defect in replication of dl5–29 prevents its successive spread to neighboring cells and because dl5–29 is impaired for establishment of latency. These properties of dl5–29 further enhance its appeal as a candidate vaccine.

What amounted to an inoculum of dl5–29 that was sufficient to be strongly immunogenic and protective in small animals may not prove achievable in humans. It is humbling to recall that a vaccine containing both recombinant gD2 and gB2 in a potent lipid emulsion adjuvant was highly effective in mice, guinea pigs, and baboons and very immunogenic in humans but failed to protect significantly against primary infection and disease, while gD2 alone in alum-monovalent phospholipid A was also highly protective in animals but protected only seronegative women (8, 31, 35). Moreover, HSV-2 with its glycoprotein H gene deleted, capable of only a single replicative cycle, induced primary humoral and cellular immune responses in mice, guinea pigs (3, 15), and humans (20; J. K. Hickling, S. E. Chisholm, I. A. Duncan, E. J. Taylor, C. Boswell, and S. C. McLean, J. Utrodge, J. S. Roberts, A. Tomasi, L. R. Stanberry, D. I. Bernstein, M. E. Boursnell, and S. C. Inglis, presented at the 8th International Congress for Infectious Disease, 1998) but failed to attenuate established recurrent genital herpes (19). Thus, there is no certainty that the dl5–29 vaccine would succeed, but its impressive immunogenicity renders it a desirable candidate for human trials, particularly for prevention of primary and recurring genital disease.

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REFERENCES


ERRATUM

Comparative Efficacy and Immunogenicity of Replication-Defective, Recombinant Glycoprotein, and DNA Vaccines for Herpes Simplex Virus 2 Infections in Mice and Guinea Pigs


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