Development of a Glycoprotein D-Expressing Dominant-Negative and Replication-Defective Herpes Simplex Virus 2 (HSV-2) Recombinant Viral Vaccine against HSV-2 Infection in Mice

Natalie V. Akhrameyeva,† Pengwei Zhang,† Nao Sugiyama,† Samuel M. Behar,2 and Feng Yao1*

Department of Surgery1 and Department of Medicine,2 Brigham and Women’s Hospital, and Harvard Medical School, Boston, Massachusetts 02115

Received 7 December 2010/Accepted 1 March 2011

Using the T-REx (Invitrogen, California) gene switch technology and a dominant-negative mutant polypeptide of herpes simplex virus 1 (HSV-1)-origin binding protein UL9, we previously constructed a glycoprotein D-expressing replication-defective and dominant-negative HSV-1 recombinant viral vaccine, CJ9-gD, for protection against HSV infection and disease. It was demonstrated that CJ9-gD is avirulent following intracerebral inoculation in mice, cannot establish detectable latent infection following different routes of infection, and offers highly effective protective immunity against primary HSV-1 and HSV-2 infection and disease in mouse and guinea pig models of HSV infections. Given these favorable safety and immunological profiles of CJ9-gD, aiming to maximize levels of HSV-2 glycoprotein D (gD2) expression, we have constructed an ICP0 null mutant-based dominant-negative and replication-defective HSV-2 recombinant, CJ2-gD2, that contains 2 copies of the gD2 gene driven by the tetracycline operator (tetO)-bearing HSV-1 major immediate-early ICP4 promoter. CJ2-gD2 expresses gD2 as efficiently as wild-type HSV-2 infection and can lead to a 150-fold reduction in wild-type HSV-2 viral replication in cells infected with CJ2-gD2 and wild-type HSV-2 at the same multiplicity of infection. CJ2-gD2 is avirulent following intracerebral injection and cannot establish a detectable latent infection following subcutaneous (s.c.) immunization. CJ2-gD2 is a more effective vaccine than HSV-1 CJ9-gD and a non-gD2-expressing dominant-negative and replication-defective HSV-2 recombinant in protection against wild-type HSV-2 genital disease. Using recall response, we showed that immunization with CJ2-gD2 elicited strong HSV-2-specific memory CD4+ and CD8+ T-cell responses. Collectively, given the demonstrated preclinical immunogenicity and its unique safety profiles, CJ2-gD2 represents a new class of HSV-2 replication-defective recombinant viral vaccines in protection against HSV-2 genital infection and disease.

Genital herpes is a chronic, lifelong disease caused by herpes simplex viruses (HSV). It is one of the most common sexually transmitted diseases worldwide and is the primary cause of genital ulcer disease in both developed and developing countries (32). Among the human herpesviruses, HSV types 1 and 2 (HSV-1 and -2) are the most closely related, with an overall incidence of identically aligned nucleotides of 83% (15). HSV can cause both an acute, productive infection and a long-term latent infection characterized by unpredictable periodic recurrences (69). HSV-2 is the primary cause of genital ulcer disease, whereas HSV-1 is often associated with orofacial infections. Approximately 70% of adults in the United States are infected with HSV-1, and 50 million to 60 million of them are infected with HSV-2 (59, 71). Although HSV infections are often asymptomatic, their clinical manifestations include genital herpes, neonatal herpes, orofacial infections, keratoconjunctivitis, and herpes encephalitis (62, 69). Individuals who are congenitally immunodeficient, clinically immunosuppressed, or suffering from disorders of skin integrity are at risk of serious complications from HSV infection, including disseminated cutaneous lesions, involvement of visceral organs, and even death (69). HSV infections are a major concern in AIDS and cancer patients, organ transplant recipients, and newborns. It has been documented that genital HSV-2 infection triples the risk for sexually acquiring HIV infection (20), and in Africa, this increase in risk conferred by HSV-2 may account for 25 to 35% of incident HIV infections (1).

Although the severity and duration of most symptomatic HSV primary infections can be reduced by oral or intravenous treatment with acyclovir, valacyclovir, or famciclovir, antiviral therapy neither prevents the establishment of latent infection from primary infection nor reduces subsequent recurrences (69). Currently, there is no effective antiviral therapy to prevent or reduce the incidence of either symptomatic or asymptomatic recurrences except for daily suppressive therapy, which again has no effect in preventing HSV latency. The failure of antiviral therapy to effectively control HSV infection and disease and the increasing incidence of HSV resistant to the current antiviral medications present a strong need for developing safe and efficacious vaccines against HSV infections (32, 61). Moreover, given that HSV suppressive therapy leads to a significant reduction in levels of HIV in the genital mucosa and plasma of women infected with both HSV-2 and HIV (50), an effective HSV vaccine could have major implications in the control of HIV infection (1).
HSV glycoprotein D (gD) is one of the most abundant viral antigens on the surface of infected cells and on the viral envelope (23). gD is essential for the entry of the virus into cells and is the major target for neutralizing antibodies against HSV infection (52). Moreover, gD is one of the dominant viral targets for CD4+ T cells, including cytotoxic CD4+ T cell and CD8+ T cells in human and murine models of HSV infection (26, 27, 30, 36, 44, 66, 77), and consequently, gD is the major focus for HSV subunit vaccine development (61). In an earlier phase 3 clinical trial, Stanberry et al. showed that vaccination with recombinant HSV-2 gD (gD2) in combination with adjuvant AS04 provided 73 to 74% efficacy in protecting against development of genital herpes disease in HSV-seronegative women, while no significant efficacy was observed in men and in subjects who were seropositive for HSV-1 (63). In recent phase 3 clinical trials, however, no positive effect was observed (10). Because the gD2/AS04 vaccine is inefficient in eliciting a CD8+ T cell response (32, 33), which plays a crucial role in controlling primary and recurrent HSV infection as well as preventing reactivation of HSV from latently infected neurons (4, 31, 35, 40, 47, 51, 58, 67, 68, 78, 79), the studies highlight the need to develop HSV vaccine candidates capable of eliciting broader and more robust humoral and CD4+ and CD8+ T-cell responses not only to gD2 but also to various other HSV viral antigens (10, 28, 32).

It is well documented that live viral vaccines capable of de novo synthesis of immunogens in the host induce broader and more durable immune responses than vaccines consisting of only peptides or proteins. Various forms of replication-defective HSV and neuroattenuated, replication-competent mutants have been tested as potential live vaccines against HSV infection in several different animal models (17). Because both replication-defective viruses and neuroattenuated mutants can coreplicate with wild-type virus or become replication competent in the context of wild-type virus, their use as a vaccine in humans poses a safety concern, particularly for individuals who harbor latent HSV infection (34). The observation that replication-defective HSV-1 mutants can reactivate the latent HSV-1 immediate-early promoter in the rodent brain raises additional safety concerns, at least theoretically, about the possibility of such vaccine vectors triggering outbreaks of productive viral infections in latently infected individuals (64). Thus, a desirable replication-defective recombinant HSV vaccine not only should possess the ability to express a broad spectrum of virus-encoded antigens but also should encode a unique function that can prevent lytic infection of wild-type HSV when encountered within the same cells. With incorporation of such a safety mechanism(s), one could minimize the potential outbreak of the vaccine virus caused by the recombination of the vaccine vector with wild-type virus in the host.

Using the tetracycline gene switch technology (T-REx; Invitrogen) developed in our laboratory (75) and the dominant-negative mutant polypeptide UL9-C535C of HSV-1 origin of viral DNA replication binding protein UL9, our laboratory has established a novel strategy for development of a safe and effective recombinant viral vaccine against HSV infection. Specifically, we constructed a replication-defective HSV-1 recombinant, CJ83193, in which both copies of the HSV-1 ICP0 gene are replaced by DNA sequences encoding UL9-C535C under the control of the tetracycline operator (tetO)-bearing human cytomegalovirus (hCMV) major immediate-early promoter (72). CJ83193 expresses high levels of UL9-C535C in non-tetracycline repressor (tetR)-expressing cells, leading to inhibition of its own viral DNA replication and that of wild-type HSV-1 and HSV-2 in coinfected cells (72, 73).

HSV gene expression has been classified into three major phases during productive infection: immediate-early (α), early (β), and late (γ), with late genes being further divided into γ1 and γ2 (56). The expression of α genes requires no de novo viral protein, and DNA synthesis and is activated by the virion-associated protein VP16 together with cellular transcription factors. While inhibition of viral DNA replication has no effect on the expression of viral β genes, expression of γ genes is highly influenced by de novo viral DNA synthesis. Specifically, de novo viral DNA replication leads to increased expression of γ1 genes, and blocking viral DNA replication inhibits expression of γ2 genes. The gene encoding gD belongs to an early-late class of HSV genes (γ1) (56). Although CJ83193 expresses viral immediate-early and early gene products, its ability to express HSV-1 glycoprotein D (gD1) is significantly impaired compared with that of wild-type HSV-1. Aiming to elevate its vaccine efficacy against HSV infections, we constructed a CJ83193-derived recombinant, CJ9-gD, by replacing the essential UL9 gene with the HSV-1 gD gene controlled by the tetO-bearing hCMV major immediate-early promoter (41). Immunization of mice with CJ9-gD elicits strong and long-lasting HSV-specific humoral and Th1 T-cell responses at levels similar to those induced in wild-type HSV-1-immunized mice (6, 41) and provides complete protection against herpetic disease in both mouse ocular and genital models of HSV-1 infection.

Many type-common and type-specific antibodies, as well as T-cell epitopes, have been identified among various HSV-1 and HSV-2 genes in both animal models of HSV infections and HSV-seropositive persons (2, 3, 9, 11, 22, 33, 53, 66). We have shown that immunization with CJ9-gD elicits stronger humoral and cellular immune responses against HSV-1 than against HSV-2 and offers significantly better protection against HSV-1 genital infection than against HSV-2 (6). Given observations that humans previously infected with HSV-2 are less likely to experience reinfection with a heterologous strain of HSV-2 than individuals with prior HSV-1 infection (8, 37, 42, 57, 70), we hypothesize that a CJ9-gD-like HSV-2 recombinant would be more effective than HSV-1 CJ9-gD in prevention of HSV-2 genital infection.

Here we describe the construction of an HSV-2 glycoprotein D (gD2)-expressing dominant-negative and replication-defective HSV-2 recombinant, CJ2-gD2, in which both copies of the HSV-2 ICP0 gene are replaced by the gD2 gene under the control of the tetO-bearing HSV-1 major immediate-early ICP4 promoter. Given that the promoter of the ICP4 gene represents one of the strongest HSV-1 promoters (13, 74) and its activity can be significantly elevated by the HSV virion-associated transactivator VP16, this design should lead to more efficient expression of gD2 than the VP16-unresponsive tetO-bearing hCMV-immediate-early promoter in the context of HSV infection (56, 65). Indeed, CJ2-gD2 expresses significantly more gD2 than CJ9-gD does for gD1. We have shown that immunization of mice with CJ2-gD2 elicited effective HSV-2-specific neutralizing antibody as well as potent memory
FIG. 1. (A) Schematic diagram of plasmids used for the construction of dominant-negative and replication-defective HSV-2 recombinants N2-C535C and CJ2-gD2. Plasmid pHSV2-ICP0 is a plasmid containing the HSV-2 ICP0 sequences covering 268 bp upstream of the HSV-2 ICP0 open reading frame (ORF) to 289 bp downstream of the stop codon of the ICP0 ORF, pHSV2.ICP0-V was constructed by replacing the XhoI-ICP0 DNA fragment containing sequences 25 bp upstream of the initiation codon of ICP0 to 397 bp upstream of the stop codon of the ICP0 ORF with an XhoI-containing multiple cloning sequence (MCS). Plasmid pHSV2.ICP0-lacZ was created by inserting the HindIII-TATA element, TATATGA. We showed that, as with CMVTO, expression of hEGF from ICP4TO could be tightly repressed by the tetracycline repressor in the absence of tetracycline (data not shown). p02ICP4-TO is a pHSV2.ICP0-V-derived plasmid, which was constructed by replacing the ICP4 promoter sequence from 377 bp to 19 bp relative to the transcriptional start site of the ICP4 gene. Similar to the tetO-bearing hCMV major immediate-early promoter (CMVTO) (Fig. 1A), it contains tandem copies of tet operator sites at 10 bp downstream of the ICPO TATA element, TATATGA. We showed that, as with CMVTO, expression of hEGF from ICP4TO could be tightly repressed by the tetracycline repressor in the absence of tetracycline (data not shown).

B. ICP0 UL US HSV-2

C. 2.664 bp 702 bp 1.364 bp 1,928 bp

CD4 + and CD8 + T-cell responses and offered complete protection against herpetic disease following intravaginal challenge with wild-type HSV-2 at a dose of 50% lethal doses (LD50). Moreover, CJ2-gD2 cannot establish detectable latent infection following subcutaneous inoculation and is more effective vaccine than HSV-1 CJ9-gD and a non-gD2-expressing dominant-negative and replication-defective HSV-2 recombinant in protection against wild-type HSV-2 genital infection and disease.

MATERIALS AND METHODS

Cells. African green monkey kidney (Vero) cells and the osteosarcoma line U2OS cells were grown and maintained in Dulbecco's Modified Eagle's medium (DMEM) (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS) in the presence of 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate (Gibco, Carlsbad, CA) (74). U2OS cells are able to complement functionally for the HSV-1 ICP0 deletion (74). U2CEP4R11 cells are tetR-expressing U2OS cells that were maintained in DMEM plus 10% FBS and hygromycin B at 50 μg/ml (75).

Plasmids. Plasmid pHSV2-ICP0 is a pUC19-derived plasmid that contains the PCR-amplified HSV-2 ICP0 sequences covering 268 bp upstream of the HSV-2 ICP0 open reading frame (ORF) to 289 bp downstream of the stop codon of the ICP0 ORF (Fig. 1A). pHSV2.ICP0-V is an HSV-2 ICP0-cloning plasmid, derived from plasmid pHSV2-ICP0 by replacing the XhoI-ICP0 DNA fragment containing sequences 25 bp upstream of the initiation codon of ICP0 to 397 bp upstream of the stop codon of the ICP0 ORF with an XhoI-containing multiple cloning sequence (MCS). Plasmid pHSV2.ICP0-lacZ was created by inserting the HindIII-TATA element, TATATGA. We showed that, as with CMVTO, expression of hEGF from ICP4TO could be tightly repressed by the tetracycline repressor in the absence of tetracycline (data not shown). p02ICP4-TO is a pHSV2.ICP0-V-derived plasmid, which was constructed by inserting the ICP4-containing fragment of pICP4TO-hEGF into pHSV2.ICP0-lacZ with the EcoRI/HindIII-containing fragment of pHSV2.ICP0-lacZ was created by inserting the ICP0 open reading frame (ORF) to 289 bp downstream of the stop codon of the ICP0 ORF (Fig. 1A). pHSV2.ICP0-V is an HSV-2 ICP0-cloning plasmid, derived from plasmid pHSV2-ICP0 by replacing the XhoI-ICP0 DNA fragment containing sequences 25 bp upstream of the initiation codon of ICP0 to 397 bp upstream of the stop codon of the ICP0 ORF with an XhoI-containing multiple cloning sequence (MCS). Plasmid pHSV2.ICP0-lacZ was created by inserting the HindIII-TATA element, TATATGA. We showed that, as with CMVTO, expression of hEGF from ICP4TO could be tightly repressed by the tetracycline repressor in the absence of tetracycline (data not shown). p02ICP4-TO is a pHSV2.ICP0-V-derived plasmid, which was constructed by inserting the ICP4-containing fragment of pICP4TO-hEGF into pHSV2.ICP0-v at the MCS sites.

pArgD-HSV-2 carries the gD2 gene from HSV-2 strain 333 (a gift of Patricia Spear, Northwestern University). p02ICP4-gD2 is a pHSV2.ICP0-V-derived plasmid containing the gD2 gene from ArgD-HSV-2 under the control of ICP4TO. p02lacZTO-gD2.C535C is a gD2 and UL9-C535C-expressing plasmid, which was created by replacing the SnaBl/PstI fragment of p02lacZTO-gD2.C535C (Fig. 1A). In p02lacZTO-gD2.C535C, the gene encoding UL9-C535C is controlled by the truncated form of the tetO-bearing hCMV immediate-early promoter (236 bp 5’ to the transcription start site of the hCMV promoter) while the gD2 gene is under the control of ICP4TO in an opposite orientation (Fig. 1A).
The plaque-forming efficiency of N2-C535C is reduced at least 1.4-fold in the absence of tetracycline and no plaques in the presence of tetracycline. Expression plaques after four rounds of plaque purification in U2CEP4R-11 cells were isolated. One of the isolates, designated N2-C535C, yielded uniformly white lac
galactopyranoside (X-Gal) 96 h postinfection. White plaques, reflecting the presence of lacZ gene by the UL9-C535C DNA sequence, were obtained in medium plus complement alone. Neutralizing titer was expressed as the final serum dilution required to achieve a 50% reduction in HSV PFU relative to the HSV PFU obtained in medium plus complement alone.

**Immunoprecipitation.** U2OS cells seeded at 7.5 × 10^5 cells per 100-mm dish at a multiplicity of infection (MOI) of 10 PFU/cell. Cell extracts were prepared at 4 h, 6 h, 9 h, or 16 h postinfection (74). Proteins in the cell extract were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9% acrylamide), transferred to polyvinylidene difluoro (PVDF) membranes, and probed with either polyclonal antibodies against HSV-1 gD (R45; a gift of Gary H. Cohen and Roselyn J. Eisenberg), UL9 (a gift of Mark Chalberg), or monoclonal antibodies specific for ICP27 and gB (Santa Cruz Biotechnology, Santa Cruz, CA).

**Mice.** Female BALB/c mice 4 to 6 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed in metal cages at four mice per cage and maintained on a 12-h-light/dark cycle. Mice were allowed to acclimatize to the housing conditions for 1 week prior to experimentation. All animal experiments were conducted according to the protocols approved by the Harvard Medical Area Standing Committee on Animals and the American Veterinary Medical Association. The Harvard Medical School animal management program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and meets National Institutes of Health standards as set forth in The Guide for the Care and Use of Laboratory Animals.

**Immunization and challenges.** Female BALB/c mice were randomly divided into several groups, and the hair on their left rear flank was trimmed. Mice were either mock infected with 2 × 10^7 PFU/mouse of CJ2-gD2, N2-C535C or CJ9-gD or sham vaccinated with DMEM in a volume of 30 μl subcutaneously (s.c.) in the left rear flank using a 1-ml syringe fitted with a 27-gauge needle. Mice were boosted after 2 weeks and challenged with wild-type HSV-2 strain G 3 weeks after secondary immunization. Five days prior to challenge, mice were injected s.c. in the neck ruff with medroxyprogesterone (Sicor Pharmaceuticals, Inc., Irvine, CA) at 3 mg per mouse in a volume of 20 μl (6). For intravaginal challenge, mice in all groups were anesthetized, preswabbed with a calcium alginate swab (sterile urethrogenital calcium alginate-tipped applicator; Puritan Medical Products Company LLC, Guilford, ME) and inoculated intravaginally with 20 μl of culture medium containing 5 × 10^5 PFU (50 LD50) of HSV-2 strain G (48). Animals were kept on their backs with their rear part elevated under the influence of anesthesia for 30 to 45 min postinfection.

**Acute infection assays and clinical observations.** On days 1, 2, 3, and 7 postchallenge, vaginal mucous was swabbed with calcium alginate (6). Infectious viruses in swab materials were assessed by standard plaque assay on Vero cell monolayers.

Following challenge with wild-type HSV-2, mice were assessed daily during a 21-day follow-up period for signs of genital lesions and systemic illness. The severity of disease was scored as follows: 0, no sign of herpetic infection; 1, slight genital erythema and edema; 2, moderate genital inflammation; 3, purulent genital lesions and/or systemic illness; 4, hind-limb paralysis; and 5, death (48).

**Detection of HSV-2-specific neutralizing antibodies.** Blood was collected from tail veins of immunized and mock-immunized mice 4 weeks after primary immunization. Neutralizing serum antibody titers were determined as previously described in the presence of complement (5, 6) with 250 PFU of wild-type HSV-2 strain 186. The neutralizing titer was expressed as the final serum dilution required to achieve a 50% reduction in HSV PFU relative to the HSV PFU obtained in medium plus complement alone.

**Immunoprecipitation.** U2OS cells seeded at 7.5 × 10^5 cells per 100-mm dish were mock transfected or transfected with 10 μg of p02.4TO-gD by using Lipofectamine 2000 at 24 h postseeding. Cell extracts were prepared at 48 h posttransfection (41). Immunoprecipitations were performed by mixing 10 μl of pooled serum collected from mock-immunized and immunized mice with 70 μl of cell extracts prepared above. The gD2/mouse IgG-specific complexes were precipitated with protein A (Pierce classic IP kit; Pierce Biotechnology, Rockford, IL) resolved on SDS-PAGE, and probed with the gD-specific polyclonal antibody, R45, following by reaction with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

**Recall response and IFN-γ ELISPOT assays.** Female BALB/c mice were sham immunized with DMEM or immunized with CJ2-gD2 at 2 doses of 2 × 10^8 PFU/mouse twice at 2 weeks apart. At 5 to 10 weeks post-secondary immunization, sham-immunized and CJ2-D2-immunized mice were mock infected or infected with wild-type HSV-2 strain 186 s.c. at a dose of 1 × 10^7 PFU/mouse. Splenocytes were isolated from individual groups of mice (n = 3) on day 4 or 5 after wild-type HSV-2 infection. The CD4+ and CD8+ T cell enzyme-linked immunospot (ELISPOT) assays were carried out as previously described (41). In brief, CD4+ and CD8+ T cells were isolated from splenocytes using Dynal mouse CD4+ and CD8-negative isolation kits and seeded in quadruplicate in a 96-well filtration plate precoated with anti-mouse gamma interferon (IFN-γ)-specific monoclonal antibody (AN18) at 7.5 × 10^5 or 1.5 × 10^6 cells/well with no further stimulation.
with HSV-2 antigens. After incubation at 37°C for 20 h, wells were washed, reacted with biotinylated IFN-γ-specific monoclonal antibody (R4-62A; Mabtech) at room temperature, and incubated with streptavidin-alkaline phosphatase (Mabtech). The IFN-γ spot-forming cells were detected by addition of the 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCP-NBT) substrate. Spots were counted in a dissecting microscope, and the number of IFN-γ spot-forming cells (SFC) was expressed as the mean ± standard error of the mean (SEM) per million CD4+ or CD8+ T cells.

Quantitative real-time PCR. The lower lumbar and sacral part of the spinal column, including the spinal cord and dorsal root ganglia (DRG), were collected 16 days after boost immunization or 21 days after intravaginal challenge with 5 × 10^5 PFU of HSV-2 strain G from 9 or 10 mice that had been immunized with either CJ2-gD2 or CJ9-gD. The spinal column was cut into 4 pieces, and each piece was kept separately in 0.5 ml of normal growth medium and stored at −80°C for further processing. Total DNA was isolated from each dorsal root ganglion using the DNeasy tissue kit (Qiagen, Santa Clarita, CA) and suspended in 400 μl AE buffer (Qiagen). The presence of HSV-2 DNA was quantified by real-time PCR (Applied Biosystems 7300 real-time PCR system) with 100 ng of ganglion DNA and primers specific to the HSV DNA polymerase (Forward, 5′ GCT CGA GTG CGA AAA AAC GTT C; reverse, 5′ GT1032 CGG GCC GCT CGG CTA AC) as previously described (7). The minimum copies of HSV-2 viral DNA that could be reliably detected were 1 copy per reaction.

Statistical analysis. For statistical analysis, unpaired Student’s t tests were performed. Results were considered to be statistically significant when the P value was less than 0.05.

RESULTS

Construction of CJ2-gD2. As the first step in generating a gD2- and UL9-C535C-expressing dominant-negative and replication-defective HSV-2 recombinant virus, we constructed a lacZ gene-containing HSV-2 ICP0 deletion mutant, N2-lacZ (Fig. 1B). We show that, similar to the HSV-1 ICP0 null mutant 7314 (74), the plaque-forming efficiency of N2-lacZ on U2OS cells was 425-fold higher than that in Vero cells, indicating that the cellular activity in U2OS cells can also functionally substitute for HSV-2 ICP0. Compared with that of wild-type HSV-2, the replication efficiency of N2-lacZ in Vero cells was reduced more than 600-fold at an MOI of 0.1 PFU/cell (data not shown). Consistent with this finding, intravaginal inoculation of N2-lacZ at 5 × 10^5 PFU/mouse led to no local or systemic illness, while mice infected with 1 × 10^6 PFU/mouse of wild-type HSV-2 developed severe genital herpes, and all died by day 11 postinfection (data not shown). These results indicate that, similar to the case with HSV-1 ICP0 (38), deletion of HSV-2 ICP0 significantly impairs the ability of the virus to initiate acute infection in vivo.

Aiming to maximize levels of gD2 expression by a dominant-negative and replication-defective HSV-2 viral recombinant, we constructed an ICP0 locus-specific shuttle plasmid, p02lacZTO-gD2.C535C. CJ2-gD2 is a fourth-round purified viral recombinant isolated from U2CEP4R-11 cells cotransfected with the linearized p02lacZTO-gD2.C535C and infectious N2-lacZ viral DNA. CJ2-gD2 yields uniform white plaques in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in U2CEP4R-11 cells in the absence of tetracycline and no plaques in the presence of tetracycline, indicating the recombinational replacement of both copies of the lacZ gene of N2-lacZ by the UL9-C535C- and gD2-encoding DNA sequence of p02lacZTO-gD2.C535C. Thus, unlike CJ9-gD, which contains a single copy of the inserted HSV-1 gD gene driven by CMVTO at the HSV-1 UL9 locus (41), CJ2-gD2 contains 2 copies of the gD2 gene under the control of ICP4TO at the ICP0 locus in an opposite orientation of the UL9-C535C-driving CMVTO (Fig. 1B). Due to the lack of the ICP0 gene and the demonstrated potent inhibitory effect of UL9-C535C on viral DNA replication, CJ2-gD2 cannot replicate in Vero cells and has to be propagated in the tetR-expressing ICP0-complementing U2CEP4R11 cells in the absence of tetracycline. We plaque assayed 6.65 × 10^6 PFU of CJ2-gD2 in 100-mm dishes of Vero cells at an MOI of ~0.1 PFU/cell and detected no viral plaque on fully confluent monolayers of Vero cells at day 4 postinfection, demonstrating that the plaque-forming efficiency of CJ2-gD2 in Vero cells is reduced at least 6.65 × 10^2-fold compared with that in U2CEP4R11 cells.

The replacement of both copies of the lacZ gene with the gD2/gD2UL9-C535C-containing DNA fragment in CJ2-gD2 at the ICP0 locus was further confirmed by PCR analysis of p02lacZTO-gD2.C535C plasmid DNA and CJ2-gD2 viral DNA with the following: (i) an ICP0 sequence-specific primer (ICP0-1) located at 377 bp upstream of the stop codon of the ICP0 ORF (Fig. 1A) and a gD2-specific primer positioned 2 bp downstream of the gD2 stop codon, which should yield a 916-bp DNA fragment (Fig. 1C, lanes 1 and 2), and (ii) an ICP0 sequence-specific primer positioned at 204 bp upstream of the start codon of the ICP0 ORF and a UL9-C535C sequence-specific primer located immediately downstream of the UL9-C535C stop codon, which should yield a 975-bp DNA fragment (Fig. 1C, lanes 4 and 5). Given the sizes of a single PCR DNA fragment detected in lanes 1 and 2 and lanes 4 and 5 and that no DNA fragment was detected when the same sets of primers were used for PCR analysis of N2-lacZ viral DNA (Fig. 1C, lanes 3 and 6), while PCR analysis of the same N2-lacZ viral DNA with the ICP0-1 primer and a lacZ-gene-specific primer positioned at 1,978 bp downstream of the start codon of the lacZ gene gave rise to a predicted DNA fragment of 1,678 bp (Fig. 1C, lane 7), the results presented in Fig. 1C confirm the predicted genome structure of CJ2-gD2.

CJ2-gD2 expresses high levels of gD2 and UL9-C535C in infected Vero cells. To examine expression of gD2 and UL9-C535C from tetO-bearing HSV-1 ICP4TO and CMVTO, respectively, Vero cells were infected with wild-type HSV-2, N2-lacZ, N2-C535C, and CJ2-gD2, and infected cell extracts were first prepared at 9 h postinfection, followed by Western blot assays with an HSV-1/2 ICP27 monoclonal antibody, an UL9 polyclonal antibody, and a gD1 polyclonal antibody (R45). Given that, like gD2, gB2 is one of the major targets for neutralizing antibody as well as T-cell responses and is a γ1 gene product, infected cell proteins were also probed with a gB-specific monoclonal antibody. Figure 2A shows that CJ2-gD2 and N2-C535C expressed levels of the HSV-2 immediate-early protein ICP27 similar to those expressed by wild-type HSV-2 and N2-lacZ. While significant amounts of UL9-C535C were detected in CJ2-gD2- and N2-C535C-infected cells, little gD2 or gB2 was detected in N2-C535C-infected cells. In contrast to N2-C535C infection, infection of Vero cells with CJ2-gD2 led to highly efficient expression of gD2 at levels similar to that of wild-type HSV-2 infection. Similar to N2-C535C, CJ2-gD2 expresses very low levels of gB2. The results also demonstrate that, as with the HSV-1 ICP0 null mutant 7134 (74), deletion of HSV-2 ICP0 in N2-lacZ greatly reduces gD2 expression at the indicated MOI. Consistent with our previous observation that the level of expression of HSV-1 UL9 from its
FIG. 3. Regulation of gD2 and UL9-C535C expression by tetR in CJ2-gD2-infected VCEP4R-28 cells. VCEP4R-28 cells were seeded at 5 × 10^5 cells per 60-mm dish. At 40 h after seeding, cells in duplicate were either mock infected or infected with wild-type HSV-2 or CJ2-gD2 at an MOI of 10 PFU/cell in either the presence or absence of tetracycline. Infected cell extracts were prepared at 9 h postinfection followed by immunoblotting (Western blotting) with polyclonal antibodies against HSV gD and UL9 and a monoclonal antibody specific for ICP27.

authentic HSV-1 early promoter was too low to be detected (72), we detected no wild-type HSV-2 UL9 protein in cell extracts prepared from cells infected by these four different viruses. We observed that levels of UL9-C535C expressed in CJ2-gD2-infected cells were consistently higher than those in cells infected by N2-C535C, suggesting that the HSV VP16-responsive elements, TAATGARAT, present in the described HSV-1 ICP4 promoter (74) 5′ to the truncated tetO-bearing hCMV immediate-early promoter in CJ2-gD2 (Fig. 1) can lead to enhanced expression of UL9-C535C. The results in Fig. 2B show that while comparable levels of gD2 and ICP27 were detected in CJ2-gD2 and wild-type HSV-2-infected cells at 9 h postinfection, CJ2-gD2 expressed much lower levels of gD2 than wild-type HSV-2 at 6 h postinfection. No significant difference was seen in levels of gD2 at 4 h postinfection among cells infected with wild-type HSV-2, N2-C535C, and CJ2-gD2. Importantly, the results showed that the kinetics of de novo expression of gD2 in CJ2-gD2-infected cells was similar to that of ICP27. Although, as with CJ2-gD2, expression of ICP27 increased significantly from 4 h to 9 h postinfection in N2-C535C-infected cells, little difference in gD2 expression was seen in N2-C535C-infected cells at 4 h, 6 h, and 9 h postinfection.

To determine whether insertion of 2 copies of the gD2 gene under the control of HSV-1 ICP4TO at the ICP0 locus renders more-efficient expression of gD2 than CJ9-gD does with gD1, we carried out an additional experiment in which Vero cells were infected with wild-type HSV-1, CJ9-gD, wild-type HSV-2, or CJ2-gD2 at an MOI of 10 PFU/cell. Infected cell extracts were prepared at 9 h postinfection and analyzed by Western blot analysis with the gD1 polyclonal antibody (R45), which is cross-reactive to gD2, and an ICP27 monoclonal antibody that reacts with HSV-1/2 ICP27. Figure 2C showed that while higher levels of gD were detected in wild-type HSV-1-infected cells than in cells infected with wild-type HSV-2, levels of gD detected in CJ9-gD-infected cells were markedly lower than those in cells infected by CJ2-gD2. Comparable levels of ICP27 were detected among cells infected with wild-type HSV-1, CJ9-gD, wild-type HSV-2, and CJ2-gD2. Taken together, the data indicate that CJ2-gD2 expresses gD2 more efficiently than gD1 is expressed by CJ9-gD.

To demonstrate that UL9-C535C and gD2 expressed in CJ2-gD2-infected Vero cells are indeed under the control of the tetO-bearing promoters, we next infected a stable tetR-expressing Vero cell line, VCEP4R-28 cells (75), with wild-type HSV-2 and CJ2-gD2 at an MOI of 10 PFU/cell in the absence or presence of tetracycline. Proteins from infected cells were harvested at 9 h postinfection and analyzed by Western blotting. As shown (Fig. 3), although similar levels of ICP27 were detected in wild-type HSV-2- and CJ2-gD2-infected VCEP4R-28 cells only when tetracycline was present, and significantly higher levels of gD2 were detected in the presence of tetracycline than in its absence.

Inhibition of wild-type HSV-2 replication by CJ2-gD2. Given the previous demonstration that overexpression of UL9-C535C by HSV-1 CJ83193 can lead to a significantly reduction in wild-type HSV-2 viral replication in coinfected cells (73), we then tested the dominant-negative effect of UL9-C535C encoded by CJ2-gD2 on the replication of wild-type HSV-2 by the coinfection assay (Fig. 4). Panel A showed that coinfection of Vero cells with CJ2-gD2 at an MOI of 5 PFU/cell and wild-type HSV-2 at an MOI of 2 PFU/cell led to a nearly 500-fold decrease in wild-type HSV-2 production compared with results for cells singly infected with wild-type HSV-2 at the same MOI, regardless of whether the virus titers were determined in Vero

FIG. 4. trans-Dominant-negative effect of CJ2-gD2 on replication of wild-type HSV-2. (A) Vero cells in triplicate were infected with either wild-type HSV-2 strain 186 at an MOI of 2 PFU/cell, 186 at an MOI of 2 PFU/cell and CJ2-gD2 at an MOI of 5 PFU/cell, or 186 at an MOI of 2 PFU/cell and N2-lacZ at an MOI of 5 PFU/cell. (B) Vero cells were either singly infected with wild-type HSV-2 at an MOI of 5 PFU/cell, coinfected with 186 and CJ2-gD2 at an MOI of 5 PFU/cell each, or singly infected with 186 at an MOI of 15 PFU/cell and coinfected with 186 at an MOI of 15 PFU/cell and CJ2-gD2 at an MOI of 5 PFU/cell. Infected cells were harvested at 18 h postinfection, and viral titers were determined on Vero cell monolayers. Viral titers are expressed as means ± SD. Numbers at the top of the graph indicate the fold reduction in wild-type virus yield between single infection and coinfection.
cells or in U2CEP4R11 cells (data not shown). Little reduction in wild-type virus yield was detected when a similar coinfection experiment was performed with N2-lacZ.

To further examine the potency of CJ2-gD2 in inhibiting the replication of wild-type HSV-2, we carried out coinfection experiments with wild-type HSV-2 and CJ2-gD2 at MOI ratios of 1:1 and 3:1, respectively. CJ2-gD2 was effective in preventing wild-type HSV-2 infection under both conditions (Fig. 4B), leading to 151- and 94-fold reduction in wild-type virus synthesis at the indicated coinfection ratios compared with results for cells singly infected with the wild-type HSV-2 at MOIs of 5 PFU/cell and 15 PFU/cell, respectively. Collectively, these results demonstrate that UL9-C535C expressed by CJ2-gD2 can exert a potent trans-inhibitory effect on the replication of wild-type HSV-2 in coinfected cells.

CJ2-gD2 is avirulent following intracerebral injection in mice. Neurovirulence is one of the hallmarks of HSV infection. To determine the ability of CJ2-gD2 and N2-C535C to replicate in the central nervous system (CNS), female BALB/c mice were randomly assigned to five groups of 8 mice each. CJ2-gD2 was effective in preventing wild-type HSV-2 infection under both conditions (Fig. 4B), leading to 151- and 94-fold reduction in wild-type virus synthesis at the indicated coinfection ratios compared with results for cells singly infected with the wild-type HSV-2 at MOIs of 5 PFU/cell and 15 PFU/cell, respectively. Collectively, these results demonstrate that UL9-C535C expressed by CJ2-gD2 can exert a potent trans-inhibitory effect on the replication of wild-type HSV-2 in coinfected cells.

**CJ2-gD2 is avirulent following intracerebral injection in mice.** Neurovirulence is one of the hallmarks of HSV infection. To determine the ability of CJ2-gD2 and N2-C535C to replicate in the central nervous system (CNS), female BALB/c mice were randomly assigned to five groups of 8 mice each. CJ2-gD2 and N2-C535C were directly inoculated into the brain of each mouse at the left frontal lobe at 2.5 × 10^6 PFU per mouse. Morbidity and mortality were monitored for 35 days. Given that the LD_{50} of wild-type HSV-2 strain 186 is around 10 PFU in female BALB/c mice after intravitreal injection (39), a group of mice was also inoculated with wild-type HSV-2 at a dose of 25 PFU/mouse. As an additional control, mice in the fifth group were inoculated with N2-lacZ at 1 × 10^6 PFU/mouse. Figure 5 shows that, like inoculation of mice with DMEM, intracerebral inoculation of mice with CJ2-gD2 and N2-C535C at a dose of 2.5 × 10^6 PFU caused no signs of neurovirulence during a 35-day follow-up, while all mice inoculated with wild-type HSV-2 at a dose of 25 PFU/mouse (a 100,000-fold-lower dose than that given to mice inoculated with CJ2-gD2) died by day 10 postinoculation. Although 100% of mice inoculated with N2-lacZ survived, all mice exhibited signs of encephalitis, including roughened fur, hunched posture, ataxia, and/or anorexia.

**Induction of HSV-2-specific neutralizing antibodies and a gD2-specific antibody response in mice immunized with CJ2-gD2.** The ability of CJ2-gD2 to elicit anti-HSV-2-specific neutralizing antibodies was determined in mice immunized with CJ2-gD2 at a dose of 2 × 10^6 PFU. As controls, groups of mice were also immunized with N2-C535C or CJ9-gD at the same dose. As shown (Fig. 6A), the HSV-2-specific neutralization antibody titer in mice immunized with CJ2-gD2 was on average 500, which is 3-fold higher than that of mice immunized with N2-C535C (P = 0.015) and is comparable to the cross-reactive HSV-2 neutralizing antibody response induced in CJ9-gD-immunized mice (P = 0.28). No specific antibody titers against HSV-2 were detected in mock-vaccinated mice at a 1:10 dilution. The finding that CJ2-gD2 failed to elicit a more-effective anti-HSV-2-specific neutralizing antibody response than CJ9-gD likely reflects the previously documented suppressive activity encoded by HSV-2, which significantly reduces the...
ability of HSV-2 to elicit a humoral immune response in HSV-2-infected mice compared with results for mice infected by HSV-1 (29, 43).

Figure 6B showed that while similar levels of gD-specific antibody response were detected between mice immunized with CJ2-gD2 and CJ9-gD when the respective immunoprecipitated gD2 complexes were probed with anti-gD1 antibodies (R45), levels of anti-gD-specific antibodies in mice immunized with CJ2-gD2 were significantly higher than in mice immunized with N2-C535C and mock-immunized control mice. Taken together, the results presented in Fig. 6 show that high-level expression of gD2 by CJ2-gD2 led to increased efficacy in eliciting anti-gD2 antibody as well as anti-HSV-2-specific neutralizing antibody responses compared with results for N2-C535C.

Induction of HSV-2-specific T-cell response in immune mice immunized with CJ2-gD2. To evaluate the effectiveness of CJ2-gD2 immunization in eliciting an HSV-2-specific T-cell response, we carried out the recall experiment to examine the memory T-cell responses in CJ2-gD2-immunized mice following s.c. immunization at a dose of 2 × 10^6 PFU/mouse in a volume of 30 μL. Given the demonstration that the HSV-2-specific CD8^+ T cells in the mouse spleen peaked on day 6 after primary HSV-2 infection (19) and the study of Milligan and Bernstein (46), we first mock challenged or challenged sham-vaccinated and CJ2-gD2-vaccinated mice with wild-type HSV-2 at 9 to 10 weeks post-boost immunization, followed by IFN-γ ELISPOT assays with CD4^+ and CD8^+ T cells isolated from individual groups of mice (n = 3) on day 5 postchallenge (Fig. 7A and B). CJ2-gD2-vaccinated mice challenged with wild-type HSV-2 had a 4.8-fold increase in IFN-γ-positive CD4^+ T cells compared with the mock-infected CJ2-gD2-immune mice (P < 0.0001). More significantly, the number of IFN-γ-secreting CD4^+ T cells detected for HSV-2-infected CJ2-gD2-immunized mice was 18-fold more than that for HSV-2-infected sham-vaccinated mice (P < 0.0001). No IFN-γ-positive CD8^+ T cells were detected in mock-infected sham-vaccinated mice under identical conditions. These findings show that immunization with CJ2-gD2 elicits strong memory CD4^+ T cell response.

While there was a greater than 2-fold increase in IFN-γ-secreting CD8^+ T cells in CJ2-gD2-vaccinated mice compared with results for the sham-vaccinated controls, similar numbers of IFN-γ-secreting CD8^+ T cells were detected in the spleens of HSV-2-infected sham-vaccinated mice and HSV-2-infected CJ2-gD2-vaccinated mice on day 5 postinfection (Fig. 7B). We thus carried out the second set of recall experiments, in which sham-vaccinated and CJ2-gD2-vaccinated mice were either mock infected or infected with wild-type HSV-2 on day 5 postchallenge. CD4^+ and CD8^+ ELISPOT assays were performed on day 4 postinfection (Fig. 7C and D). An 8.6- and 5.7-fold increase in IFN-γ-secreting CD4^+ and CD8^+ T cells, respectively, was detected in wild-type HSV-2-infected CJ2-gD2-immune mice compared with mock-infected CJ2-gD2-immune mice (for CD4^+ T cells, P = 0.035; for CD8^+ T cells, P = 0.01). Moreover, IFN-γ-secreting CD4^+ and CD8^+ T cells were 8- and 9.5-fold higher, respectively, in wild-type HSV-2-infected CJ2-gD2-immune mice compared with wild-type HSV-2-infected sham-vaccinated mice (for CD4^+ T cells, P = 0.036; for CD8^+ T cells, P = 0.01). Collectively, these studies demonstrate that immunization with CJ2-gD2 can elicit robust HSV-2-specific memory CD4^+ and CD8^+ T-cell responses, which can be efficiently recalled during HSV-2 infection.

Protection against HSV-2 genital infection and disease in immunized mice. Five to 6 weeks after the initial immunization, mice were challenged intravaginally with HSV-2 strain G at 50 LD₅₀ (5 × 10^6 PFU/mouse). Vaginal swabs were taken on days 1, 2, 3, 5, and 7 after challenge. Mice were observed during a 21-day follow-up period for the incidence of genital and disseminated HSV-2 disease. HSV-2 titers were reduced more than 200-fold on day 1 (P < 0.001) and 130-fold on day 2 (P < 0.0001) in mice immunized with CJ2-gD2 (n = 9) compared with those of sham-immunized controls (n = 10) (Fig. 8A). Although there was no significant difference in HSV-2 shedding between CJ2-gD2- and N2-C535C-vaccinated mice (n = 10) on days 1, 2, and 3 postchallenge, CJ2-gD2 was more effective than CJ9-gD in reducing HSV-2 shedding on days 1 (P = 0.03), 2 (P = 0.025), and 3 (P < 0.007). By day 5 postchallenge, little or no HSV-2 virus was detected in mice vaccinated with CJ2-gD2, N2-C535C, or CJ9-gD, whereas all sham-vaccinated mice continued to shed virus at an average yield of more than 5 × 10^6 PFU/ml. No HSV-2 was detected in the vaginal swabs collected on day 7 postchallenge in three vaccinated groups. In a separate experiment, virus shedding was eliminated by day 5 postchallenge in CJ2-gD2-immunized mice; in contrast, HSV-2 was detected in 5 out of 7 N2-C535C-vaccinated mice.

Mice immunized with CJ2-gD2 were completely protected...
from development of local genital lesions and exhibited no signs of systemic disease after challenge with wild-type HSV-2 (Fig. 8B). All mock-immunized mice developed severe genital lesions and succumbed to wild-type HSV-2 infection by day 11 postchallenge (Fig. 8C). Although immunization with N2-CS53C and CJ9-gD protected mice against lethal challenge by wild-type HSV-2, 20% and 30% of mice experienced a transient local genital disease, and a third mouse had signs of systemic illness and died on day 14 postchallenge. Similarly, 3 of 7 N2-CS53C-immunized mice (43%) had local genital disease (score = 1). No signs of local or systemic herpetic disease were seen in CJ2-gD2-immunized mice.

Given the demonstrated vaccine efficacy of CJ2-gD2 at a dose of 2 × 10⁶ PFU/mouse, we determined whether vaccination of mice with a lower dose of CJ2-gD2 (5 × 10⁵ versus 2 × 10⁶ PFU/mouse) would be effective. Although CJ2-gD2 was again more effective than N2-CS53C in preventing HSV-2 genital challenge, its efficacy in preventing herpetic disease at a dose of 5 × 10⁵ PFU was only 42% (Table 1). Among 7 mice vaccinated with 5 × 10⁵ PFU of CJ2-gD2, two died by day 19 postchallenge. In this experiment, 3 of 7 mice immunized with 2 × 10⁶ PFU of N2-CS53C developed local and systemic illness and succumbed to disease. Immunization with UV-inactivated CJ2-gD2 at a dose of 2 × 10⁶ PFU/mouse was insufficient in protection against wild-type HSV-2 genital infection, and under these conditions, 4 of 6 mice developed genital disease and 2 mice died by day 14 postchallenge (data not shown). Taken together, these studies demonstrate that CJ2-gD2 is a vaccine superior to N2-CS53C and CJ9-gD in protecting mice against genital disease following intravaginal challenge with wild-type HSV-2, and de novo viral gene expression is required for CJ2-gD2 to elicit fully protective immunity. Last, the effectiveness of CJ2-gD2 in eliciting protective immunity against wild-type HSV-2 genital disease in mice is dose dependent.

**Latent CJ2-gD2 DNA is not detectable in the dorsal root ganglia of mice following s.c. immunization.** We have previously shown that no CJ9-gD viral DNA could be detected in the mouse trigeminal ganglia after intranasal and ocular inoculation (41) and in the dorsal root ganglia (DRG) after s.c. immunization (6). To investigate whether CJ2-gD2 can establish latent infection in immunized mice, DRG DNA plus spinal cord DNA were isolated from sham-immunized mice and CJ2-gD2-immunized mice at a dose of 2 × 10⁶ PFU at 2 weeks after boost immunization. Additionally, DRG DNA was also isolated from CJ2-gD2- and CJ9-gD-immunized mice following challenge with wild-type HSV-2, described in the legend for Fig. 8. Using quantitative real-time PCR analysis, no CJ2-gD2 viral DNA was detected in DRG DNA isolated from CJ2-gD2-immunized mice (Fig. 9). The average amount of latent viral DNA load of challenge HSV-2 in CJ2-gD2-immunized mice was 1,435 copies/mouse, 10-fold lower than that detected in CJ9-gD-immunized mice (P = 0.24). Since all sham-vaccinated mice succumbed to HSV-2 by day 11 postchallenge, the latent viral DNA load in sham-vaccinated mice following challenge

### TABLE 1. Vaccine efficacy against herpetic disease in mock-immunized and immunized mice following intravaginal challenge with wild-type HSV-2

<table>
<thead>
<tr>
<th>Expt no. (n)</th>
<th>% protection with vaccine and dose (PFU/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CJ2-gD2</td>
</tr>
<tr>
<td>1 (9–10)</td>
<td>0 (10) 100  ND  70</td>
</tr>
<tr>
<td>2 (7–8)</td>
<td>0 (8) 100  ND  57</td>
</tr>
<tr>
<td>3 (7)</td>
<td>0 (7) 100  ND  57</td>
</tr>
<tr>
<td></td>
<td>a no. of mice challenged.</td>
</tr>
</tbody>
</table>
with HSV-2 could not be analyzed in the described experiments.

**DISCUSSION**

Using a new strategy, we have constructed an HSV-2 glycoprotein D (gD2)-expressing dominant-negative and replication-defective HSV-2 recombinant, CJ2-gD2, which contains 2 copies of the gD2 gene at the HSV-2 ICP0 locus driven by the tetO-bearing HSV-1 major immediate-early ICP4 promoter, while the gene encoding UL9-C535C is under the control of the tetO-containing hCMV major immediate-early promoter in an orientation opposite that of the inserted gD2 gene. Because the native ICP4 promoter is subject to downregulation by ICP4 due to the presence of the ICP4-specific DNA binding sequence spanning the transcription start site of the ICP4 gene (14, 55), we designed the tetO-containing ICP4 promoter, consisting of the HSV-1 ICP4 promoter sequence from base pair −377 to base pair −19 relative to the transcriptional start site of the ICP4 gene. We hypothesized that the absence of the ICP4 binding site in the described tetO-containing ICP4 promoter would eliminate downregulation of the gD2 gene from the tetO-bearing ICP4 promoter by HSV-2 ICP4 following CJ2-gD2 infection. We demonstrate that although CJ2-gD2 is replication defective in Vero cells, it expresses gD2 as efficiently as wild-type HSV-2 in Vero cells and expresses significantly more gD2 than CJ9-gD does for gD1. We showed further that *de novo* expression of UL9-C535C by CJ2-gD2 exerted a potent trans-Inhibitory effect on the replication of wild-type HSV-2 in coinfected cells, leading to an ~150-fold reduction in wild-type HSV-2 virus synthesis in cells coinfected with CJ2-gD2 at an MOI ratio of 1:1 of wild-type HSV-2 to CJ2-gD2. This demonstrated that a potent trans-Inhibitory effect of CJ2-gD2 on wild-type HSV-2 viral replication should offer a significant safety advantage over the conventional replication-defective HSV-2 vaccine approach in clinical applications, especially as a therapeutic vaccine in individuals who have been latently infected with HSV.

The effectiveness of CJ2-gD2 as a vaccine against HSV-2 infection was examined in a mouse model of HSV-2 intravaginal infection. Immunization with CJ2-gD2 induced more-efficient anti-gD-specific antibody and anti-HSV-2 neutralizing antibody responses than did a non-gD2-expressing dominant-negative and replication-defective HSV-2 recombinant, N2-C535C. Similar to CJ9-gD, CJ2-gD2 is capable of eliciting both HSV-2-specific CD4 + and CD8 + T-cell responses. Moreover, using recall experiments, we demonstrate that the magnitude of HSV-2-specific CD4 + and CD8 + T-cell responses in HSV-2-infected CJ2-gD2-immunized mice is significantly greater than that in HSV-2-infected sham-immunized mice on day 4 postchallenge (Fig. 7). The HSV-2-specific CD4 + T-cell recall response could also be detected in wild-type HSV-2-infected CJ2-gD2-immune mice on day 3 after wild-type HSV-2 infection (data not shown). Collectively, the results indicate that the onset of the HSV-2-specific CD4 + T-cell response in CJ2-gD2-immune mice is at least 2 days faster than that of the primary CD4 + T-cell response induced in the wild-type-HSV-2-infected sham-immunized control.

Mice immunized with 2 × 10^6 PFU/mouse of CJ2-gD2 were completely protected from development of local genital disease and exhibited no signs of systemic illness after challenge with wild-type HSV-2 at a dose of 50 LD_{50} (Fig. 8 and Table 1). In contrast, immunization with N2-C535C or CJ9-gD at the same dose provided 65 to 67% protection against HSV-2 genital disease (Table 1). Thus, these studies demonstrate that CJ2-gD2 is a more effective vaccine than N2-C535C and CJ9-gD in protecting mice against genital disease following intravaginal challenge with wild-type HSV-2. Last, we find that immunization with CJ2-gD2 offers full protection against HSV-2 genital disease when mice are challenged 5 months after vaccination with 50 LD_{50} of wild-type HSV-2 (data not shown).

Like CJ9-gD (41), CJ2-gD2 is avirulent following intracerebral injection and cannot establish a detectable latent infection following s.c. inoculation. Thus, CJ2-gD2 represents a unique class of HSV-2 recombinant viral vaccine that is both replication defective and dominant negative and expresses high levels of gD2 independent of viral replication. It should be noted that because of the nature of its replication defectiveness, CJ2-gD2 retains the capability of expressing a wide array of HSV-2 antigens, which are known targets of the CD8 + T-cell response in HSV-2-seropositive persons. These antigens include the viral immediate-early antigens ICP4, ICP27, and ICP22, and the early antigens UL23, UL29, UL39, and UL50, and the early-late antigens UL18, UL19, UL49, UL55, and US8 (26, 45, 54). The Western blot analyses shown in Fig. 2 illustrate that CJ2-gD2 expresses the HSV-2 viral immediate-early gene product ICP27 as efficiently as wild-type HSV-2.

HSV evolves several different strategies to evade host immune responses (16, 18, 21, 24, 25, 49, 60, 76). ICP0 and ICP47 are the two HSV immediate-early proteins directly involved in host immune evasion following viral infection. While ICP47 inhibits major histocompatibility complex (MHC) class I peptide loading on the surface of infected cells (21, 25, 76), ICP0 plays a key role in suppressing IFN-induced inhibition of viral infection (18, 49). A recent study demonstrated that the ICP0
gene also encodes an immunosuppressive function that inhibits the Toll-like receptor 2 (TLR2)/TLR9-induced inflammatory cytokine response to HSV infection (12). Thus, an ICPO null mutant-based HSV recombinant viral vaccine candidates, such as CJ2-gD2, would likely be more effective in inducing host innate immunity, leading to enhanced vaccine efficacy against HSV infection compared with that of an HSV recombinant viral vaccine expressing functional ICPO. Collectively, given the demonstrated preclinical immunogenicity and its unique safety profiles, CJ2-gD2 possesses several advantages (including being dominant negative, overexpressing gD2, and lacking ICPO-mediated suppression of IFN-induced inhibition of viral infection and TLR signaling) over traditional replication-defective recombinant viral vaccines and the monovalent gD2 subunit vaccine in protecting against HSV-2 genital infection and disease in humans. Whether CJ2-gD2 is indeed more effective than the gD2/AS04 subunit vaccine in protection against HSV-2 genital infection and disease in naive and HSV-1-seropositive guinea pigs is currently being investigated.

ACKNOWLEDGMENTS

This work was supported by the Partners Innovation Fund, Partners HealthCare, intramural funds from the Department of Surgery, Brigham and Women’s Hospital, and Public Health Service grant 5R01AI05088 from the National Institutes of Health.

REFERENCES


Downloaded from http://jvi.asm.org/ on October 2, 2017 by guest


