Construction and Characterization of a Replication-Defective Herpes Simplex Virus 2 ICP8 Mutant Strain and Its Use in Immunization Studies in a Guinea Pig Model of Genital Disease

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A replication-defective mutant of herpes simplex virus 2 (HSV-2) was engineered by replacing the ICP8 gene of HSV-2 strain 186 with an ICP8–lacZ fusion gene from the herpes simplex virus 1 (HSV-1) HD-2 mutant strain. The resulting virus, HSV-2 5BlacZ, is defective for growth in Vero cells but is capable of growth in a cell line that expresses HSV-1 ICP8. In Vero cells, the mutant virus is defective for DNA synthesis but is able to express many viral proteins at levels similar to those of wild-type virus, including several of the late kinetic class. SDS–PAGE and Western blot analysis demonstrated the expression of glycoproteins B and D by 5BlacZ in Vero cells. Initial studies have shown that immunization with 5BlacZ protects guinea pigs from intravaginal HSV-2 challenge. Immunized animals had less severe genital skin disease and reduced replication of the challenge virus in the genital tract during primary infection and reduced episodes of recurrent disease. Thus, HSV-2 ICP8 shows gene regulatory properties similar to those of HSV-1 ICP8, and this HSV-2 ICP8 mutant virus shows a phenotype similar to those of HSV-1 ICP8 mutant strains. Replication-defective mutants of HSV-2 offer a potential vaccine approach for immune intervention against HSV-2 genital disease and latent infection.

INTRODUCTION

Productive infection by herpes simplex virus proceeds through a cascade of events in the order of immediate early (IE) gene expression, early (E) gene expression, viral DNA replication, and late (L) gene expression. The expression of E proteins requires the IE protein ICP4 and, in some cases, the IE ICP27 protein. The expression of viral late proteins requires IE proteins ICP4 and ICP27 and viral DNA replication. Seven HSV-1 gene products, including the viral DNA polymerase (UL30), the polymerase accessory factor (UL42), the origin-binding protein (UL9), the single-stranded DNA-binding protein (UL29 or ICP8), and the helicase–primase complex (UL5, UL8, and UL52) are required for viral DNA replication (Challberg, 1986; Knipe, 1989; Weller, 1991). The ICP8 protein also plays a role in regulating viral gene expression in that ICP8 ts mutants show overexpression of viral gene products at the nonpermissive temperature (Godowski and Knipe, 1985, 1986), and a dominant mutant form of ICP8 can inhibit late gene transcription (Chen and Knipe, 1996; Gao and Knipe, 1991). A notable phenotype of viral mutants bearing ts and deletion mutations in the ICP8 gene is the expression of substantial amounts of late viral proteins in the absence of viral DNA replication. Therefore, HSV-1 ICP8 plays a role in suppressing transcription of viral late genes from input or parental viral DNA genomes. This novel phenotype of HSV-1 ICP8 mutants has been exploited to provide replication-defective mutant viruses for vaccine strains that can express a broad spectrum of viral proteins, including late proteins, in the absence of viral DNA replication. The HSV-1 ICP8 mutant d301 has been used to protect against lethal challenge infection (Nguyen et al., 1992) and against establishment of latent infection in a mouse corneal challenge model (Morrison and Knipe, 1994, 1996). The lack of viral DNA synthesis in cells infected with a vaccine virus would provide greater safety in preventing viral DNA exposure to the host and the cellular mutagenic events that have been associated with viral DNA replication, including cellular DNA amplification (Heilbronn and Zur Hausen, 1989) and chromosomal aberrations (Chenet-Monte et al., 1986).

Herpes simplex virus 2 (HSV-2) is responsible for most cases of genital herpes disease. Pregnant women with genital herpes are at risk of transmitting the virus to their newborns at parturition, which may result in severe, potentially fatal neurologic or disseminated disease in the newborn. Several vaccines against HSV-2, including subunit glycoprotein preparations (Meignier et al., 1987; Mertz et al., 1990; Stanberry et al., 1987), have been tested for their ability to prevent genital disease in animal models as well as in clinical trials. In addition, viral vectors such as human adenovirus expressing HSV-2 glycoprotein B (gB) (McDermott et al., 1989) and vaccinia virus expressing HSV-1 or HSV-2 glycoprotein D (gD) (Aurelian et al., 1991) have been tested in mice and, in the latter case, in guinea pigs also. Recombinant HSV-1 viruses...
expressing HSV-2 glycoproteins D, E, G, and part of I have been tested in several animal models including guinea pigs and owl monkeys (Meignier et al., 1988a, 1988b, 1990). This vaccine has also been tested in clinical trials, the results of which have been reviewed recently (Stanberry, 1996). In addition, an HSV-2 gH gene deletion mutant virus that undergoes a single round of replication in infected cells has been tested in guinea pigs for its ability to protect against an HSV-2 challenge (Boursnell et al., 1997). More recently, nucleic acid vaccines expressing HSV-2 gD have been evaluated in mice or guinea pig models of genital herpes (Bourne et al., 1996; Ghiassi et al., 1995; Kriessel et al., 1996).

HSV-2, although less well studied than HSV-1, is highly homologous to HSV-1 and shows many common gene regulatory patterns. The HSV-2 ICP8 is also required for viral DNA synthesis (Spang et al., 1983) and can substitute for HSV-1 ICP8 (Conley et al., 1981; Morse et al., 1978). However, nothing is known about the effects of HSV-2 ICP8 on viral gene expression. To determine if HSV-2 ICP8 exerted regulatory effects similar to those of the HSV-1 protein and to determine if an HSV-2 ICP8 mutant virus could provide a safe, live virus vaccine against genital herpes, we have engineered an HSV-2 ICP8 mutant virus, characterized the effect of HSV-2 ICP8 on viral gene expression, and conducted initial tests of this mutant virus for its ability to protect guinea pigs against vaginal HSV-2 challenge. We selected the guinea pig model because it has been used to evaluate several vaccine candidates that are currently in clinical trials and because the model mimics genital HSV infection in humans using a natural route of inoculation (intravaginal) to produce a self-limited primary infection which results in establishment of latency and subsequent spontaneous recurrent infections (Stanberry, 1991).

MATERIALS AND METHODS

Cells and viruses

Vero (ATCC, Rockville, MD) and S2 cells which contain the HSV-1 ICP8 gene were grown and maintained as described previously (Gao and Knipe, 1989). The HSV-1 ICP8 mutant virus strain HD-2, which contains the lacZ coding sequences fused to the ICP8 ORF, has been described previously (Gao and Knipe, 1989). The wild-type HSV-2 strain 186 syn^-1 virus, used as the parental wild-type (wt) HSV-2 strain, was originally isolated as a non-syncytial plaque (Spang et al., 1983) from HSV-2 strain 186 (Rawls et al., 1968) stock kindly provided by P. Schaffer. The HSV-2 strain MS (Stanberry et al., 1982) was used as the vaginal challenge virus in immunized and control animals. All ICP8^- mutant viruses were grown on the complementing S2 cell line. The wild-type HSV-2 strains 186 syn^-1 and MS were grown on Vero cells and primary rabbit kidney (PRK) cells, respectively. Virus stocks were prepared and stored as described by Morrison and Knipe (1996), except for challenge virus stocks which were prepared as described by Stanberry (1982). In experiments in which inactivated virus was used to immunize animals, virus stocks were inactivated with β-propiolactone, resulting in a 5-log reduction in virus titer.

Plasmids

Plasmid pICP8-lacZ was constructed by digesting HSV-1 HD-2 DNA with BamHI and cloning the largest fragment, approximately 12.2 kbp in size, into the BamHI site of plasmid pNEB193 (New England Biolabs, Beverly, MA). This HD-2 BamHI fragment extends from upstream of the ICP8 promoter (BamHI V/R junction of HSV-1; bp 62,653 from the sequence of McGeoch et al. (1988)) to downstream of the gB gene (BamHI G/E' junction; bp 52,588 (McGeoch et al., 1988)). Following transformation into bacterial cells, white colonies selected on medium containing the chromogenic substrate X-Gal were screened for the presence of the insert. The identity of desired clones was confirmed by restriction endonuclease analysis. Plasmid pEH60 (Spang et al., 1983), which contains an EcoRI–HindIII DNA fragment from HSV-2 strain 186 syn^-1 from approximately 0.31–0.40 map units, that was cloned into pBR322 was used to rescue the HSV-2 ICP8^- mutant, 5BlacZ, constructed in this study. All restriction enzymes were obtained from New England Biolabs.

Marker transfer using cotransfection

Viral recombinants were isolated by homologous recombination in cells cotransfected with infectious HSV-2 DNA and cloned segments of viral DNA as described previously (Spang et al., 1983). The method used was a modification (Knipe et al., 1979; Ruyechan et al., 1979) of the calcium phosphate coprecipitation method (Graham and Van der Eb, 1973). Infectious HSV-2 viral DNA was purified by sodium iodide density gradient centrifugation (Walboomers and ter Schegget, 1976). Cloned viral DNA sequences for cotransfection were cleaved from recombinant plasmids, isolated by gel electrophoresis, purified by phenol–chloroform extraction and ethanol precipitation, and resuspended in TE buffer. Alternatively, recombinant plasmids were linearized outside of the HSV sequences and purified. The cloned sequences were co-transfected with 1 µg of infectious viral DNA at plasmid:viral DNA molar ratios ranging from 5:1 to 30:1 into subconfluent S2 cell monolayers in 25-cm² flasks (Corning Inc., Corning, NY). Each flask was transfected with a total of 16 µg of DNA with salmon sperm DNA serving as carrier. After viral CPE was apparent, infected cell lysates were prepared by the addition of 0.5 vol of sterile reconstituted milk (nonfat powdered milk; Carnation Co., Los Angeles, CA), three cycles of freeze–thawing, and sonication. The transfected cell lysate containing the progeny virus was plated in 10-fold dilutions on confluent S2 or Vero cell monolayers in 6-well trays (Corning). The infected monolayers were overlaid with 199-
DNA replication

G. Cohen and Dr. R. Eisenberg) were used as primary glycoproteins gB and gD. Rabbit polyclonal sera R1161 formed using a random-primer labeling kit (Boehringer Memmheim, Indianapolis, IN). Plaques formed by viruses expressing \( \beta \)-galactosidase showed blue staining within 6-12 hr.

Southern hybridization

To isolate viral genomic DNA, confluent cell monolayers in tissue culture flasks were infected with virus at an m.o.i. of 1 and incubated for 2 to 3 days at 34\(^\circ\) C. When infected cells became loosely attached to the substrate, they were shaken off, transferred to 50-ml polystyrene centrifuge tubes (Corning), and collected by centrifugation at 30 g, 4\(^\circ\) C in an IEC centrifuge (Model CRU-5000, Needham Heights, MA). The cells were gently lysed in buffer (100 mM Tris – Cl, pH 8.0; 10 mM EDTA, 1% SDS), and the lysate was digested with Proteinase K (200 \( \mu \)g/ ml). Viral DNA was isolated using sodium iodide gradients, dialyzed against TE buffer, quantitated by UV spectrophotometry, enzymatically restricted with EcoRI and Kpnl, Sall, or Xhol, resolved by electrophoresis in 0.7% agarose gels, and transferred to nylon membranes (Nitran; Schleicher and Schuell, Keene, NH) for Southern hybridization. The membranes were probed with linearized, \( ^{32}P \)-labeled pICP8 – lacZ plasmid DNA. Labeling with \( \alpha^{32}P \)dCTP (New England Nuclear, Boston, MA) was performed using a random-primer labeling kit (Boehringer Mannheim, Indianapolis, IN).

DNA replication

To examine viral DNA replication, Vero cells were infected with wild-type virus or 5BlacZ at an m.o.i. of 20 or were mock-infected. Cells were overlaid with 199 – 1% CS medium and over laid with a 1:1 mix of 2\( \times \)199 – 1% CS medium and 1% agarose containing 300 \( \mu \)g/ml X-Gal (Boehringer Mannheim, Indianapolis, IN). Plaques formed by viruses expressing \( \beta \)-galactosidase were observed within 2 days after plating. For in situ detection of \( \beta \)-galactosidase expression, the infected cell monolayers were then washed twice with 199 – 1% CS medium and overlaid with a 1:1 mix of 2\( \times \)199 – 1% CS medium and 1% agarose containing 300 \( \mu \)g/ml X-Gal (Boehringer Mannheim, Indianapolis, IN). Plaques formed by viruses expressing \( \beta \)-galactosidase showed blue staining within 6-12 hr.

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DNA replication

To examine viral DNA replication, Vero cells were infected with wild-type virus or 5BlacZ at an m.o.i. of 20 or were mock-infected. Cells were overlaid with 199 – 1% CS medium and incubated at 37\(^\circ\) C. DNA was labeled from 3 to 7 hr postinoculation by changing the overlay medium to 199 – 1% CS containing 25 \( \mu \)Ci/ml \( [\text{H}] \)thymidine (New England Nuclear). Cells were harvested at 7 hr postinoculation, and total DNA was isolated as described by Challberg (1986). Equivalent amounts of labeled DNA were digested with BamHI and resolved by electrophoresis in 0.5% agarose gels. The gel was fluorographed (Entensify, New England Nuclear), dried, and exposed to film (Kodak XAR) at -80\(^\circ\) C for 3 days.

Cell viability

The viability of infected cells was assayed by trypan blue exclusion (Fuller et al., 1995). Briefly, confluent Vero cell monolayers in T25 flasks were infected with wild-type virus or 5BlacZ at an m.o.i. of 5 or mock-infected and harvested at 24, 48, or 72 hr postinoculation. Cells were resuspended in a 0.5% (w/v) solution of trypan blue in phosphate-buffered saline and counted in a hemocytometer. Nontiable cells were enumerated by dye uptake, and their numbers were expressed as a percentage of the total cell count.

Analysis of viral proteins

The pattern of gene expression in virus-infected cells was examined by isotopic labeling of proteins and SDS – PAGE. Vero cells were infected with virus at an m.o.i. of 20 or were mock-infected. Infections were carried out either in the presence or in the absence of a viral DNA synthesis inhibitor, phosphonoacetic acid (PAA), at a concentration of 400 \( \mu \)g/ml. Virus or mock-infected cells were pulsed with \( [\text{S}] \)methionine, \( [\text{S}] \)cysteine (Tran35S-label, ICN, Irvine, CA) in methionine-free Eagle's modified MEM medium (ICN) for 30 min (2 ml, 15 \( \mu \)Ci/ml) and harvested at various times postinoculation. Cell lysates were subjected to SDS – PAGE in 9.25% gels. Following electrophoresis, gels were fixed, stained with Coomassie blue, dried, and exposed to Kodak SB-5 film for autoradiography. To quantify the levels of viral proteins expressed in infected cells, the autoradiograms were scanned using an LKB Bromma Ultrascan XL Laser densitometer, and peak areas representing specific viral protein bands were determined.

Immunoblots

The levels of expression of specific viral glycoproteins were determined by immunoblot assay. Infected cell lysates were resolved by electrophoresis using a mini protein gel apparatus (Bio-Rad, Richmond, CA), electroblotted onto nitrocellulose membranes, and probed for viral glycoproteins gB and gD. Rabbit polyclonal sera R1161 (provided by Dr. R. Courtney) and R-45 (provided by Dr. G. Cohen and Dr. R. Eisenberg) were used as primary antibodies to detect glycoproteins gB and gD, respectively. Goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI) was used to detect the immune complexes. The enzymatic substrates nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) were used in the reaction to detect alkaline phosphatase activity as recommended by the supplier (Promega).

Animals

Female Hartley guinea pigs were obtained from Charles River Breeding Laboratories (Wilmington, MA) and housed under AAALAC-approved conditions. All procedures and protocols were approved by Children's Hospital Research Foundation Animal Care and Use Committee (Cincinnati, OH).

Immunization studies

Female guinea pigs were randomized into three groups of 12 animals each and were immunized with 0.5
ml of a suspension containing $10^7$ PFU of 5BlacZ by subcutaneous injection on the back or into the two rear footpads or were left unimmunized. In other experiments, animals were immunized with 5BlacZ either vaginally with live virus or in the rear footpads with live or inactivated virus. Viral challenge was performed on Day 33 postimmunization. The animals were inoculated with virus by rupturing the vaginal closure membrane with a moistened calcium alginate-tipped swab (Calgiswab 3, Spectrum Labs, Los Angeles, CA) and instilling 0.1 ml of a virus suspension containing $5 \times 10^5$ PFU of HSV-2 strain MS into the vaginal vault using a plastic catheter (Abbocath, Abbott Labs, North Chicago, IL). To maximize the number of animals infected, the inoculation procedure was repeated 30 min later. Vaginal swab samples were collected on Days 1, 2, 3, 5, 7, and 10 postinoculation and stored frozen at $-70^\circ$ until assayed for the presence of virus by titration on primary rabbit kidney cells. Guinea pigs were evaluated daily and the severity of primary genital skin disease was quantified using a lesion score scale described previously (Stanberry et al., 1982). Primary genital skin disease was defined as any primary episode clinical disease beginning before Day 10 postinoculation. Following recovery from primary infection, animals were examined daily from Days 15 to 45 postinoculation for evidence of spontaneous recurrent herpetic disease (Stanberry et al., 1985a).

RESULTS

Construction of an HSV-2 ICP8 gene mutant

To introduce a mutation into the HSV-2 ICP8 gene, we chose to replace the HSV-2 ICP8 gene with an ICP8-lacZ gene fusion that we had previously engineered in the HSV-1 HD-2 viral strain (Gao and Knipe, 1989). The feasibility of this approach was suggested by the extensive homology between the HSV-1 and HSV-2 genomes and the previous ease of isolation of intertypic recombinants in general (Marsden et al., 1978; Morse et al., 1978) and around the ICP8 gene in particular (Conley et al., 1981). To this end, we cloned a BamHI DNA fragment encompassing the ICP8-lacZ gene fusion from HD-2 virus DNA into plasmid vector pNEB193 to generate plasmid pICP8-lacZ (Fig. 1). Because this BamHI fragment contains other viral genes including the gB gene of HSV-1, a 6.4-kbp KpnI subfragment containing the ICP8-lacZ gene fusion but excluding the gB gene was isolated and used to construct the HSV-2 ICP8 mutant virus (Fig. 1). This fragment, which extends from 233 bp downstream of the ICP8 ATG initiation codon to approximately 1.3 kbp upstream of the gB gene promoter, was cotransfected with HSV-2 186 syn-1 viral DNA into S2 cells. From the progeny viruses, we isolated viruses that formed blue plaques under an X-Gal overlay and contained the ICP8-lacZ gene fusion inserted into the ICP8 gene (Fig. 2). In the initial screen for viruses that formed blue plaques under the X-Gal overlay, approximately 1 in every $10^3$ plaques appeared blue. Several independently isolated blue plaques were screened for the presence of the ICP8 mutation by the ability to grow on the S2 complementing cell line and the failure to grow on Vero cells. Two such isolates, 5BlacZ and 20BlacZ, were plaque-purified thrice and their genomes were checked by Southern analysis.

Analysis of the mutant viral genomes

The growth of 5BlacZ on S2 cells, which express ICP8 upon HSV infection and complement the growth of ICP8 mutant viruses, provided the initial evidence that the mutation in 5BlacZ was in the ICP8 gene. To confirm that the ICP8 gene was mutated in 5BlacZ, we performed Southern blot analysis on viral DNA from 5BlacZ, its sibling 20BlacZ, the HSV-1 HD-2 strain, and the parental HSV-2 186 wt strain. Viral DNA was digested with EcoRI and KpnI, resolved by gel electrophoresis, transferred to nylon membranes, and probed with a $^{32}$P-labeled BamHI DNA fragment from HD-2 shown in the second line of Fig. 2D. The probe hybridized with two fragments of 6.4 and 7.8 kbp (Fig. 3, lane 1) of HSV-2 wt DNA, which map as shown in Fig. 2D. In contrast, the probe hybridized strongly with four fragments of 1.9, 3.6, 2.8, and 4.7 kbp of HD-2 DNA (Fig. 3, lane 4), which map as shown in Fig. 2D. With 5BlacZ and 20BlacZ DNA, the probe hybridized to new fragments of 5.5 and 2.9 kbp as well as to a 7.8-kbp fragment similar to that of HSV-2. The new fragments in 5BlacZ and 20BlacZ were consistent with a recombinational event inserting the ICP8-lacZ gene fusion into the 6.4-kbp fragment of HSV-2 or within the ICP8 gene (Fig. 2D). No other bands were observed with...
5BlacZ or 20BlacZ (Fig. 3, lanes 2 and 3), providing evidence that the lacZ sequences were inserted only within the ICP8 gene locus. Other digests with EcoRI and SalI plaques could have been detected, and the efficiency of plating of 5BlacZ on Vero cells compared to S2 cells was calculated to be \( < 3 \times 10^{-7} \). In contrast, wild-type virus formed plaques equally well in Vero cells and in S2 cells (Table 1).

Viral DNA synthesis. HSV-1 ICP8 is an essential gene product for viral DNA replication. To examine the ability of the HSV-2 ICP8 mutant virus to replicate its DNA, \([^{3}H]thymidine\)-labeled DNA was isolated from Vero cells infected with either wt HSV-2 or 5BlacZ or mock-infected and enzymatically restricted and resolved in agarose gels (Fig. 4). From cells infected with wt virus, a series of viral DNA bands was labeled (Fig. 4, lane 1), consistent with viral DNA synthesis, while a smear of bands was labeled in mock-infected cells due to cellular DNA replication (Fig. 4, lane 3). In contrast, no viral DNA bands were labeled in cells infected with 5BlacZ (Fig. 4, lane 2), indicating that no viral DNA synthesis occurred in 5BlacZ or 20BlacZ (Fig. 3, lanes 2 and 3), providing evidence that the lacZ sequences were inserted only within the ICP8 gene locus. Other digests with EcoRI and SalI plaques could have been detected, and the efficiency of plating of 5BlacZ on Vero cells compared to S2 cells was calculated to be \( < 3 \times 10^{-7} \). In contrast, wild-type virus formed plaques equally well in Vero cells and in S2 cells (Table 1).

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HSV-1 ICP8 mutant viruses grow only on a complementing cell line and do not replicate viral DNA in normal cells (Gao and Knipe, 1989; Orberg and Schaffer, 1987). The HSV-2 ICP8 mutant isolated in this study, 5BlacZ, was also examined for its phenotypic properties.

Growth properties. The 5BlacZ mutant virus formed plaques and grew to titers comparable to those of wt virus on S2 cells but did not form plaques on Vero cells (Table 1). At low dilutions, a general cytopathic effect was observed on Vero cells, preventing detection of any replication-competent virus at these dilutions. Therefore,
Southern blot analysis of recombinant viral genomes. Viral DNA was isolated and restricted with EcoRI and KpnI. Digests were resolved by electrophoresis in 0.7% agarose gels, blotted onto nylon membranes, and probed with a \[^{32}P\]dCTP-labeled pICP8-lacZ BamHI fragment. Lane 1, wt HSV-2 186 syn^-1; lane 2, HSV-2 ICP8^-mutant 5BlacZ; lane 3, HSV-2 ICP8^-mutant 20BlacZ; lane 4, HSV-1 ICP8^-mutant HD-2. Sizes of DNA fragments in kbp are indicated at the right of the figure.

Measurement of viral DNA replication. Vero cells were infected with wt HSV-2 186 syn^-1 (lane 1) infected with HSV-2 ICP8^-mutant 5BlacZ (lane 2), or mock-infected (lane 3) and were labeled with \[^{3}H\]thymidine from 4 to 7 hr post-infection. Total DNA was isolated, equivalent amounts of each DNA sample (4 \(\mu\)g) were digested with BamHI, and the fragments were resolved by electrophoresis in a 0.5% agarose gel. A fluorogram of the dried gel is shown.

Gene expression. Initial comparisons of protein expression by 5BlacZ and wild-type virus showed some differences in levels and patterns of protein expression (results not shown). To prove that the protein expression phenotype of 5BlacZ was due to the mutation in the ICP8 gene, we used marker rescue to restore a wild-type ICP8 gene into the 5BlacZ genome. The ICP8 mutation in 5BlacZ was rescued with plasmid pEH60, which contains the ICP8 gene of the parental HSV-2 186 syn^-1 strain. Mutant virus was rescued at a frequency between 0.04 and 2%. One of the rescuants, 5BR, was chosen for characterization. The 5BR strain grew equally well on Vero and S2 cells (Table 1). Southern blot analysis of the rescued virus and wt HSV-2 virus using the HSV-1 ICP8 plasmid p8B-S (Gao and Knipe, 1989) as probe revealed identical hybridization patterns for the two viruses (results not shown).

TABLE 1

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genotype</th>
<th>Vero titer (PFU/ml)^a</th>
<th>S2 titer (PFU/ml)</th>
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<tr>
<td>186 syn^-1</td>
<td>wt</td>
<td>3.2 (\times) 10^6</td>
<td>3.1 (\times) 10^6</td>
</tr>
<tr>
<td>5BlacZ</td>
<td>lacZ insertion in ICP8</td>
<td>&lt;10^2</td>
<td>8.9 (\times) 10^6</td>
</tr>
<tr>
<td>5BR^c</td>
<td>wt ICP8 gene restored in 5BlacZ</td>
<td>9.3 (\times) 10^6</td>
<td>9.9 (\times) 10^6</td>
</tr>
</tbody>
</table>

a Dilutions of a stock of each virus were plated in duplicate on both Vero cells and S2 cells in T25 flasks.

b Wild-type parental strain from which 5BlacZ was derived.

c Rescued 5BlacZ.
We then examined the patterns of protein synthesis in Vero cells infected with the mutant 5BlacZ, the rescued 5BR, or wild-type virus in the presence and in the absence of PAA, a specific inhibitor of viral DNA synthesis. At 5 or 9 hr postinoculation, we labeled the cultures with \([^{35}S]\)methionine and \([^{35}S]\)cysteine for 30 min. The cells were harvested, and infected cell-lysates were prepared and resolved by SDS–PAGE (Fig. 5). Examination of autoradiograms revealed that major proteins of all kinetic classes were expressed by 5BlacZ at levels similar to or slightly less than those expressed by the wild-type parental virus (Fig. 5), including \(\gamma\) genes such as ICP5, gB, and ICP15. Densitometric measurements of the band intensities of the late gene products at 9 hr postinoculation revealed that 5BlacZ expressed late proteins at 25–79% of the levels of the rescued virus in cells infected in the absence of PAA (Table 2). When PAA was added to all cultures to inhibit viral DNA synthesis in both mutant and rescued-mutant infected cultures, 5BlacZ expressed late proteins at levels similar (gB) to or 2- to 2.5-fold greater (ICP5, ICP15, ICP25) than the levels expressed by the rescued virus (Table 2). Therefore, similar to HSV-1 ICP8, HSV-2 ICP8 decreases late gene expression in the absence of viral DNA synthesis. Also, relevant to the use of the mutant virus for immunization studies, 5BlacZ expressed gB at 67% of the level of the rescued virus in control cells (−PAA). Thus, although the mutant virus did not replicate its genome, it expressed gB, an important target for anti-HSV immunity, at levels nearly equivalent to those of the rescued virus.

Levels of accumulation of glycoproteins gB and gD. The above results using analysis of labeled protein bands resolved by SDS–PAGE indicated that 5BlacZ expressed significant levels of late proteins, in particular gB, at 9 hr postinoculation. To ensure the specificity of detection of the viral glycoproteins, we used Western blot analysis to determine the levels of accumulation of the gB and gD glycoproteins (Fig. 6). Comparisons of equivalent amounts of lysates revealed that cells infected with 5BlacZ accumulated gB (Fig. 6, lanes 5–8) and gD (Fig. 6, lanes 1–4) at levels only slightly lower than those in cells infected with wt virus. Therefore, 5BlacZ expressed significant levels of authentic gB and gD although it did not replicate viral DNA.

Table 2

<table>
<thead>
<tr>
<th>Virus</th>
<th>ICP5</th>
<th>gB</th>
<th>ICP15</th>
<th>ICP25</th>
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<tbody>
<tr>
<td>5BR</td>
<td>142</td>
<td>100</td>
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<td>5BlacZ</td>
<td>61</td>
<td>67</td>
<td>36</td>
<td>27</td>
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<td>5BR + PAA</td>
<td>18</td>
<td>40</td>
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</tr>
<tr>
<td>5BlacZ + PAA</td>
<td>36</td>
<td>51</td>
<td>23</td>
<td>23</td>
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\(a\) Numbers represent units of protein at 9 hr postinoculation as determined by densitometry of autoradiogram bands. Numbers in parentheses represent the percentage of 5BR values in cells infected in the absence of PAA.

**FIG. 5.** Polypeptide profiles of Vero cells infected with the HSV-2 ICP8- mutant 5BlacZ (lanes 1, 4, 7, and 10), rescued strain 5BR (lanes 2, 5, 8, and 11) or wt HSV-2, 186 syn−1 (lanes 3, 6, 9, and 12), or mock-infected (lanes 13 and 14) either in the absence or in the presence of 400 μg/ml PAA as indicated. Cells were labeled for 30 min prior to being harvested at 5 hr postinfection (lanes 1 - 6) or 9 hr postinfection (lanes 7 - 14). The migration positions of some representative viral polypeptides are indicated on the left.

**FIG. 6.** Western blot analysis of gB and gD in infected cell lysates. Proteins from 5BlacZ- (lanes 1, 2, 5, and 6) or HSV-2 wt- (lanes 3, 4, 7, and 8) infected cell lysates were electroblotted from SDS–polyacrylamide gels onto nitrocellulose membranes and probed with either anti-gD (lanes 1 - 4) or anti-gB (lanes 5 - 8) antibody. Lanes 1, 2, 5, and 7 were loaded with 20 μl of cell lysate and lanes 2, 4, 6, and 8 were loaded with 40 μl. Arrowheads indicate the specific glycoprotein bands. The molecular weight markers are in lane 9 with the corresponding weights shown on the right.
Viability of cells infected with mutant virus

A potential concern about the use of a replication-defective mutant virus as a vaccine is that the infected cell might survive and the mutant virus could establish a persistent infection in the host. We therefore examined the fate of cells infected with the mutant virus. Vero cells were infected with either wt HSV-2 or 5BlacZ and harvested at 1, 2, or 3 days postinoculation. Loss of cell viability was assessed by uptake of trypan blue and expressed as a percentage of total cell count (Fig. 7). Infection of cells with either virus resulted in a 2- to 5-fold increase in the percentage of nonviable cells by Days 1 and 2 and a 10-fold increase by Day 3, at which time 100% of infected cells were nonviable. Over the same 3-day period, the percentage of nonviable cells among the uninfected controls remained at approximately 10%. Thus, it is clear that, despite the defect in its ability to replicate in Vero cells, 5BlacZ is still able to kill cells at a level comparable to wild-type virus. Like wt virus infection (Leopardi and Roizman, 1996), cells infected with 5BlacZ appeared to die from necrosis in that cellular DNA fragmentation was not observed (results not shown).

Immunization studies with 5BlacZ

Given the ability of 5BlacZ to express nearly wild-type levels of gB and gD in normal cells but not produce infectious progeny in those cells, we investigated its ability to elicit protective immunity against HSV-2 genital disease in a guinea pig model. Animals were immunized with 5BlacZ by one of three routes: subcutaneous injection in the back, subcutaneous injection in the rear footpad, or intravaginal immunization. Immunizations were performed with either live or inactivated virus stocks. Following immunization, all animals were challenged with wt HSV-2. Two challenge stocks of the same HSV-2 MS strain were used: one a high-virulence stock used mainly to study primary disease and the other a low-virulence stock used mainly to study recurrent disease.

Effects of immunization on primary infection and disease. Three groups of 12 female Hartley guinea pigs were immunized with a single dose of $10^7$ PFU of 5BlacZ virus administered subcutaneously in the footpads or in the back, or left untreated. At Day 33 postimmunization, the animals were challenged intravaginally with a high-virulence stock of HSV-2 strain MS to achieve an efficient, synchronous primary vaginal infection (Stanberry, 1982) and evaluated daily for signs of disease (Table 3). The amount of virus shed in the genital tract of all animals was also determined from vaginal swabs taken at various times postinoculation (Fig. 8). A single immunization with 5BlacZ afforded guinea pigs significant protection from genital herpes. In contrast to the control group in which 11 of 12 animals exhibited lesions, only 3 of 12 animals immunized in the footpad developed the cutaneous lesions characteristic of primary genital herpes, and disease in these animals was very mild. In addition, 6 of

<table>
<thead>
<tr>
<th>Immunization group</th>
<th>Primary genital skin disease</th>
<th>Recurrent genital skin disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Number&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Severity&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>6/12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.50 ± 0.52&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Footpad</td>
<td>3/12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.0 ± 0.29&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of animals with clinical disease/number of animals in which virus could be isolated from the genital tract.
<sup>b</sup> Mean ± SE. Severity measured as the area under the lesion score curve calculated using only symptomatic animals.
<sup>c</sup> Number of animals with recurrent disease/number of infected animals which could be assessed for recurrences from Days 15 to 42 postinoculation.
TABLE 4
Effect of Prophylactic Vaginal and Footpad Inoculation of 5BlacZ Mutant Virus on Primary Genital Herpes Following Intravaginal HSV-2 Challenge

<table>
<thead>
<tr>
<th>Immunization Group</th>
<th>Number</th>
<th>All</th>
<th>Symptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>15/15</td>
<td>6.3 ± 0.8</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>Vaginal</td>
<td>4/10</td>
<td>1.2 ± 0.6a</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td>Footpad &quot;inactive&quot;</td>
<td>4/10a</td>
<td>0.4 ± 0.2b</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>Footpad &quot;live&quot;</td>
<td>0/15a</td>
<td>0 ± 0b</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

a Number of animals with symptomatic primary genital skin disease/number inoculated.
b Mean ± SE. Severity measured as area under the lesion score-day curve.
c P < 0.005.
d P < 0.01 by ANOVA with Bonferroni correction.
e P < 0.0001.

FIG. 8. Virus shedding in the genital tract after HSV-2 challenge. Guinea pigs were left untreated or immunized with 5BlacZ by either the footpad or the subcutaneous route. Following a vaginal challenge with HSV-2 strain MS, animals were swabbed to determine levels of virus shed in the genital tract. Samples were collected on Days 1, 2, 3, 5, 7, and 10 postinoculation and titrated on primary rabbit kidney cells. Mean viral titers are presented as log10 PFU/ml.

To examine the effects of immunization on viral shedding in the genital tract, all animals were swabbed on Days 1, 2, 3, 5, 7, and 10 postchallenge, and titers of virus shed in the genital tract were determined by assay on primary rabbit kidney cells. Virus was recovered from vaginal swab specimens of all animals at Day 1, indicating that the vaccine did not protect against infection. However, by Day 2, there was a 10-fold decrease in virus shed by the immunized animals compared to control unimmunized animals (Fig. 8). This difference increased to 100-fold and 1000-fold by Days 5 and 7, respectively. Thus, parenteral immunization with 5BlacZ virus elicited protective immunity that reduced viral replication and/or spread at a mucosal site.

Protection against latent and recurrent infection. The study of recurrent disease in the guinea pig model requires that animals recover from the primary infection well enough to be used to score recurrent infections. To ensure an adequate number of animals for evaluation of recurrent disease in a second experiment, a less virulent stock, HSV-2 strain MS, than had been used in the initial study of primary disease was used to challenge animals. Prior to challenge, groups of guinea pigs were immunized with two doses of 10⁷ PFU 5BlacZ intravaginally or in the footpad or with an equivalent amount of virus that had been inactivated by 5 logs with β-propiolactone. Immunization with 5BlacZ provided significant protection against disease caused by primary infection as measured by the number and severity of lesions compared to the untreated controls (Table 4). None of the 15 animals that received an inoculation of live virus via the footpad developed primary genital skin disease compared to 4 of 10 animals that received inactivated virus by the same route that did develop primary disease. Most importantly, the number of animals showing recurrent infections was significantly reduced by footpad immunization (Table 5), and the frequency of recurrences was

TABLE 5
Effect of Prophylactic Vaginal and Footpad Inoculation of 5BlacZ Mutant Virus on Recurrent Genital Herpes Following Intravaginal HSV-2 Challenge

<table>
<thead>
<tr>
<th>Immunization Group</th>
<th>Number</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>All</td>
</tr>
<tr>
<td>Untreated</td>
<td>11/11</td>
<td>5.9 ± 1.2</td>
</tr>
<tr>
<td>Vaginal</td>
<td>6/9</td>
<td>1.8 ± 0.8c</td>
</tr>
<tr>
<td>Footpad &quot;inactive&quot;</td>
<td>3/14d</td>
<td>0.4 ± 0.2e</td>
</tr>
<tr>
<td>Footpad &quot;live&quot;</td>
<td>2/15d</td>
<td>0.1 ± 0.1e</td>
</tr>
</tbody>
</table>

a Number of animals experiencing recurrences/number which could be evaluated.
b Mean ± SE. Recurrent lesion days between Day 15 and Day 42 postinoculation.
c P < 0.01 by ANOVA with Bonferroni correction.
d P < 0.001 by ANOVA with Bonferroni correction.

P < 0.0005 by Fisher’s exact test.
reduced significantly by footpad or vaginal immunization (Table 5). For all parameters of challenge virus infection, live virus provided better protection than inactivated virus. The protection against recurrent disease afforded to animals immunized with 5BlacZ was significant compared to untreated controls (Table 5). Fewer of the immunized animals developed recurrent lesions, and among those that did, a significant reduction in the frequency of recurrence during the period of evaluation was observed compared to control animals. Thus immunization with 5BlacZ provided significant protection against primary and recurrent infection, and live virus provided better protection than killed virus.

**DISCUSSION**

We have constructed and characterized a replication-defective HSV-2 mutant and shown it to protect against genital herpes in a guinea pig model of HSV-2 disease. The virus has a mutation in an essential early gene, ICP8, and grows only on cells that express functional ICP8. In normal cells infected with this HSV-2 ICP8 mutant virus, expression of IE, E, and at least some L genes occurs although there is no viral DNA replication. In the absence of viral DNA replication, 5BlacZ can express significant levels of the late proteins ICP5, ICP15, and ICP25 and the glycoproteins gB and gD. Previous studies have shown that HSV-1 ICP8 not only serves as an essential component of the viral DNA replication apparatus but also is involved in the regulation of viral gene expression. Specifically, HSV-1 ICP8 plays a role in repressing late gene expression from parental genomes while stimulating late gene expression from progeny genomes (Chen and Knipe, 1996; Gao and Knipe, 1991; Godowski and Knipe, 1985). The regulatory effects exerted by ICP8 are independent of its role in DNA synthesis; therefore, ICP8 mutants showed altered gene expression compared to wild-type virus in the presence of a viral DNA synthesis inhibitor. The ICP8 mutation in HSV-2 resulted in a similar phenotype to that observed for HSV-1 ICP8 mutant viruses. The HSV-2 5BlacZ mutant virus was unable to replicate its DNA and demonstrated altered gene expression compared to wild-type virus or the rescued strain 5BR. Based on these observations, it appears that ICP8 exerts similar effects on gene expression in both HSV-1 and HSV-2. The similarity in the functions of HSV-1 and HSV-2 ICP8 is reinforced by the ability of the HSV-1 ICP8 expressed by the S2 cell line to complement the growth of the HSV-2 mutant virus. Growth of 5BlacZ in S2 cells has the potential for rescue of the mutant virus by recombination with the resident HSV-1 ICP8 gene in the S2 cell line. Nevertheless, although the HSV-1 ICP8 gene can functionally complement the defect in HSV-2 virus, the HSV-2 genome and the HSV-1 gene do not readily recombine with each other as evidenced by the absence of detectable wild-type recombinants (<3 x 10^-5).

The ICP8 mutant studied in this work showed nearly normal inhibition of host cell DNA synthesis; therefore, ICP8 is not required for this effect of the virus. HSV ICP8 is required for the redistribution of sites of host cell DNA synthesis in the nuclei of infected cells (de Bruyn Kops and Knipe, 1988). Thus, the redistribution of host cell DNA synthesis sites and the inhibition of host cell DNA synthesis appear to be separate effects of HSV infection on the host cell. Consistent with the host cell shut-off properties of the 5BlacZ mutant, cells infected with this mutant showed CPE similar to that of cells infected with wt virus as well as similar kinetics of loss of viability. This indicates that although 5BlacZ does not replicate its DNA, it completes enough of the replicative cycle to cause cell death. It is unlikely that any significant amount of 5BlacZ will spread to sensory neurons to establish a latent infection (Katz et al., 1990). The implication of these considerations is that, in host organisms immunized with 5BlacZ, infected cells will not survive and 5BlacZ is not likely to persist in the host after inoculation of this virus for immunization. Experiments are in progress to measure the ability of HSV-2 replication-defective mutants to establish latent infection.

The immunization studies in this report were designed to provide an initial test of the feasibility of using the 5BlacZ mutant virus to elicit protective immunity against genital herpes infection in the guinea pig model. Immunization with one dose of 5BlacZ did reduce the incidence and severity of genital disease and the shedding of virus from primary infection of the genital tract. Immunization did not prevent infection by the challenge virus in that virus shedding was the same for immunized and unimmunized animals on Day 1. Thus, immunization with the mutant virus reduced disease and virus spread, and further studies are needed to optimize the dose and frequency to further increase the protection against primary infection. A second experiment to measure protection against recurrent infection involved two immunizations with 5BlacZ and challenge with a less virulent stock of HSV-2. This showed better protection against primary disease, probably due to the increased number of immunizations. These immunized animals showed decreased recurrent infections compared to the control animals, indicating that immune effector mechanisms are acting to decrease establishment of latent infection or reactivation or both. Studies of immune protection against establishment of latent infection by HSV-1 in murine trigeminal ganglia have shown that CD4 T cells play a major role in protection from latent infection (Morrison and Knipe, manuscript in preparation), but little is known about immune mechanisms protecting against latent infection by HSV-2. Further studies are needed to define the stage of HSV-2 infection or reactivation blocked by the host immune response and the necessary components of that response.

One of the primary concerns about any live-virus vaccine must be safety. Live attenuated HSV-2 strains have given some viral disease in animal models (McDermott...
et al., 1984; Stanberry et al., 1985b), raising concerns about their safety. The 5BlacZ mutant virus has elicited no disease in inoculated guinea pigs (Bourne and Stanberry, unpublished results), immunocompetent mice, or even immunoodeficient mice (Morrison, Da Costa, and Knipe, unpublished results). Thus, although additional muta- tions need to be incorporated into any potential replication-defective HSV-2 mutant strain for vaccine purposes (Knipe et al., 1996) and further testing needs to be performed, these initial results indicate that a replication- defective HSV-2 strain may show a high level of safety.

It is worth noting that parenteral immunization with 5BlacZ was used to elicit protection against a mucosal challenge. Optimal mucosal immunity is believed to require mucosal immunization; thus, it will be important to determine the mechanism(s) of the immunity elicited by parenteral immunization as well as whether mucosal immu-

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References


