INTRODUCTION

The morbidity associated with human genital infection with herpes simplex virus 2 (HSV-2) and the uncommon but devastating complication of neonatal disease are the impetus toward development of more effective therapies against HSV-2. The high incidence of unrecognized clinical HSV-2 disease and asymptomatic shedding post-genital infection both suggest that the only way to significantly impact upon transmission is through a prophylactic vaccine. Recent reports of an increase in prevalence of HSV-2 in the United States (Fleming et al., 1997) and of a putative role for HSV-2 in facilitating the worldwide pandemic of human immunodeficiency virus emphasize that the development of a vaccine against HSV-2 continues to be a high priority (reviewed by Corey and Handsfield, 2000).

A number of vaccine strategies have been or are being tested against HSV, including attenuated viruses, replication-impaired viruses, heterologous viral vectors expressing HSV proteins, inactivated virus, viral protein subunits, and DNA vaccines (reviewed in Stanberry et al., 2000). Many of these approaches can prevent local disease or encephalitis. However, to reduce transmission to uninfected individuals, an effective vaccine should not only protect against local disease in the genital region but also reduce or prevent latent infection of the sensory ganglia (Roizman, 1991).

The ability of HSV-2 vaccine candidates to protect against latency has been reported in terms of reactivation of challenge virus from explanted ganglia and/or detection of the latency-associated transcripts (LATs) in sensory ganglia (Berman et al., 1988; Boursnell et al., 1997; Burke et al., 1991; Cremer et al., 1985; Dix and Mills, 1986; Meignier et al., 1988; Schneweis et al., 1981; Spector et al., 1998; Stanberry et al., 1986; Walz et al., 1977). However, a more stringent demonstration of a reduction in latent viral load within the sensory ganglia using more sensitive assays for latent infection, such as quantitative polymerase chain reaction (Q-PCR) for HSV-2 viral DNA, was previously reported in only a few cases (Bourne et al., 1996; Da Costa et al., 1999). Even less well described is the ability of the vaccine candidate itself to establish latent infection in the nervous system (Klein et al., 1984; Meignier et al., 1988; Robertson et al., 1992; Thompson et al., 1986) or its ability to persist at the site of inoculation (Bourne et al., 1996).

Replication-impaired mutant viral vaccines have been designed as replication-defective mutants (Nguyen et al., 1992) and as single-cycle mutant viruses (Farrell et al., 2000).
1994). As a test of the use of replication-defective HSV-2 strains for immunization, we previously showed that immunization with the HSV-2 replication-defective mutant 5BlacZ, which is defective for the gene encoding ICP8, can elicit protection against acute and recurrent genital disease in both the mouse and guinea pig models of genital infection (Da Costa et al., 1997; Morrison et al., 1998) and is capable of eliciting an HSV-specific systemic and mucosal immune response (Morrison et al., 1998). A double-deletion mutant of HSV-2 is also capable of inducing protective immunity against genital challenge (Da Costa et al., 1999). In this study, we developed an intranasal mouse model of HSV-2 infection to examine the HSV-2 replication-defective mutants not only in terms of their ability to protect against latent infection but also in terms of the in vivo behavior of the mutants at the site of inoculation, in trigeminal ganglia (TG) and in the central nervous system (CNS).

RESULTS

Intranasal model of HSV-2 infection

We developed a mouse model of nasal HSV-2 infection with which to examine the biological properties of various HSV-2 strains and to test the ability of the HSV-2 replication-defective mutants to protect against latent infection by a challenge virus. We chose to use the intranasal route to inoculate the viruses because (1) the intranasal route is used frequently for immunization to elicit mucosal immunity, especially in the genital tract; (2) the trigeminal ganglia are more accessible in mice than are the sacral ganglia, the site of latency after intravaginal inoculation; and (3) the intranasal route is a major route for HSV-2 infection of newborns. We constructed an attenuated, thymidine kinase-negative mutant of the HSV-2 strain 186syn-1, 186ΔKpn (Fig. 1A), for latency studies because wild type HSV-2 causes lethal encephalitis when inoculated into mice.

Dose curve of HSV-2 latency after intranasal inoculation. To optimize conditions for establishment of latent infection, we infected mice intranasally with doses of 10^1 to 10^6 plaque-forming units (PFU)/mouse of HSV-2 186ΔKpn, 10^1 to 10^5 PFU/mouse of wt virus, or an equivalent amount of uninfected cell extract (CE) as a negative control. The mice were examined daily for signs of acute infection, neurological disease, and moribundity. Thirty days postinfection (p.i.), we sacrificed the surviving mice and removed the TG. To determine the abundance of latently infected neurons in the TG, we performed in situ hybridization for HSV-2 LATs using the LAT probe pBP2 (Fig. 1B).

We observed that the HSV-2 TK- strain 186ΔKpn was sufficiently attenuated to use in our studies of HSV-2 latency, as none of the mice inoculated with this strain showed signs of CNS disease or lesions at the external nares, and all survived until sacrifice at Day 30. In contrast, only mice infected with low doses (<10^4 PFU/mouse) of wt virus survived (Table 1). The lethal dose (LD50) of 186ΔKpn after intranasal inoculation was calculated to be >10^5 PFU/mouse and the LD50 for wt HSV-2 was 5 x 10^3 PFU after intranasal inoculation.

When we examined LAT expression by in situ hybridization in the 186ΔKpn-infected ganglia at 28 days p.i., we observed a roughly linear relationship between numbers of LAT-positive neurons and the dose of 186ΔKpn virus, between doses of 10^3 and 10^5 PFUs per mouse (Table 1). Mice that survived inoculation with wt virus did not show any LAT+ neurons (Table 1). We demonstrated the specificity of the pBP2 plasmid for LAT by showing a lack of hybridization to mock-infected tissue (Table 1) and by performing DNase incubation of the slides, which did not affect the signal, and RNase digestion, which obliterated the signal (C. A. Jones and D. M. Knipe, unpublished results). Thus, intranasal (i.n.) inoculation of 186ΔKpn (TK-) virus resulted in latent infection of the TG. We are uncertain why, at doses of 10^3 PFU, 186ΔKpn infection yielded more LAT+ neurons than did wt virus.
This may be due to experimental variability between the two groups of animals; alternatively, the TK<sup>−</sup> phenotype may increase the numbers of latently infected cells or levels of LAT expression.

**Site of replication after intranasal inoculation.** To determine the site of primary viral replication after i.n. inoculation, we inoculated mice with 1 × 10<sup>6</sup> PFU/mouse of wt 186, 186ΔKpn, or an equivalent volume of CE. On Days 1, 2, and 3 p.i., animals from each group were sacrificed and the nasal septa, trachea, lung, nasopharynx, and stomach were removed, homogenized, and assayed for infectious virus by standard plaque assay. We observed that the nasal septa were the major tissue sites of replication after intranasal inoculation of both wt and 186ΔKpn viruses (Fig. 2). Some infectious wt virus was detected from Day 1 p.i. in the trachea and lungs, as well. Stomach homogenates were toxic to the monolayers at dilutions less than 1:100, and no infectious virus was detected in homogenates diluted more than this. Viral titers were, on average, 10- to 100-fold greater in all tissues in wt virus-infected mice than in tissues of animals inoculated with 186ΔKpn virus. Therefore, a significant proportion of the inoculum remains in and infects cells in the nasal septa after intranasal inoculation, and nasal septa homogenates can be used effectively to monitor acute viral replication after inoculation at this site. Infectious virus was not detected in nasal swabs collected at any time post i.n. inoculation (C. A. Jones and D. M. Knipe, unpublished results).

**HSV-2 replication-defective mutants protect against latent infection by a challenge virus**

Protection against latency by the HSV-2 replication-defective mutant 5BlacZ. To test if immunization with 5BlacZ, an HSV-2 ICP8<sup>−</sup> replication-defective mutant (Da Costa et al., 1997), would protect against latency after intranasal challenge with 186ΔKpn, mice were immunized twice s.c. in the rear flank with 2 × 10<sup>6</sup> PFU/mouse.

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**TABLE 1**

Survival and Numbers of LAT-Positive Neurons in Trigeminal Ganglia in Mice Infected with Various Doses of HSV-2 wt or 186ΔKpn Viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Dose (PFU/mouse)</th>
<th>Percentage survival (no. mice)</th>
<th>No. TG scored</th>
<th>No. sections scored</th>
<th>Total No. LAT&lt;sup&gt;+&lt;/sup&gt; neurons</th>
<th>Mean No. LAT&lt;sup&gt;+&lt;/sup&gt; profiles/TG ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>186ΔKpn</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>100 (5)</td>
<td>10</td>
<td>540</td>
<td>3423</td>
<td>342 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>100 (3)</td>
<td>5</td>
<td>208</td>
<td>300</td>
<td>60 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>100 (4)</td>
<td>5</td>
<td>254</td>
<td>168</td>
<td>33.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>100 (4)</td>
<td>6</td>
<td>501</td>
<td>28</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>100 (4)</td>
<td>4</td>
<td>191</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>100 (4)</td>
<td>1</td>
<td>73</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>100 (4)</td>
<td>5</td>
<td>185</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>100 (4)</td>
<td>4</td>
<td>327</td>
<td>0</td>
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<tr>
<td></td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>100 (4)</td>
<td>6</td>
<td>463</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CE</td>
<td>100 (5)</td>
<td>9</td>
<td></td>
<td>461</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

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**FIG. 2.** Viral titers in nasal septa, trachea, and lung after intranasal inoculation. Mice were inoculated with 2 × 10<sup>6</sup> PFU of wt HSV-2 (strain 186) or 186ΔKpn, i.n. (N = 3 mice/timepoint/virus strain). On Days 1, 2, and 3 p.i., the nasal septa, trachea, lungs, and stomach were removed, homogenized in assay media, and assayed for infectious virus by standard plaque assay on Vero cells. Stomach homogenates were cytotoxic to the cell monolayers at dilutions less than 1:100, at and above which no infectious virus was detected. Symbols: ●, 186 WT; □, 186ΔKpn.
of 5BlacZ or an equivalent volume of CE, at 0 and 3 weeks and given an i.n. challenge of $2 \times 10^6$ PFU/mouse of 186ΔKpn or an equivalent volume of CE at 6 weeks p.i. The TG were removed at 4 weeks postchallenge, and in situ hybridization was performed, probing for LATs. We observed that immunization of mice with 5BlacZ reduced the number of neurons expressing LATs (as detected by in situ hybridization) by approximately ninefold after intranasal challenge with the replication-competent TK⁻ virus 186ΔKpn (Table 2). The numbers of LAT-positive neurons in the immunized animals were significantly less than the number of positive neurons in the control animal TG ($P < 0.01$; Mann–Whitney U test).

**Immunization with 5BlacZ virus reduces challenge viral burden in the TG.** To test if immunization with 5BlacZ reduced the amount of latent viral DNA in the TG, and not just the expression of the LAT transcripts, we harvested TG of immunized and mock-immunized mice 30 days after i.n. challenge with $2 \times 10^6$ PFU/mouse of 186ΔKpn and quantified the number of HSV-2 DNA molecules in whole TG homogenates by quantitative PCR. Figure 3A shows representative standards generated by PCR amplification of known amounts of HSV-2 DNA in ganglion homogenates, and Fig. 3B shows PCR products from ganglia of mock-immunized and immunized animals. We found that mice immunized with 5BlacZ had a 43-fold reduction in the number of viral DNA molecules in the TG compared to that of mock-immunized mice (Table 2). This indicated that there was a reduction in the total amount of latent challenge virus in the TG and not just a change in the expression of the viral gene products.

To test if the HSV-2 replication-defective mutant could elicit protection against latent infection after intranasal challenge by a virulent strain of HSV-2, a second experiment was performed in which mice were immunized twice s.c. with $2 \times 10^6$ PFU of 5BlacZ or an equivalent volume of cell lysate, and again challenged with $2 \times 10^6$ PFU/mouse of 186ΔKpn. In addition, a group of 5BlacZ-immunized mice was challenged with $2 \times 10^6$ PFU/mouse of wt HSV-2 virus. The mock-immunized animals were not challenged with the wt strain because we had shown in earlier experiments (Table 1) that wt HSV-2 causes lethal encephalitis in mice, so there were no survivors with latent infection. In this experiment we found a 1000-fold reduction in the number of viral DNA molecules in mice immunized with 5BlacZ and challenged with 186ΔKpn, compared to that of mock-immunized mice (Table 3). The level of viral DNA in TG from immunized mice was significantly less than the level of DNA in TG from mock-immunized mice ($P < 0.01$; Mann–Whitney U test). Mice that had been immunized prior to challenge with the virulent wt strain did not develop clinical signs of HSV-2 CNS disease and had 100-fold fewer DNA molecules than mock-immunized

### Table 2

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Challenge</th>
<th>Number of ganglia analyzed by ISH</th>
<th>Mean no. LAT⁺ neurons/ganglion</th>
<th>Number of ganglia analyzed by PCR</th>
<th>Log₁₀ mean no. HSV DNA molecules/ganglion</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE</td>
<td>186ΔKpn</td>
<td>6</td>
<td>75.8 ± 22.4</td>
<td>5</td>
<td>4.02 ± 0.21</td>
</tr>
<tr>
<td>5BlacZ</td>
<td>186ΔKpn</td>
<td>6</td>
<td>8.3 ± 2.4</td>
<td>6</td>
<td>2.39 ± 0.39</td>
</tr>
<tr>
<td>CE</td>
<td>CE</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*All available sections were analyzed by in situ hybridization for LAT.*

*Contralateral ganglia were analyzed by quantitative PCR for HSV DNA.*

*Values represent log₁₀ geometric means ± SEM. Level of detection was 10 molecules/TG.*

*CE = uninfected cell extract.*

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**FIG. 3.** Effect of immunization with 5BlacZ on the latent viral load of challenge virus. Mice were immunized twice s.c. with either 5BlacZ or CE and challenged i.n. with 186ΔKpn at 3 weeks postboost. The latent viral load was determined by PCR detection of HSV-2 DNA. (A) An autoradiograph of a probed Southern blot of PCR products of HSV-2 DNA standards in TG homogenates. Numbers above the blot represent the number of HSV-2 DNA molecules in each reaction. (B) An autoradiograph of a probed Southern blot of PCR products of TG homogenates from mice immunized with 5BlacZ (5BlacZ/186ΔKpn: six samples from six mice) or from mice mock-immunized with CE (CE/186ΔKpn: five samples from five mice), CE/CE lanes (three samples from three mice) show products from mice mock-immunized and mock-challenged with cell extract. Each lane contains PCR products of one-half of a TG homogenate of a single mouse. M, markers.
mice challenged with 186ΔKpn (Table 3). The levels of DNA were again significantly different (P < 0.01; Mann–Whitney U test). Therefore, immunization with 5BlacZ protected against latent infection by attenuated or virulent challenge viruses.

An important safety concern of live-attenuated HSV vaccines is whether they could recombine with a wild-type (wt) virus in vivo to form a virulent strain. For this reason, an HSV-2 replication-defective double-deletion mutant, d/5-29 (defective in two essential genes for HSV-2 replication: UL5 and UL29), has been constructed (Da Costa et al., 1999). The preceding experiment was performed together with a subset of mice that had been immunized with either the double mutant 5BlacZ, and challenged intranasally with 186ΔKpn or wt HSV-2. Immunization with d/5-29 reduced latent infection by challenge virus DNA by several thousand-fold (Da Costa et al., 1999). Thus d/5-29, the double-deletion mutant, reduced the latent viral DNA burden in mouse TG at least as well as the single mutant 5BlacZ, against wt or 186ΔKpn intranasal challenge.

HSV-2 replication-defective mutants protect against challenge virus infection by reducing viral replication at the site of inoculation and in the trigeminal ganglia

To determine the stage of infection at which the vaccine-induced immune response acted to reduce latent infection by the challenge virus, a subgroup of mice in the preceding experiment were euthanized on Days 1 to 5 postchallenge, and their nasal septa, TG, and brains were removed. Infectious virus in homogenates of these tissues was titrated. We observed that mice immunized with either the double mutant d/5-29 or the single mutant 5BlacZ had a 10- to 100-fold reduction in replication in the nasal septa, as early as Day 1 postchallenge with wt186 virus, and a faster rate of clearance of productive infection at this site (Fig. 4A). A 100-fold reduction in viral titer was also detected when immunized mice were challenged with the 186ΔKpn strain (C. A. Jones and D. M. Knipe, unpublished results) (Fig. 4A). Therefore, the immune response induced by the double mutant d/5-29 acted to reduce latent infection by reducing replication at the site of inoculation of the challenge virus. Although the immunity induced by d/5-29 appeared to clear challenge virus from the nasal tissue faster than the immunity induced by 5BlacZ (Fig. 4A), we have not observed differences between immunity induced by single and double mutants in other experiments (Da Costa et al., 1999).

In trigeminal ganglia, thymidine kinase-deficient strains are characteristically replication-defective; thus, only the trigeminal ganglia, thymidine kinase-deficient strains were assayed for infectious virus at this site. We observed that immunized mice had low levels of infectious virus in the TG on Day 2 postchallenge, which represented a 2.5 log10 reduction in titer compared to that of mock-immunized mice. Infectious virus in the TG of immunized mice was cleared by 4 days postchallenge (Fig. 4B). Therefore, the HSV-2 replication-defective mutants protected against latent infection by reducing replication of the challenge virus both at the site of inoculation and in the TG.

In brain tissue we detected infectious wt virus only in mock-immunized mice, observed first on Day 3 postchallenge and peaking on Day 5, after which time the mice died. No infectious wt virus was detected in the brains of mice immunized with either the single or double HSV-2 replication-defective mutant (Fig. 5), indicating that immunization also protected against virus reaching the CNS.

Do the replication-defective mutants establish latency in the TG after intranasal inoculation?

The ability of a potential vaccine strain to establish latent infection is important to determine as a safety feature and as a control for the immunization experiments. To determine if 5BlacZ could establish latent infection in the TG as detected by in situ hybridization for LATs, a group of mice was mock-immunized with CE at 0 and 3 weeks and challenged with 5BlacZ intranasally 6 weeks later. TG were harvested 30 days p.i. One trigeminal ganglion per mouse was sectioned and probed for LATs at 4 weeks postchallenge. We observed that 5BlacZ

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Challenge</th>
<th>No. TG</th>
<th>DNA molecules/ganglion*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5BlacZ</td>
<td>186ΔKpn</td>
<td>6</td>
<td>1.1 × 10^3 ± 2.0 × 10^0</td>
</tr>
<tr>
<td>5BlacZ</td>
<td>wt</td>
<td>6</td>
<td>1.4 × 10^2 ± 3.9 × 10^1</td>
</tr>
<tr>
<td>CE</td>
<td>186ΔKpn</td>
<td>6</td>
<td>8.8 × 10^3 ± 4.9 × 10^4</td>
</tr>
</tbody>
</table>

* Lower limit of detection was 10 molecules/TG and values represent geometric means + SEM.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>2TK-1</td>
<td>TGG ATT ACG ATC AGT CGC C</td>
</tr>
<tr>
<td></td>
<td>2TK-2</td>
<td>ACA CCA CAC GAC AAC AAT GC</td>
</tr>
<tr>
<td></td>
<td>2TK-3</td>
<td>CCA TCG CGG AGA TAC GCG AC</td>
</tr>
<tr>
<td>Mouse adipsin</td>
<td>MT-1</td>
<td>AGT GTG CGG GGA TGC AGT</td>
</tr>
<tr>
<td></td>
<td>MT-2</td>
<td>ACG CGA GAG CCC CAC GTA</td>
</tr>
</tbody>
</table>

* Sequences were reported by Katz et al. (1990).
did not establish latent infection in the TG, as determined by in situ hybridization for LAT expression (Table 3).

To test if 5BlacZ DNA could be detected in the TG, we assayed the other ganglion of these same mice for viral DNA by Q-PCR 30 days post i.n. inoculation. We observed that 5BlacZ DNA could be detected in only one of five TG tested (of five different mice) to the order of 1 to a few molecules (Fig. 6). No DNA was detected in mice given CE i.n. (Fig. 6) or in the water blanks (C. A. Jones and D. M. Knipe, unpublished results). Therefore, 5BlacZ, the single mutant rarely and inefficiently establishes latency in the TG.

To test whether infectious d5-29 reaches the TG, we inoculated mice i.n. with $1 \times 10^6$ PFU per naris of d5-29.
186ΔKpn, or wt virus, and harvested the TG at 4 h, 20 h, and on Days 1 to 5 and 7 p.i. The titer of infectious virus in the TG was determined on a complementing cell line (Fig. 7). No infectious dl5-29 virus was detected at any timepoint in the TG, whereas wt virus could be detected by Day 3 p.i., peaking on Day 4 p.i. Low levels of infectious 186ΔKpn could be detected only on Day 6 p.i. Assays of dl5-29 DNA in the TG showed that it accumulated there in low levels only transiently at Days 2 and 3 p.i. (Da Costa et al., 1999).

**Lack of persistence of replication-defective mutant DNA at the site of inoculation**

We previously showed that the HSV-1 replication-defective mutants elicit durable immune protection in the mouse eye model of disease (Morrison and Knipe, 1994). The mechanism of this long-lived immunity is unclear. One possible explanation is the presence of a persistent antigenic stimulus. Intranasal immunization with 5BlacZ induced protective immunity in mice (Morrison et al., 1997). To test if the HSV-2 double-mutant dl5-29 DNA persists at the site of inoculation, mice were given i.n. 2 × 10^6 PFU/mouse of dl5-29, 186ΔKpn, wt virus, or an equivalent volume of CE. Animals were sacrificed on Days 1 to 7 p.i., and the nasal septa were removed. The tissues were homogenized and ½ of each sample was assayed for infectious virus (on a complementing cell line) while ½ was used to determine the abundance of viral DNA by Q-PCR. We reported elsewhere that the titer of infectious dl5-29 declined rapidly to undetectable levels by Day 1 p.i. (Da Costa et al., 1999). The presence of dl5-29 DNA in the nasal septa was detected until Day 3 p.i. WT and 186ΔKpn DNAs were detected through at least Days 5 and 7, respectively (Fig. 8). Therefore, dl5-29 DNA either did not persist at the site of inoculation in nasal septa tissues or persisted at levels too low to be detected.

**DISCUSSION**

We used a mouse nasal model of HSV-2 infection to examine the in vivo behavior of several HSV-2 strains and to examine the ability of HSV-2 replication-defective mutants to protect against latent infection by challenge virus. Infection of HSV-2 by the intranasal route is of significance because this is a frequent route of virus entry in the newborn and because it may be used as a route of immunization to induce mucosal immunity, particularly in the genital tract. Prior to this work, intranasal HSV-2 infection had not been characterized in adult mice. The inoculation of HSV-1 into the nares of animals was previously studied extensively as a model of HSV-induced encephalitis (Beers et al., 1993; Drummond et al., 1994; Esiris et al., 1995; Walev et al., 1995) and pneumonia (Adler et al., 1997; Davis et al., 1997). In
contrast, reports of intranasal inoculation of HSV-2 had been limited to a rabbit model of keratitis (Stroop et al., 1994), neonatal models of mice (Kern et al., 1986) and guinea pigs (Bravo et al., 1994; Mani et al., 1996), or in studies of passive immunotherapy in mice (Erlich and Mills, 1986). In these reports, the initial events after inoculation of virus into the nares were not extensively described. Therefore, we chose to describe the events after intranasal inoculation of HSV-2 and to evaluate nasal septa homogenates as a tool for monitoring replication at this site.

We demonstrated that after intranasal inoculation of HSV-2 in a small volume, the majority of the inoculum remains in the nasal passage, with only small amounts being swallowed or aspirated, because the major site of replication is in the nasal tissue. This has important implications for use of this route as a vaccine. In addition, nasal septa homogenates proved a valuable tool for monitoring replication at the site of inoculation, given the absence of infectious virus in nasal swabs in mice. The lack of infectious virus in nasal swabs was likely due to the small diameter of murine nasal passages. This contrasts with the results with rabbits (Stroop et al., 1994) or guinea pigs (Bravo et al., 1994; Mani et al., 1996), in which nasal swabs readily detect infectious virus.

HSV-2 strains have an enhanced capacity to enter and replicate in the central nervous system of mice (Richards et al., 1981) and newborn guinea pigs (Mani et al., 1996) when compared to that of HSV-1. Thus, we isolated and utilized a thymidine kinase-negative mutant virus to study latent infection following intranasal inoculation. HSV-2 strains deficient for the expression of the thymidine-kinase gene were previously reported to be neuroattenuated when given via the vaginal route in mice (McDermott et al., 1984) and guinea pigs (Stanberry et al., 1985), and via the corneal route in rabbits (Stroop et al.,

FIG. 6. Lack of persistence of 5BlacZ DNA in trigeminal ganglia. The levels of HSV-2 viral DNAs in TG were determined at 30 days postchallenge in mice inoculated i.n. with 5BlacZ (five TG samples from five mice) or CE (3 TG from 3 mice). Shown is an autoradiograph with a long exposure (>2 t1/2's) of a probed Southern blot of Q-PCR products of one-half of TG homogenates run in duplicate. M, markers.

FIG. 7. Acute viral replication in trigeminal ganglia. Infectious virus was titrated in TG of mice at 4 h, 20 h, and on Days 1–5 and 7 post i.n. inoculation with dl5-29, 186ΔKpn, or wt virus (N = 8/timepoint/group). Values shown are the geometric means of the viral titers ± SEM. Assay was performed on a cell line (V5-29) that complements the defective genes in dl5-29. Symbols: ◆, 186 wt virus; □, 186ΔKpn TK− virus; X, dl5-29 virus.
1994). Others reported HSV-2 TK− strains with single missense mutations in the nucleotide-binding site that retained neurovirulence in mice (Tanaka et al., 1993). In these experiments we demonstrated that an HSV-2 TK− construct in the background of a virulent HSV-2 strain (186) was neuroattenuated, despite being administered in close proximity to the CNS. This is consistent with the findings of Nishiyama et al. (1991), who inoculated mice with a TK− strain in the same background as that of our study virus (although the location and size of the mutation were different) via the intracerebral route and showed an LD50 of 10^5 PFU/mouse. After intranasal inoculation, this TK− strain, like the wt strain, grew predominantly in the nares, but did so 10- to 100-fold less efficiently than wt. We also observed some low-level replication in the TG, which was not reported for other HSV-2 TK− mutants in the TG, but seen with the same mutation in another genetic background (strain 333) in the sacral ganglia (McDermott et al., 1984). It is possible that within the HSV-2 background, the block to acute infection in the TG is more easily overcome by complementation with cellular factors.

Latent infection of trigeminal ganglia by HSV-2 was previously described by Mitchell et al. (1990) using an attenuated strain (HG52) given via the corneal route. Our studies showed that the number of LAT-positive neurons was proportional to the dose of virus inoculated intranasally, thus providing a quantitative assay for the number of cells that are latently infected.

Protection against latent infection as measured by two assays

Having shown that the number of LAT-positive cells was proportional to the virus inoculum dose, we examined the effect of prior immunization with a replication-defective mutant virus, 5BlacZ, on the number of cells that become latently infected with an HSV-2 challenge strain following intranasal inoculation. Prior immunization reduced the number of LAT-positive cells by approximately ninefold. In contrast, PCR measurement of viral DNA in the latently infected ganglia showed a 43-fold reduction in viral DNA in the latently infected ganglia as a result of prior immunization. This suggests that there is a reduction in the number of latently infected cells and a reduction in the number of viral DNA molecules in the neurons that do become latently infected as a result of preexisting immunity.

It remains to be determined whether the number of latently infected cells or the DNA content per cell—or both factors—is the more relevant determinant in defining the likelihood of reactivation and recurrent disease. Several studies have shown a relationship between the level of latent infection and the probability of reactivation, but these studies did not distinguish between DNA content and number of latently infected cells as being the important factor in defining reactivation ability. Perng et al. (1996) used a rabbit ocular infection model to show that an ICP34.5-deletion mutant showed wild type levels
of spontaneous reactivation at high doses. Both Maggoncalda et al. (1996) and Gordon et al. (1996) correlated decreased rates of reactivation as determined by explant cocultivation with a reduced number of latently infected rabbit TG after corneal inoculation of LAT− mutants. Lekstrom-Hines et al. (1998) correlated the recurrence rate of disease of HSV-1 and HSV-2 strains from the sacral ganglia of guinea pigs to the level of DNA in the ganglia at this site and not to the strain or concentration of LATs. The importance of a reduction of latent viral burden in humans was previously shown indirectly by clinical studies that correlate the frequency of recurrence of genital herpes with the severity of the initial lesion and, hence, the burden of latent DNA in humans (Corey et al., 1983). Whether there exists in humans, as Gordon et al. (1995) hypothesized to exist in rabbits, a threshold level of latency required for efficient reactivation has not yet been established.

Prior immunization reduced challenge virus replication in both nasal tissue and in the trigeminal ganglia. Immune effector mechanisms may function at both primary and secondary sites of viral replication or just at the primary site of infection. The extent to which primary-site replication versus secondary-site replication is affected may vary with different experimental systems because, in another system, we observed that reduced viral replication in the ganglia may be the major effect of preexisting immunity (M. Kramer and D. M. Knipe, manuscript in preparation).

The immune mechanism(s) responsible for reducing acute viral replication and latent infection also remains to be defined. Clearance of virus from infected tissue was previously shown to be predominantly CD4+ T-cell dependent in mice after intravaginal inoculation of HSV-2 (Lekstrom-Hines et al., 1998). In contrast, passage of virus from the TG to the CNS was previously shown to be reduced by the presence of HSV-specific antibody (Kapoor et al., 1982; Simmons and Nash, 1985).

Lack of persistence of the replication-defective mutants in the CNS and in the periphery

HSV-2 vaccine candidates have only rarely been assessed for their ability to establish latent infection in their own right (Klein et al., 1984; Meignier et al., 1988; Thompson et al., 1986). Lack of ability to establish latent infection would be an important characteristic, from a safety standpoint, to reduce the possibility of recombination with a wild type strain in vivo. We showed that the single mutant 5BlacZ rarely and inefficiently established latent infection, as detected by quantitative PCR for viral DNA and in situ hybridization for LAT. We used this same system to show that the double-deletion mutant dl5-29 shows low, transient accumulation of viral DNA in the trigeminal ganglia but does not persist in that tissue (Da Costa et al., 1999). These results show that these mutants establish latent infection to a greatly reduced degree or that they do not establish a stable latent infection. Further studies on the time course and nature of the mutant viral DNA in the ganglia are underway in our laboratory.

HSV-1 replication-defective mutants were previously shown to elicit a durable immune response (Morrison and Knipe, 1994). Durable antibody responses could be due to long-lived plasma cells and thus independent of antigenic stimulus and T-cell activation. Protective immunity, which is highly dependent on CD4+ T cells, is also durable (Morrison and Knipe, 1996). This could be due to a memory T-cell response or to persistence of an antigenic stimulus. HSV DNA was previously reported to be present at a number of sites outside the CNS (reviewed in Hill, 1985). For HSV-2, this has largely been within the footpad of mice (Clements and Subak-Sharpe, 1988) and within the vagina of guinea pigs (Scriba, 1981), although the frequent recurrences seen in guinea pigs has made the phenomenon of peripheral persistence contentious in that system. Simmons et al. (1997) recently showed the presence of HSV-1 viral DNA in keratinocytes of mouse footpads after scarification. This DNA was detected for more than 2 weeks after the disappearance of infectious virus and viral antigens, and the location of the DNA became more superficial with time. We previously showed that an HSV-1 replication-defective mutant virus persists in an infectious form up to 7 days post-inoculation in the footpad (Morrison and Knipe, 1994). Infectious HSV-2, however, is rapidly cleared from the nasal septa after intranasal inoculation of the HSV-2 replication-defective mutant (Da Costa et al., 1999). We demonstrated in these experiments that the viral DNA of the HSV-2 replication-defective mutants does not persist beyond 5 days p.i. in the nares after intranasal inoculation. In addition, we were also unable to demonstrate persistence of dl5-29 (the HSV-2 double-replication-defective mutant) DNA in the skin after intradermal inoculation at Days 3 and 30 p.i. and in muscle at Day 30 p.i. after intramuscular injection (C. A. Jones and D. M. Knipe, unpublished results). Nor did we demonstrate dl5-29 DNA in the regional lymph nodes at Day 30 after intradermal inoculation into the face (C. A. Jones, M. F., Kramer, and D. M. Knipe, unpublished results). This suggests that the durable immune protection elicited by these replication-defective strains is due to a memory immune response that can be rapidly mobilized to provide protective immunity. The lack of persistence of these replication-defective mutant strains combined with their ability to induce long-lived immune responses provide unique features for their use as vaccines and vaccine vectors.

MATERIALS AND METHODS

Viruses and cells

The origin of the 186 syn+1 strain of HSV-2 used as the wild-type (wt) strain in these experiments was de-
scribed previously (Spang et al., 1983). The construction of 5BlacZ, the replication-defective mutant of HSV-2 strain 186 syn^-1 defective for the gene encoding ICP8 (UL29) due to insertion of lacZ coding sequences, was described previously (Da Costa et al., 1997), and the construction of dl5-29, a replication-defective mutant of HSV-2 strain 186syn^-1 defective in UL29 and UL5, is reported elsewhere (Da Costa et al., 1999, 2000).

HSV-2 strains 186 and 186ΔKpn were propagated and assayed on Vero cells as described previously (Gao and Knipe, 1989), and 5BlacZ and dl5-29 were propagated on V5-29 cells that were stably transfected with UL5 and UL29 (Da Costa et al., 1997, 1999, 2000). For immunization experiments, all stocks, including wild-type strains, were titered on the complementing cell line.

Construction of 186ΔKpn. A thymidine kinase-negative mutant of HSV-2 strain 186 syn^-1 was constructed by an approach similar to that of McDermott et al. (1984), by deleting a 213-bp KpnI–KpnI fragment from the plasmid pEH48 (Spang et al., 1983), which contains the HindIII–EcoRI fragment of HSV-2 strain 186 from approximately 0.28–0.31 map units inserted into pBR322. The resulting plasmid, pEH48ΔKpn, was cotransfected with HSV-2 wt strain 186 DNA (Fig. 1), as described elsewhere (Da Costa et al., 1997). The mutant virus was selected using 100 μM acyclovir and, after three rounds of plaque purification, the presence of the mutation was confirmed by Southern hybridization (Da Costa et al., 1997). Digestion of the mutant DNA with PvuII demonstrated a lack of the 1.5-kbp fragment from the TK gene and a new, faster-migrating fragment (results not shown). This mutant virus was named 186ΔKpn.

Construction of the HSV-2 LAT probe plasmid pBP2. The HSV-2 LAT probe plasmid pBP2 was constructed by digesting HSV-2 strain 186 syn^-1 DNA with BamHI (New England Biolabs, Beverly, MA) and cloning fragments in the range of 3–5 kbp which would contain the BamHI–P fragment (shown to be 3.4 kbp in strain 333 by Mitchell et al., 1990) into pUC19. The presence of the desired insert was confirmed by restriction endonuclease analysis. To ensure that the probe did not contain DNA that hybridized to ICP0 gene transcripts, the 876-bp BamHI–PvuII fragment within the BamHI–P fragment was subcloned into pZERO 2.1 (Invitrogen, San Diego, CA), to give the plasmid pBP2 (Fig. 1B). The presence of the correct insert was again confirmed by restriction digest patterns and by comparing the DNA sequence of the insert to a published sequence of the LAT-encoding region in HSV-2 strain HG52 (Davison et al., 1981).

Animal protocols

Six-week-old female Balb/c mice (Taconic Farms, Germantown, NY) were acclimated for 1 week prior to use. All experiments were conducted in accordance with the institutional guidelines for animal welfare.

Intranasal inoculation of virus. Mice were anesthetized with metophane, and 5 μl of virus stock was delivered into each nostril with the aid of a micropipettor. The inoculum was subsequently inhaled by the mouse. The dose of virus administered was 1 × 10^6 PFU/naris, unless stated otherwise. For immunization studies, uninfected cell extract from the same cell line on which the immunogen was derived was administered in the same volume.

Subcutaneous immunization. The fur was clipped at the base of the tail to expose the skin, and a dose of virus equal to 1 × 10^6 PFU or an equivalent volume of uninfected cell extract (CE) in a total volume of 20 μl of saline was injected subcutaneously (s.c.) into the left rear flank near the base of the tail with a 26-gauge needle.

Harvesting of tissue samples. All mice were euthanized prior to harvesting of tissue samples. Trigeminal ganglia, brains, nasal septa, and internal organs for standard plaque assay were removed by sterile technique and placed into 1.0 ml of assay media. Nasal septa were harvested by stripping skin and subcutaneous tissue from the face of the mouse, dissecting through each external nasal orifice to the ipsilateral orbit and then extending the incision on both sides from the orbit to the frontal lobe to form a diamond-shaped plate of bone. The bone flap was carefully removed, and the rudder-shaped nasal septa were identified in the midline. The nasal septa, complete with epithelial lining, were removed using sterile technique after dissecting the tissue from its anterior attachment.

Assays of acute infection

Tissue samples that were used solely for assay of infectious virus were placed in sterile vials containing 1.0 ml of assay media and then frozen at −80°C until use. At the time of assay, the tissue was thawed on ice and homogenized using a sterile Kontes pestle (Garber et al., 1997). Standard plaque assay was performed on the appropriate cells as described elsewhere (Knipe and Spang, 1982).

In situ hybridization

Trigeminal ganglion samples for in situ hybridization were placed into sterile vials and frozen at −80°C in OCT compound embedding medium until they were cryosectioned. No evidence of tissue desiccation was present when the samples were sectioned. The TG were sectioned at 8-μm thickness and placed serially on a slide to allow correction for double counting of LAT-positive profiles within the one neuron. TG sections were probed using the double-stranded DNA LAT probe pBP2, labeled with [α-35S]dATP and -dCTP (Amersham, Arlington Heights, IL) by nick translation (Boehringer-Mannheim, Indianapolis, IN). Occasional lost sections were recorded and corrected for in the final count, although all
remaining sections were examined and counted. The method used for in situ hybridization was described previously (Garber et al., 1997; Kosz-Vnenchak et al., 1993).

Preparation of ganglionic DNA and PCR methods

Trigeminal ganglia were collected under stringent conditions to avoid contamination and were stored at −80°C until digestion. Tissues were handled and assays were performed as outlined by Kramer and Coen (1995), with a few modifications. Specimens were digested with proteinase K (0.2 mg/ml) in a volume of 50 μl. Digestion was at 50°C for 12–18 h. The enzyme was inactivated by heating the mixture to 95°C for 10 min. One-half (25 μl) at 50°C for 12–18 h. The enzyme was inactivated by heating the mixture to 95°C for 10 min. One-half (25 μl) of the digested TG sample was assayed directly for viral DNA using the Advantage-GC Genomic Polymerase Mix (Clontech, Palo Alto, CA) as per the manufacturer's instructions together with Perfect Match Polymerase Enhancer (Stratagene, La Jolla, CA) in a final volume of 100 μl. Primers and the probe for the HSV-2 gene are listed in Table 4. PCR mixtures were denatured at 94°C for 7 min, cycled at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles. Each set of samples was assayed with a series of HSV-2 DNA standards composed of TG DNA and known amounts of HSV-2 186 DNA, prepared as described elsewhere (Katz et al., 1990), in parallel with appropriate mock-infected tissue and water blanks. PCR products were then resolved on an 8% polyacrylamide gel, stained with SYBR-Green 1 (Molecular Probes, Eugene, OR), and visualized with UV light. Visible DNA products were analyzed using the Fluor-S Multilimage (Bio-Rad, Richmond, CA) system. As the majority of products were of low abundance, the DNA was transferred to Genescreen Plus (NEN Research Products, Boston, MA), prehybridized, probed, and washed as previously described (Kramer and Coen, 1995). The internal oligonucleotide 2TK-3 (Table 4) was end-labeled with [32P]ATP (ICN, Irvine, CA) to a specific activity greater than 1 × 106 cpm/μg. The labeled filter was then exposed to a Molecular Imager cassette (Bio-Rad) for 5 to 24 h. The amount of DNA was quantified after either analysis using the MultiAnalyzer/Macintosh software (Bio-Rad). When the entire TG was not directly digested and analyzed, the recovery was normalized for the amount of cellular DNA by coamplification for the single-copy mouse adipsin gene as described elsewhere (Kramer and Coen, 1995). Primers for the mouse adipsin gene (Table 4) were as reported by Katz et al. (1990).

Statistics

For the immunization studies, the Mann–Whitney U test was used to determine the significance of the differences in (1) the numbers of LAT-positive neurons by ISH or (2) the abundance of HSV-2 viral DNA in the TG by Q-PCR in latently infected trigeminal ganglia of mice immunized with 5BlacZ or mock-immunized with CE.

ACKNOWLEDGMENTS

This work was supported by Public Health Service Grant Al20410 from the National Institutes of Health. C.A.J. was supported by the Infectious Diseases Society of America—Connaught Laboratories Fellowship for Pediatricians in Infectious Diseases. The technical assistance of David Spezzano is gratefully acknowledged. Instruction in the technique of quantitative PCR by Martha Kramer and nasal septa dissection by Brian Ackerley is greatly appreciated. We thank the members of the Knipe lab for their helpful comments on this work and Lisa Holik for her assistance in the preparation of the manuscript.

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