Maternal Immunization with a Herpes Simplex Virus Type 2 Replication-Defective Virus Reduces Visceral Dissemination but Not Lethal Encephalitis in Newborn Mice after Oral Challenge

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Herpes simplex virus (HSV) causes devastating infections in newborns. Maternal immunization is one potential strategy to reduce neonatal HSV disease. Female mice were immunized with an HSV type 2 (HSV-2) replication-defective mutant (HSV-2 dl5-29, which is defective for the genes UL5 and UL29) and then mated. Protection was evaluated in newborn mice after a virulent HSV-2 oral challenge. Heightened neonatal susceptibility was observed to a thymidine kinase-negative HSV-2 strain (HSV-2 186ΔKpn) that is highly attenuated in adult mice. Maternal immunization with HSV-2 dl5-29 and HSV-2 186ΔKpn reduced visceral spread of infectious challenge virus in pups after challenge at either 1 day or 1 week of age but did not prevent replication at the site of entry, spread to the central nervous system, or lethal encephalitis. No protection was seen in pups born to mock-immunized mothers or to mothers immunized with a UV-inactivated wild-type HSV-2 strain. Levels of protection correlated with levels of passively transferred maternal HSV-2-specific IgG antibody.

Herpes simplex virus (HSV) is a common human pathogen that can have devastating effects if transmitted to newborns. The incidence of neonatal HSV ranges from 1:3500 to 1:8700 live births in the United States [1–3] and from 1:30,000 to 1:60,000 live births in Australia [4] and the United Kingdom [5]. Neonatal infection can manifest as disease localized to the skin, eye, or mouth as encephalitis or pneumonitis or as disseminated infection with multiorgan failure. Long-term sequelae include developmental delay, seizures, and motor and visual impairment. The development of preventive or therapeutic strategies against HSV in this age group remains a priority, because morbidity and mortality remain unacceptably high despite the availability of antiviral agents.

Maternal immunization is a strategy that has been used effectively to protect mothers and newborns against a range of life-threatening diseases, including tetanus [6], influenza [6–8], and poliomyelitis infection [8–10]. Transmission of HSV to neonates occurs primarily through an infected birth canal, although it also can result from intrauterine infection or can be caused postpartum by contact with an infected caregiver.

The capacity of maternal antibody to protect the neonate against HSV disease has been controversial. Early clinical studies found no correlation between levels of maternal anti-HSV antibody and neonatal disease [11, 12], unlike data from animal studies [13, 14]. Subsequent epidemiologic studies found that high neonatal neutralizing antibody titers were associated with a reduction in disease severity [15–17], and a recent report showed that the highest neonatal attack rate is in the offspring of women with an initial HSV infection during pregnancy who have not seroconverted by the time of delivery (30%–50% risk vs. 3%–5% risk for the offspring of women with recurrent infection) [18]. Possible ways in which prior maternal immunization against HSV may ameliorate newborn disease are by reducing the risk of maternal genital infection, reducing the titer and duration of viral shedding in the genital tract if maternal infection occurs, reducing the amount of virus to which the neonate is exposed, and/or by providing protection through the passive transfer of HSV-specific antibodies [19].

An effective vaccine against HSV has eluded researchers for decades, although a number of strategies show promise (reviewed in [19]). These include attenuated viruses [20], replication-defective viruses [21, 22], heterologous viral vectors expressing HSV proteins [23, 24], inactivated virus, viral protein subunits [25], and DNA vaccines [26, 27]. The use of replication-defective HSV mutants is a vaccine strategy that has been safe and immunogenic in a range of adult animal models of HSV disease [28–31]. Replication-defective HSV mutants are defective in gene(s) essential for viral replication and are propagated on cell lines expressing the missing protein(s). The HSV type 2 (HSV-2) rep-
lication-defective mutant 5Blac Z, which is defective for the gene encoding ICP8, protects against acute and recurrent genital disease in mouse and guinea pig models [28, 31] and is capable of eliciting an HSV-specific systemic and mucosal immune response [31]. The double deletion HSV-2 186syn-1 mutant dl5-29 (HSV-2 dl5-29), which is defective for the genes UL5 (encoding the helicase primase complex) and UL29 (encoding DNA polymerase) also elicits protective immunity against genital challenge and reduces the burden of latent infection by wild-type (wt) virus [29]. One potential application of these safe immunogenic vaccine candidates is as a maternal vaccine against genital HSV infection, to prevent or ameliorate neonatal HSV disease. In this study, we sought to determine whether maternal immunization with HSV-2 dl5-29 could confer protection against disease to newborn mice after a high-dose HSV-2 oral challenge at ages 1 day and 1 week.

Materials and Methods

Viruses and cells. The construction of the replication-competent, thymidine kinase (TK)-negative mutant strain of HSV-2 186syn-1 (HSV-2 186ΔKpn) has been described elsewhere [30]. Stocks of the wt HSV-2 strain 186syn-1 [32] and HSV-2 186ΔKpn were propagated and assayed on Vero cell monolayers and stored at −80°C as infected cell extracts. UV-inactivated 186syn-1 virus (HSV-2 UV-186) was made by exposing wt virus stock to 254 nm of UV light for 3 h. The titer after UV inactivation was <10 pfu/mL. The construction of HSV-2 dl5-29 has been described elsewhere [33]. Stocks of HSV-2 dl5-29 were propagated and assayed on V5-29 cells that were stably transfected with UL5 and UL29 [28]. For immunization experiments, all stocks were titered on the complementing cell line. Uninfected cell extract was prepared in the same manner as for virus stocks and used for mock immunization.

Immunization procedures. Female 5-week-old BALB/c mice were purchased from the Animal Resource Centre and acclimated for 1 week before use. Series of mice were immunized subcutaneously with 2 × 10^6 pfu/mouse of either HSV-2 186ΔKpn, HSV-2 dl5-29, HSV-2 UV-186, or an equivalent amount of uninfected V5-29 cell extract in a 30-μL volume near the base of the tail. Mice were boosted in the same manner 3 weeks later and mated the next day. Pregnant mice were observed daily, and the day of birth was recorded as the day the litter was found. Pups were challenged orally at either 1 day or 1 week of age with 2 × 10^6 pfu/mouse of HSV-2 186ΔKpn in 10 μL. Pups were examined daily for signs of lethal disease (pallor or cyanosis, rapid breathing, decreased activity, or paralysis).

Assays for infectious virus titer. At 12 h and on postchallenge (pc) days 1, 2, 3, and 4, pups were euthanized, and the brain, lungs, stomach, liver, kidneys, and spleen were collected and frozen in 0.5 mL of assay medium (PBS containing 0.1% fetal bovine serum, 0.1% glucose, and 0.1% CaCl2). Organs were stored at −80°C until assayed for infectious virus titer by standard plaque assay, as described elsewhere [34].

Quantitative polymerase chain reaction (PCR) analysis. At a series of pc time points, pups were euthanized, and we collected the trigeminal ganglia (TG) and blood under stringent conditions to avoid contamination. Samples were stored at −80°C until digestion. Specimens were digested with proteinase K (0.2 mg/mL) in a volume of 50 μL for TG or double the volume of the blood sample at 50°C for 18 h (blood) or 3 h (TG) and then at 95°C for 10 min. Quantitative PCR was done on 15 μL of each sample in a final volume of 50 μL with the Advantage-GC genomic polymerase mix (Clontech) by using 2 primers from the region of the HSV-2 TK gene not deleted in HSV-2 186ΔKpn, as described elsewhere [30]. PCR mixtures were denatured at 94°C for 5 min and then cycled at 94°C, 60°C, and 72°C for 1 min each for 30 cycles. Each sample set was assayed with a series of HSV-2 strain 186 DNA standards of known concentration in parallel with appropriate mock-infected tissue and PCR mix controls. The amount of HSV-2 DNA per sample was normalized for the amount of cellular DNA by coamplification with the single-copy mouse adipsin gene, as described elsewhere [30]. Primers for the mouse adipsin gene were as reported by Katz et al. [35]. PCR products were resolved on an 8% polyacrylamide gel, stained with SYBR-Green I (Molecular Probes), and visualized with UV light. DNA was transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech), probed with a specific internal oligonucleotide probe [30], and end labeled with [γ-32P]dATP (GeneWorks) to a specific activity >10^8 cpm/μg. The labeled membrane was exposed to a storage phosphor screen (Molecular Dynamics) for 14–18 h, and the amount of DNA was quantified on a phosphorimager with ImageQuant software (Molecular Dynamics).

Determination of IgG antibody titers. Blood was collected from female mice of all immunization groups at the time of delivery and from pups at the time of challenge. Serum samples from each group were assayed for HSV-2–specific IgG1 and IgG2a antibody levels by standard ELISA. Immuno-pure MaxiSorp microwell plates (Nunc) were coated with 100 μL of UV-inactivated HSV-2–infected cell lysate in carbonate buffer (pH 9.5) or 1 μg/mL anti–mouse IgG1 or IgG2a (PharMingen) in PBS and incubated overnight at 4°C. The wells were washed with PBS and 0.5% Tween 20 and blocked for 1 h with blocking buffer (PBS and 10% heated fetal bovine serum [HBFS]). Standard mouse IgG1 or mouse IgG2a (PharMingen) was added to the anti-IgG1– or anti-IgG2a–coated wells at a concentration of 100 ng/mL and serially diluted 2-fold. Serum samples were added to the UV–HSV-2-coated wells and diluted 3-fold in blocking buffer. All plates were incubated overnight at 4°C. The wells were washed and then incubated with 100 μL of a mixture of biotin-labeled rat anti–mouse IgG1 or IgG2a and avidin–horseradish peroxidase conjugate (Pharmingen) in blocking buffer at room temperature for 1 h. Wells were washed and incubated with 100 μL of tetramethyl benzidine substrate solution (Pharmingen) in the dark at room temperature for 15 min. The reaction was stopped with 50 μL of 2N H2SO4, and plates were read on a plate reader (EL312; Bio-Tek Bio-kinetics) at 450 nm. The quantity of antibody in the serum was determined by comparison with the simultaneously run standard curve.

Assay of antibody neutralization activity. Blood was collected from female mice of all immunization groups at the time of delivery and from pups at the time of challenge. Serum samples from each group were pooled and assayed for HSV-2–specific neutralization activity, as described elsewhere [21]. In brief, serial 2-fold dilutions of serum in PBS or PBS alone were mixed with an equal volume of HSV-2 186ΔKpn at 10^5 pfu/mL and incubated for 2 h at 4°C. In all, 200 μL of the mixture was adsorbed to Vero cell monolayers in du-
After intranasal inoculation [30]. To determine whether HSV-2 186ΔKpn was also attenuated in newborn mice and hence could be used as a less virulent challenge to pups in the maternal immunization studies, series of naive pups were inoculated orally with a range of doses up to $2 \times 10^5$ pfu/mouse at either age 1 day or 1 week. Pups infected at age 1 day showed signs of lethal disease from age 3 days when given doses as low as $2 \times 10^2$ pfu/mouse (figure 1A). Survivors displayed no signs of disease up to 30 days after infection. In contrast, $>80\%$ of pups inoculated at age 1 week tolerated doses of HSV-2 186ΔKpn up to $2 \times 10^7$ pfu/mouse (figure 1B). Thus, newborn mice display a heightened susceptibility, even to HSV-2 strains that are highly attenuated in adult mice. This susceptibility was still present at age 1 week.

To test whether HSV-2 dl5-29 causes disease in newborn mice, series of newborn pups were also inoculated orally with the HSV-2 replication-defective mutant at doses of up to $2 \times 10^6$ pfu/mouse. No morbidity or mortality was observed up to the highest administered dose at any time point (data not shown). Thus, an HSV-2

**Results**

Immunization with HSV-2 dl5-29 was shown previously to induce protective immunity against acute genital HSV disease and latent infection in adult mice [29]. To determine whether maternal immunization would confer protection against neonatal disease, series of adult female mice were immunized with either HSV-2 dl5-29, HSV-2 186ΔKpn, HSV-2 UV-186, or uninfected cell extract. Their newborn pups were challenged at age 1 day or 1 week with a high dose of HSV-2 186ΔKpn. Newborn mice were challenged at age 1 day to mimic the response of a premature human newborn and at 1 week to represent a human newborn at term [36]. Reduction of challenge virus replication at the site of entry, in the nervous system, and in distal organs were the end points used in these experiments because they are thought to be better human correlates of vaccine efficacy than protection against mortality. Furthermore, we euthanized the newborn mice at the onset of signs of HSV disease because this was invariably rapidly fatal in this age group. The level of infectivity in the various organs of euthanized mice was included with the data from mice culled for that time point. We chose the challenge dose of $2 \times 10^6$ pfu/mouse because we wanted to mimic the level of virus to which a mouse pup or human newborn would be exposed in the birth canal after primary genital maternal HSV-2 infection (i.e., $\approx 10^7–10^8$ pfu/mL) [37].

Newborn mice have an age-related susceptibility to TK-negative HSV-2 mutants but HSV-2 dl5-29 is nonlethal in newborn mice. wt HSV-2 causes lethal encephalitis even in adult mice. Studies of newborn mice have shown that HSV-2 rapidly disseminates to the brain and distal organs with subsequent death to the animal [38, 39]. We previously showed that HSV-2 186ΔKpn is highly neuroattenuated in adult mice with no morbidity or mortality observed up to the highest dose available ($10^6$ pfu/mouse) after intranasal inoculation [30]. To determine whether HSV-2 186ΔKpn was also attenuated in newborn mice and hence could be used as a less virulent challenge to pups in the maternal immunization studies, series of naive pups were inoculated orally with a range of doses up to $2 \times 10^5$ pfu/mouse at either age 1 day or 1 week. Pups infected at age 1 day showed signs of lethal disease from age 3 days when given doses as low as $2 \times 10^2$ pfu/mouse (figure 1A). Survivors displayed no signs of disease up to 30 days after infection. In contrast, $>80\%$ of pups inoculated at age 1 week tolerated doses of HSV-2 186ΔKpn up to $2 \times 10^7$ pfu/mouse (figure 1B). Thus, newborn mice display a heightened susceptibility, even to HSV-2 strains that are highly attenuated in adult mice. This susceptibility was still present at age 1 week.
mutant defective for replication does not cause disease in newborn mice.

**Immunization of adult mice with HSV-2 dl5-29 elicits a strong HSV-2–specific, Th1-mediated T cell response and HSV-2–specific antibody response.** Ideally, a vaccine against genital HSV disease would induce both high levels of neutralizing antibody and a strong Th1 CD4 T cell response if it were to protect against HSV infection and disease in the newborn. To evaluate the level and nature of HSV-2–specific antibody and the nature of the splenocyte cytokine response in the immunized adult female mice at the time of delivery, we collected blood postpartum from the mothers and harvested their spleens after all their pups were culled. As shown in table 1, immunization with HSV-2 dl5-29 elicited a strong IgG response in the adult mice. Analysis of the IgG2a:IgG1 ratios showed that this was a predominantly IgG2a response, which is indicative of Th1-driven immunity. This was supported by the strong IFN-γ response to all maternal immunogens. Mice immunized with HSV-2 dl5-29 also produced significantly higher levels of IL-5 than those immunized with HSV-2 186ΔKpn (P < .05) and higher levels of IL-4 and IL-5 than those immunized with HSV-2 UV-186 (P < .013, for both cytokines). Thus, HSV-2 dl5-29 induced a mixed Th1/Th2 cytokine response in adult female mice, compared with the predominantly Th1 response induced by HSV-2 186ΔKpn and HSV-2 UV-186.

**Maternal immunization with HSV-2 dl5-29 does not prevent or reduce replication of a replication-competent TK-negative HSV-2 challenge virus at the site of entry in pups.** One means by which a maternal immunogen may protect against perinatal transmission of HSV is by reducing the level of virus to which the newborn is exposed in the birth canal. This situation cannot be mimicked in mice, because intravaginal infection of pregnant dams fails to infect their offspring during the short delivery time [40]. Therefore, we challenged the newborn pups orally, because it was more akin to the mode of infection in human newborns, rather than with an intraperitoneal injection, the route most commonly used in previous studies of HSV infection in newborn mice.

To test whether maternal immunization with HSV-2 dl5-29 protected newborn pups against replication of the challenge virus at the site of inoculation, the titer of infectious virus was determined in the lungs and stomachs of pups that had been infected on age 1 day or 1 week. The lungs were chosen to represent inhaled virus, and the stomach was chosen to represent ingested virus. We determined previously that no infectious virus could be detected in mouth swabs of naive newborn mice after oral inoculation up to 5 days after infection (data not shown). Time points are listed only to day 3 after challenge at age 1 day and to day 4 after challenge at age 1 week, because we observed in an earlier pilot experiment that all newborn mice developed pallor, cyanosis, inactivity, and paralysis indicative of systemic HSV disease from these time points, regardless of maternal immunogen. Infectious virus was detected from pc day 1 in the lungs of all 1-day-old pups and in the stomachs of all pups, regardless of maternal immunogen (figure 2). No significant difference was demonstrated in the mean titer of virus in these organs between pups of mock-immunized mothers and any other group (Student’s t test). Thus, maternal immunization with HSV-2 dl5-29 does not prevent infection with the challenge virus at the portal of entry or reduce the level of replication at these sites.

**Maternal immunization with HSV-2 dl5-29 reduces visceral dissemination of virulent HSV-2 challenge virus in newborn pups.** If neonatal HSV infection occurs either during delivery or postnatally, a maternal immunogen may also attenuate the disease in the neonate by limiting spread to distal organs. Therefore, we next examined the effect of maternal immunization with HSV-2 dl5-29 on the dissemination of infectious virus in pups. Maternal immunization with HSV-2 dl5-29 protected newborn pups from visceral dissemination of infectious virus.

### Table 1. Herpes simplex virus type 2 (HSV-2)–specific IgG antibody levels and splenocyte cytokine responses following maternal immunization.

<table>
<thead>
<tr>
<th>Maternal immunogen</th>
<th>HSV-2–induced cytokine production, pg/mL ± SEM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Maternal immunoglobin geometric mean titer, ng/mL ± SEM&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-γ</td>
<td>IL-4</td>
</tr>
<tr>
<td>HSV-2 dl5-29</td>
<td>2885 ± 41</td>
<td>458 ± 88</td>
</tr>
<tr>
<td>HSV-2 186ΔKpn</td>
<td>2887 ± 45</td>
<td>273 ± 165</td>
</tr>
<tr>
<td>HSV-2 UV-186</td>
<td>3009 ± 77</td>
<td>60 ± 15&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CE</td>
<td>1035 ± 226</td>
<td>30 ± 24</td>
</tr>
</tbody>
</table>

<sup>a</sup> Adult female BALB/c mice (<i>n</i> = 4 mice/group) were immunized subcutaneously with 2 × 10⁶ pfu/mouse of HSV-2 replication-defective mutant (HSV-2 dl5-29), the thymidine kinase–negative HSV-2 strain (HSV-2 186ΔKpn), or a UV-inactivated HSV-2 strain (HSV-2 UV-186) or were mock immunized with uninfected cell extract (CE). Mice were given 2 doses 3 weeks apart and then mated. Blood was collected at the time of delivery and assayed for HSV-2–specific IgG1 and IgG2a antibodies by capture ELISA. Data are representative of 2 independent experiments.

<sup>b</sup> Splenocytes were harvested from immunized adult female mice 3 weeks after boosting, restimulated in vitro for 72 h with HSV-2 UV-186, and the cytokine concentration in the culture supernatant was determined by ELISA. Data are representative of 2 independent experiments.

<sup>c</sup> P < .05, vs. HSV-2 dl5-29 (Student's t test).

<sup>d</sup> P < .013, vs. HSV-2 dl5-29 (Student's t test).
newborn pups. To this end, the level of infectious virus in the liver, kidney, and spleen of pups born to mothers mock immunized or immunized with either HSV-2 dl5-29, HSV-2 186ΔKpn, or HSV-2 UV-186 were determined at a series of time points up to the onset of signs of systemic disease. Given that all pups were developing lethal HSV disease at the last time point shown for each age group, regardless of maternal immunogen, we had anticipated that there would be little difference in the spread of virus to distal organs. However, surprisingly, we observed that although infectious virus was detected only in the livers of pups born to mock-immunized mothers from pc day 2, it was not detected at any time point in the livers of mice born to mothers immunized with either HSV-2 dl5-29, HSV-2 186ΔKpn, or HSV-2 UV-186 and that were challenged orally with 2 × 10^6 pfu/mouse of HSV-2 186ΔKpn at either age 1 day (A and C) or 1 week (B and D). At 12 h and on postchallenge days 1–4, the lungs (A and B) and stomach (C and D) were harvested and assayed for infectious virus titer by standard plaque assay. Data are mean ± SEM titers. Dashed line, lower limit of detection. Two independent experiments gave similar results.

![Figure 2](https://academic.oup.com/jid/article-abstract/185/11/1550/834762)

Figure 2. Maternal immunization with the herpes simplex virus type 2 (HSV-2) replication-defective mutant (HSV-2 dl5-29) and reduced replication of HSV-2 challenge virus at site of entry in pups. Pups (n = 4–5/group) born to mothers immunized subcutaneously with 2 doses of HSV-2 dl5-29, HSV-2 thymidine kinase–negative mutant (HSV-2 186ΔKpn), or a UV-inactivated HSV-2 strain (HSV-2 UV-186), 3 weeks apart (2 × 10^6 pfu/mouse), or mock immunized with uninfected cell extract (CE) were challenged orally with 2 × 10^6 pfu/mouse of HSV-2 186ΔKpn at either age 1 day (A and C) or 1 week (B and D). At 12 h and on postchallenge days 1–4, the lungs (A and B) and stomach (C and D) were harvested and assayed for infectious virus titer by standard plaque assay. Data are mean ± SEM titers. Dashed line, lower limit of detection. Two independent experiments gave similar results.

Maternal immunization with HSV-2 dl5-29 does not protect newborn mice against viremia. To test whether the observed protection against spread of infectious virus to distant organs in the offspring of mice immunized with HSV-2 dl5-29 correlated with protection against viremia, we determined the amount of HSV-2 DNA in the blood of pups from 12 h to pc day 4 by quantitative PCR. No HSV-2 DNA was detected in the blood of pups born to mothers immunized with HSV-2 186ΔKpn at any of the time points tested to the level of detection of our assay. In contrast, low levels of HSV-2 DNA appeared in the blood of some pups born to mothers immunized with HSV-2 dl5-29 and not at all in those born to mothers immunized with HSV-2 186ΔKpn. Differences in mean titers between offspring of mock-immunized mice and the other groups only reached significance in the spleens of 1-day-old mice on day 3 (P < .01). Thus, maternal immunization with HSV-2 dl5-29 reduced early visceral dissemination of the challenge virus in newborn pups as effectively as HSV-2 186ΔKpn. Furthermore, the lethal HSV disease observed in the newborn mice born to mothers immunized with dl5-29 was not due to disseminated HSV infection.
This result suggests that the lack of challenge virus replication in distal organs that we previously observed in pups born to mothers immunized with HSV-2 dl5-29 was not through protection against release of virus into the bloodstream from the site of infection. Protection from viral dissemination correlates with levels of passively transferred HSV-2–specific antibody in newborn mice. Next, we sought to determine whether the levels of neutralizing antibody and HSV-2–specific IgG antibody transferred to the pups correlated with the observed protection against disseminated infection seen in the offspring of mice immunized with HSV-2 dl5-29. Serum collected prior to challenge at either day 1 or day 7 was analyzed for IgG1 and IgG2a levels by capture ELISA. We found that IgG1 levels were 10-fold lower than those in adults (table 2), but that relative levels of antibody between maternal immunization groups were comparable to those observed in mothers. Neutralizing antibody titers were equivalent in mothers and pups.
immunized with HSV-2 dl5-29 and HSV-2 186ΔKpn (table 3) and were detected at similar but lower levels in their pups. Neutralizing antibodies were not detected in the mothers or offspring of mice immunized with HSV-2 UV-186 or mock immunized, correlating with the observed lack of protection in these groups against visceral dissemination of challenge virus.

To confirm that the level of passively transferred maternal antibody corresponded with protection against visceral dissemination, series of naive, 6-day-old pups (4 pups/group) were injected with 100 μL of maternal serum (from the adult mice described in table 2 and figure 3) that had been diluted 10^2 or 10^4 with low endotoxin, sterile saline, or left untreated by use of the protocol described by Kohl et al. [13]. The pups were challenged orally 1 day later with 2 × 10^6 pfu/mouse of a UV-inactivated HSV-2 strain (HSV-2 UV-186) (lanes 5–8 and 21–23), dl5-29 (lanes 9–12 and 24–27), or HSV-2 thymidine kinase–negative mutant (HSV-2 186ΔKpn) (lanes 13–16 and 28–31) or mock immunized with uninfected cell extract (CE) (lanes 1–4 and 17–20). Each lane represents blood from 1 pup. M, markers.

Figure 4. Maternal immunization with herpes simplex virus type 2 (HSV-2) replication-defective mutant (HSV-2 dl5-29) and protection of newborn mice against viremia following oral challenge with a virulent HSV-2 challenge virus. A, Phosphorimage of a probed Southern blot of polymerase chain reaction (PCR) products of HSV-2 DNA standards. The no. above each blot is the no. of HSV-2 molecules in each reaction (×5). No viral DNA was detected in blood from uninfected pups (U) or PCR mix alone (BL). B, Phosphorimage of a probed Southern blot of PCR products of blood collected postchallenge days 3 (d3) and 4 (d4) from pups of mothers immunized twice subcutaneously with 2 × 10^6 pfu/mouse of a UV-inactivated HSV-2 strain (HSV-2 UV-186) (lanes 5–8 and 21–23), dl5-29 (lanes 9–12 and 24–27), or HSV-2 thymidine kinase–negative mutant (HSV-2 186ΔKpn) (lanes 13–16 and 28–31) or mock immunized with uninfected cell extract (CE) (lanes 1–4 and 17–20). Each lane represents blood from 1 pup. M, markers.

HSV replication-defective mutants are safe and immunogenic in a variety of animal models of HSV disease [28–31]. In this study, we showed that maternal immunization with an HSV-2 dl5-29 protected offspring from replication of challenge virus in distal organs, it did not protect against spread to or replication within the peripheral nervous system (PNS) or CNS.

Discussion

HSV replication-defective mutants are safe and immunogenic in a variety of animal models of HSV disease [28–31]. In this study, we showed that maternal immunization with an HSV-2 dl5-29 [± SEM], respectively) were 1–1.5 log-fold less than the titer in untreated pups after challenge (3.17 ± 0.14 log_{10} pfu/organ). Thus, increasing the concentration of maternal antibody corresponded with increased protection against visceral dissemination. However, given the small numbers of pups used, the differences in mean titers between groups were not statistically significant (Student’s t test, HSV-2 dl5-29 vs. untreated, HSV-2 186ΔKpn, or HSV-2 UV-186). Future larger scale studies will be performed to confirm this finding.

Maternal immunization with HSV-2 dl5-29 does not prevent viral spread to the central nervous system (CNS) or encephalitis in pups challenged orally with virulent HSV-2 strains. Regardless of the level of protection against spread of the challenge virus to the liver, kidneys, and spleen and of maternal immunogen, newborn mice pups succumbed to lethal HSV disease by pc days 3–4 and 4–5 after challenge at age 1 day or 1 week, respectively. Assay of infectious virus in the brain confirmed that the cause of death was encephalitis, with infectious virus detected in the CNS of all pups from pc day 3 (figure 5). Similarly, we observed no difference in the rate of appearance of HSV-2 DNA in the TG between offspring of immunized mice or controls with low levels of HSV-2 DNA present at pc day 2, increasing in all pups to >10^4 HSV-2 DNA molecules/TG by pc day 3 (figure 6). Thus, although maternal immunization with HSV-2 dl5-29 protected offspring from replication of challenge virus in distal organs, it did not protect against spread to or replication within the peripheral nervous system (PNS) or CNS.

Table 2. Herpes simplex virus type 2 (HSV-2)–specific IgG antibody levels in pups following maternal immunization.

<table>
<thead>
<tr>
<th>Age at viral challenge, maternal immunogen</th>
<th>Geometric mean titer, ng/mL ± SEM</th>
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<tbody>
<tr>
<td>HSV-2 dl5-29</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>HSV-2 186ΔKpn</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>CE</td>
<td>6 ± 0</td>
</tr>
<tr>
<td>HSV-2 dl5-29</td>
<td>29 ± 1</td>
</tr>
<tr>
<td>HSV-2 186ΔKpn</td>
<td>36 ± 2</td>
</tr>
<tr>
<td>HSV-2 UV-186</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>CE</td>
<td>14 ± 5</td>
</tr>
</tbody>
</table>

NOTE. Blood was collected from pups (n = 4/group) at time of viral challenge (1 day or 1 week of age) and assayed for HSV-2–specific IgG antibody by capture ELISA. Mothers were immunized subcutaneously with 2 doses of the HSV-2 replication-defective mutant (HSV-2 dl5-29), the replication-competent thymidine kinase–negative HSV-2 strain (HSV-2 186ΔKpn), or a UV-inactivated HSV-2 186 wild-type strain (HSV-2 UV-186) or were mock immunized with uninfected cell extract (CE).
replication-defective mutant mitigated against disseminated disease in newborn mice after a virulent oral challenge with a replication-competent TK-negative HSV-2 strain, but did not protect against spread of virus to the CNS.

We used HSV-2 186ΔKpn as the challenge virus in neonatal mice because we sought a virus that would replicate in the site of entry but spread less rapidly to and within the nervous system. Most wt HSV-2 strains are highly neurovirulent, even in adult mice. We and other researchers previously showed that TK-negative HSV-2 strains are avirulent after intranasal [30] or intravaginal [41] inoculation of adult mice. TK-negative HSV type 1 (HSV-1) and HSV-2 strains are virulent in neonatal mice after intranasal [42] and intracerebral [43, 44] inoculation, but we hypothesized that the spread to the CNS would be less rapid after oral inoculation. However, we observed that oral infection of newborn mice with HSV-2 186ΔKpn was highly lethal up to age 1 week and possibly beyond, in contrast to the situation in adult mice. Our findings are consistent with the observation that acyclovir-resistant, TK-negative HSV strains can cause disseminated disease in human neonates after the initiation of antiviral therapy [45, 46]. The mechanism of this increased neurovirulence of TK-negative HSV strains in the newborn is unclear. Although there is a decline in neuronal cell division and TK activity in mice from birth to age 3 weeks [44], Hay et al. [42] excluded immune dysfunction and cellular complementation of HSV TK as likely mechanisms of the neuronal permissiveness to TK-negative HSV-1 strains in newborn mice.

Intravaginal infection of pregnant mice with HSV-2 results in low transmission rates to pups accompanied by high maternal mortality [40]. Therefore, we used an oral route of inoculation in our mouse model because we considered it to be more representative of human infection, compared with intraperitoneal or intracerebral inoculation, and because it allowed us to control for the amount of virus to which the newborn was exposed.

Unlike humans, most of the protective maternal antibody in mice is transferred through colostrum and adsorbed through the neonatal intestine, with minimum transfer through the placenta [47]. Therefore, it is possible that some direct neutralization of challenge virus may have occurred with the ingestion of maternal colostrum containing HSV-specific antibody in the offspring of immunized mothers. In this model, this could be taken to represent transudation of HSV-2–specific IgG antibody into the vaginal lumen, which provides early protection against vaginal challenge [48], and potentially to reduce the amount of virus to which the newborn is exposed. Intranasal challenge offered no advantage in this regard because the inoculum is also largely distributed into the stomach and inhaled into the lungs after infection by this route [30].

We observed that HSV-2 186ΔKpn reduced but did not prevent replication at the site of entry in newborn pups. This result was not unexpected, because no vaccine to date protects the host against infection by the wt organism. In addition, control of initial HSV replication occurs through innate immune mechanisms, which are invariably defective in newborn humans and rodents and are not influenced by the level of antibody [14, 15, 49, 50].

We chose the challenge dose of 2 × 10^6 pfu/mouse because we wanted to mimic the level of virus to which a mouse pup or human newborn would be exposed in the birth canal after primary genital maternal HSV-2 infection [29, 37]. We used replication in distal organs, not mortality, as an end point, because it is now clear that the latter does not predict efficacy in humans [19]. The absence of protection against mortality was not surprising given that we used a challenge dose many times above the LD_{50} value. It is possible that use of a lower dose of HSV-2 TK-negative virus to challenge the pups may have allowed for better discrimination of degrees of protection against bloodborne or neural spread between maternal immunogens; however, we have ob-
observed that challenge of pups at a dose at which all mice survived (2 × 10^5 pfu/mouse) fails to induce dissemination (authors’ unpublished data). Nevertheless, there was a striking difference in the degree of visceral replication in offspring born to mothers immunized with either the replication-defective virus or the live-attenuated strains, compared with mock-immunized and killed vaccine (HSV-2 UV-186) controls where no protection against CNS spread was seen, suggesting different requirements for protection against spread to each site.

The levels of maternal antibody transferred were modest; however, there appeared to be a correlation between the degree of protection against dissemination and both the level of HSV-specific antibody and the level of neutralizing antibody in the mothers and newborn pups. It is possible that higher levels of antibodies may bring about greater protection. Further studies are underway to confirm this point. In addition, our findings are consistent with the observation that the level of transferred neutralizing antibody correlates with the degree of visceral spread of HSV in human neonates but not with the presence of CNS disease [15–17].

In humans, the frequency of viremia in the natural history of neonatal HSV infection has only recently been recognized. HSV DNA has been detected in the blood or serum and cerebrospinal fluid of neonates with skin, eye, mouth, or CNS disease and disseminated infection [51–53]. In our model, we hypothesized that the observed reduction of disseminated infection was due to the prevention or reduction of viremia. Although some pups of mothers immunized with the replication-defective vaccine had low levels of HSV DNA present in the bloodstream from pc day 3, no viral DNA was detected in pups born to HSV-2 186ΔKpn-immunized mothers whose IgG titers were higher than those of HSV-2 dl5-29–immunized mothers. This suggests that there is a maternal antibody threshold above which viremia is prevented. Further studies are underway to explore this possibility.

Despite the observed protection against infection in distal organs, all our mice were dying of encephalitis by pc days 3–5, as shown by high levels of infectious virus in the brain from pc day 3. Viral DNA was also detected in the TG of all pups from pc day 2, regardless of maternal immunogen. This suggests that maternal antibody may not protect the neonate against spread to the PNS, in contrast to observations in B cell–deficient adult mice [54], in cultured neuronal cells [55], and in epidemiologic studies of persons with agammaglobulinemia (where an effect for the use of intravenous immunoglobulin was controlled for) [56].

Possible reasons why maternal antibody failed to prevent access to the PNS in neonates include increased neuronal permissiveness in neonates, whereby a lower level of virus has a greater effect, or levels of antibody insufficient to overcome the rapid replication at the site of virus entry in the periphery. Whether HSV must first enter an extracellular space before it gains access to the CNS so that there is an opportunity for antibody to neutralize the virus or whether there are fused areas of neurons with epidermal cells, especially in the olfactory region, remains to be resolved. In the periphery, HSV spreads cell-to-cell via intracellular bridges, thus suggesting a mechanism of immune evasion by avoiding exposure to neutralizing antibody in the extracellular fluid [57]. Nevertheless, our data suggest that, although an immunogenic maternal vaccine that generates high levels of neutralizing antibody may protect against disseminated infection, it may not protect against spread to the PNS and the brain if the newborn becomes infected with HSV.

The important question remains: how well does this extrapolate to the human situation? The guinea pig is usually thought to be a better model of neonatal HSV disease than the mouse. Given the paucity of immune reagents available for guinea pigs, we could not extend our observations into the mechanism of protection as we are doing with mice. However, extension of our observations on the pathogenesis of HSV infection by using lower doses of HSV-2 186ΔKpn may best be studied in newborn guinea pigs. Another important caveat is that our model of an oral postnatal challenge does not include other potential protective effects of a maternal vaccine. These effects include reduction of the likelihood of acquiring the wt virus, the rate of recurrences if wt infection occurs, and the duration and amount of viral shedding to which the infant is exposed.

In summary, we observed that maternal immunization against HSV-2 with a replication-defective mutant is associated
with a modest transplacental transfer of antibody reduced of vis-
ceral dissemination after a virulent challenge in newborn mice but
not spread to the ganglia or lethal encephalitis. Although
further gains may be obtained by augmenting the level of trans-
ferred antibody, it is clear that we must continue to develop post-
exposure immunotherapeutic strategies against HSV disease
targeted to this age group. Our finding that an HSV-2 TK-negative
strain that is highly attenuated in adult mice had heightened
susceptibility in the newborn cautions against the use of live-at-
tenuated HSV strains as vaccines in this age group.

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